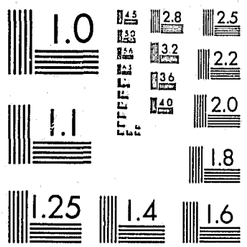

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Sourcebook in Forensic Serology, Immunology, and Biochemistry

Unit IX: Translations of Selected Contributions to the Original Literature of Medicolegal Examinations of Blood and Body Fluids

a publication of the **National Institute of Justice**

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James K. Stewart
Director

U.S. Department of Justice
National Institute of Justice

Sourcebook in Forensic Serology, Immunology, and Biochemistry

Unit IX: Translations of Selected Contributions to the Original Literature of Medicolegal Examinations of Blood and Body Fluids

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FOREWORD

Translations of Selected Contributions to the original Literature of Medico-legal Examinations of Blood and Body Fluids is Unit IX of a larger work with the title, *Sourcebook in Forensic Serology, Immunology and Biochemistry.*

Unit IX contains 50 translated papers arranged in four sections. Section I, on Identification of Blood, consists of 16 papers beginning with the early work of Orfila, using chemical means to identify blood, and differentiating rust, blood stains and stains from lemon juice on iron in resolving the medical-legal matter of wounds. The microscope was found useful by Mandl, Robin and Salmon identifying the formed elements, red cells with and without a nucleus (human and certain species of animal blood) and differentiating menstrual blood from blood drawn from a vessel by noting the mixture of blood cells with epithelial cells and leukocytes. In 1853, Teichman described hemin crystals, a crystalline chloride of heme, which he obtained from human blood and blood of the dog, rabbit, steer and other animals. Hoppe's 1862 paper gives the early report on the behavior of hemoglobin in the spectrum of sunlight.

In 1912, Takayama reported and expanded on the medicolegal applications of hemochromogen crystals; Donogany described the production of hemochromogen crystals in 1893.

In 1862, van Deen described the guaiacum test for detection of blood which was replaced with the more sensitive benzidine test reported by the Adlers in 1904. The last paper in Section I is the 1937 publication of Specht on the chemiluminescence of hemin in detecting blood stains.

Section II, on Body Fluids has 10 papers, mainly dealing with the detection of semen by microscopy and stains, the crystal test of Florence and finally, Lundquist's acid phosphatase test reported in 1945, which is useful in aspermic individual stains.

Section III, Determination of Species of Origin has 16 papers dealing with the applications of immunology in forensic serology. Essentially, these were biological protein differentiation and eventually, forensic blood differentiation reported by Uhlenhuth, and Wassermann and Schütze, within several weeks time, by use of the precipitin test. Neisser and Sachs reported on the application of the complement-fixation test as control and a supplement to the precipitin test.

Section, IV, Blood Grouping—Medicolegal Applications has 8 papers. Notable are the papers of Leone Lattes in which he brought to the attention of the "forensic camp" the Landsteiner rule sitting in the literature at that time for 12 years. Namely, that human bloods can be divided into four groups based on cells with or without A and/or B antigen; and serum with or without anti-A and/or anti-B antibody. One can determine this relationship from Landsteiner's study, but Lattes further explains that the division was expressed in this form only in the works of v. Dungern and Hirszfeld. Lattes also noted that the percentages of the single type (A-B-O gene frequencies), quoted independently of one another, by v. Dungern and Hirszfeld in Heidelberg, and by Moss in Philadelphia, were just about identical. The figures showed that 40% of all bloods tested had no A or B antigens on the red cells, but did contain two agglutinins, alpha and beta in the serum. These bloods were called group O.

In early articles, Lattes used the A-B-O nomenclature of Landsteiner: $A\beta$, $B\alpha$, AB, and $O\alpha\beta$ but in the 1927 paper he used the Landsteiner and Jansky nomenclatures (classifications) together: Group II ($A\beta$), Group I ($O\alpha\beta$). This is noted because during the 1930's the Moss, Jansky and Landsteiner classifications were used which resulted in some confusion to blood group serologists, physicians and blood bank personnel in general. The situation was finally corrected when the National Research Council recommended sole use of the Landsteiner classification to the Armed Services at the

beginning of World War II which was also the beginning of large scale blood transfusion.

Siracusa reported (1923) on his absorption experiments with stains to identify the group by allowing the A and B antigens to absorb A and/or B agglutinin, respectively. He further confirmed the specificity of the absorption by eluting at 45°C and testing the eluted agglutinins with A and B red cells.

Franz Holzer was an internationally know forensic pathologist. His contributions are well cited in the last three papers of Section 4. Holzer was one of Landsteiner's five students. His passing, followed by that of Wiener, leaves only three remaining: Philip Levine, Merrill W. Chase, and J. L. Jacobs. In our lifetime, their contributions have illuminated the scientific literature.

Professor Gaensslen is to be commended for his scholarly achievement and contribution to the English literature in bringing together this extensive series of translations with direct bearing on forensic serology, immunology and biochemistry. The translators, specialists, involved in this project have done an outstanding job.

The late A. S. Wiener described P. B. Candela's efforts in identifying the blood groups of ancient bones (mummies) as a labor of love. Certainly the same can be said of the Sourcebook and translation series which Professor Gaensslen and associates have worked so diligently to bring to a successful completion. The translations and Sourcebook will provide additional reference material for operational crime laboratories, academic institutions and research libraries. This availability to the workers, teachers, and research oriented staffs in the fields of forensic serology, immunology and biochemistry provides a means to achieve new excellence in medicolegal studies.

Frank R. Camp, Jr.
Louisville, Kentucky
September, 1978

PREFACE

In the course of preparing the *Sourcebook in Forensic Serology, Immunology and Biochemistry,* I became aware of set of translations of selected important papers in blood grouping and immunohematology. Selected and edited by Col. Frank R. Camp, Jr., Col. Frank R. Ellis and Col. Nicholas F. Conte at the Blood Bank Center of the U. S. Army Medical Research Laboratory, Fort Knox, KY, the collection contains 36 original papers and monographs by Landsteiner, Hirszfeld, Bernstein, Friedenreich, Schiff, Dahr and others covering the most important developments in blood grouping serology and immunohematology. In addition, two theses written in other languages were translated. One was an extensive study of weak subgroups of A by Arne Gammelgaard; the other an extensive study of secreted group substances in body fluids by Grethe Hartmann. A few papers originally written in English, but old and virtually inaccessible, were included as well. This compilation was of enormous help to me in preparing the Sourcebook, since it was necessary to consult most of the papers which were contained in it. I was greatly impressed by the set of translations, and realized that, but for the efforts of Camp, Ellis and Conte and their collaborators, much of this now classical literature would not be available to those of us whose abilities in foreign languages are less than noteworthy.

I thought that a somewhat similarly conceived set of translations might be of some value in connection with the Sourcebook. There are a number of important papers in the original literature of the medicolegal examination of blood and body fluids which were written in languages other than English. The history and development of the field interested me, and I thought that it might interest others as well. I consulted with a number of colleagues and friends, and received encouragement to carry forward with the translations set. In addition, I wrote to Col. Camp in Kentucky and asked for his opinion about my plan. He was most encouraging, enthusiastic and helpful, not only in his response to my initial inquiry, but throughout the course of the project. This Unit of the Sourcebook is the result of the translations project. It was my strong feeling from the outset that the continuity between the set of translations prepared at Fort Knox and this project could best be expressed by asking Col. Camp to write a Foreword to this set. I am most grateful to him for having very kindly agreed to do so.

The selection of papers for inclusion in this set was not always easy, and for better or for worse, I alone am responsible for the choices. I wanted to include some of the very earliest works on the subject, those of the renowned Orfila and his collaborators in early 19th Century France. I wanted to include those papers which may now be regarded as classical, in that they represented the first reports of methods or techniques, some of which have survived to the present time. Other papers were chosen because they represented the viewpoints about certain procedures in various places at various times in the history of the field. The translations set is divided into four sections, corresponding approximately to the divisions of the main Sourcebook: Identification of Blood; Identification of Body Fluids; Determination of Species of Origin; and Blood Grouping. In some cases, the papers discussed more than one of these subjects, and the decision concerning which section they should be placed in was somewhat arbitrary. Each section has a brief introduction, the purpose of which is to explain something about the basis for the selection of papers and the way in which the different works relate to one another. I have also included such information as I have been able to discover about the authors of the papers, some of whose names are synonymous with tests that are still used today. Where possible, I have included photographs.

All of the papers which appear in the translations set are cited in the main Sourcebook. The work is discussed in connection with other work on the same or

similar subjects. I have not, therefore, gone into the nature of the work in very much detail in the short introductions to the sections of this Unit.

An effort was made in preparing the translations to keep the language fairly literal, as close as reasonably possible to what was written by the original author. At times, this practice results in English which is not in the best syntax, or is somewhat convoluted in construction. We nevertheless thought that there was merit in following this practice.

A few conventions have been followed in setting out the translations: The original pagination has been included as a page number, followed by a slash, at the right-hand margin. All page number references in the texts are to the original page numbers, and not to ours. In some cases, the formats, especially of the title pages, have been rearranged to reflect more modern style. In the older literature, first name(s) or initials were sometimes omitted. In cases where the first initials or names of the authors were known, we have added them. In papers where there were very many footnotes, or references appearing as footnotes, we have placed these at the end of the paper in a reference list. In some cases, the reference numbers do not correspond to the originals, because it was the convention in some older journals to number the footnotes from Number 1 on each new page. But all the numbers have been adjusted so that the correct reference matches the reference number. It was necessary in a few places to add comments or remarks which did not appear in the original article. This we have done in square brackets. Photographs and drawings have not been included in the translations. The references in the papers have been left in their original form for the most part. They have not been edited, and may not all be accurate.

It is a pleasure to acknowledge the interest and assistance of a number of people who have helped to see to it that this project was completed. I want to thank the PSC-BHE Research Award Program and the Research Foundation of the City University of New York as well as the National Institute of Law Enforcement and Criminal Justice for financial support. Without this support, the work could not have been done.

All the translators did very excellent work, which is very much appreciated: Hannah DeVegh, Timothy Miller, Hugo Twaddle, Emile Capriotti, Rebecca Guenther and Kaori Kato.

A number of people were helpful with difficult passages in foreign languages, and with bibliographic material, including John Broadwyn and Dr. Stephen Kim at the National Library of Medicine.

I am very grateful to Prof. Dr. Hiroshi Hirose of Kyushu University, Fukuoka, Japan, for supplying me with a great deal of information about Dr. Takayama, for sending me the photograph of Dr. Takayama, and for assistance in obtaining permission to reprint the translation of the pyridine hemochromogen paper. Dr. George Sensabaugh of the University of California, Berkeley, was not only generally supportive, but helped to arrange for one of the translations.

A number of people were helpful in obtaining the photographs which appear in this unit, as well as permission to reprint them. I am indebted to Lucinda Keister, Curator of the Portrait Collection at the National Library of Medicine, for a number of the photographs. Prof. Dr. Hiroshi Hirose sent me the photograph of Takayama, and was extremely cooperative in many ways. I thank Prof. Antonio Fornari and Prof. Sergio Perugini in Pavia for obtaining the photograph of Prof. Leone Lattes, which originally appeared in *Haematologica,* volume 38, number 9 for 1954, and I am grateful to the editors of *Haematologica* for permission to use the picture. I want to thank Prof. Dr. Angelo Fiori in Rome for sending the photograph of Prof. Siracusa, and for obtaining the latter's permission to publish it. Verlag Franz Deuticke kindly extended permission to reprint a photograph of Prof. F. J. Holzer which originally appeared in *Beiträge zur Gerichtlichen Medizin.*

The kind permission of many publishers of the original articles which were protected by the copyright laws and agreements for the reprinting of the translations is gratefully acknowledged: Nordisk Medicin, Stockholm; Walter de Gruyter & Co.

Verlag, Berlin; J. F. Bergmann Verlag, Munich; Verlag Schmidt-Römhild, Lübeck; Georg Thieme Verlag, Stuttgart; Springer Verlag, Heidelberg and New York; Edizioni Minerva Medica, Torino; Masson, S. A. in Paris; Verlag Franz Deuticke, Vienna; Verlagsgesellschaft Otto Spatz, Munich. A special word of thanks to Fiametta Lattes Treves of Milano, Italy, for giving permission on behalf of the family to reprint the papers of Prof. Dr. Leone Lattes. In this connection, I would also like to thank Prof. Dr. H. Jahrmärker, Prof. Dr. Friedrich Geerds, Prof. Dr. J. Gerchow, Prof. Dr. A. Fornari and Prof. Dr. Diethard Gemsa for handling various correspondence, and for their help in obtaining the permissions. I am grateful to Mr. V. Borsodi and Mr. W. Bergstedt of Springer Verlag for help in straightening out several problems with the permissions.

Maureen Swift typed the entire manuscript, accurately and efficiently, often from very difficult drafts, without once losing her good humor. Without her assistance, the work could surely not have been completed.

Washington, D.C.
June, 1978

Col. Frank R. Camp, Jr. (USA, Ret.) died in 1983 before the final publication of this book. The earlier, similar work of Col. Camp and his colleagues served as a motivating factor in the undertaking of this project, and his assistance with and enthusiasm for it were important in bringing it to completion. Accordingly, this work is dedicated to the memory of Col. Camp and to his many contributions to immunohematology and serology.

Prof. Mattieu Joseph Bonaventure Orfila 1787-1853
Courtesy National Library of Medicine

Prof. Ludwik Karol Teichmann 1823-1895
Courtesy National Library of Medicine

Prof. Dr. Masao Takayama 1872-1943
Courtesy National Library of Medicine

Section 1. Identification of Blood

The application of chemical methods to the identification of blood for medico-legal purposes has its essential beginnings in the studies of Mathieu-Joseph-Bonaventure Orfila. At the time the studies were published, he was one of the most highly regarded medical scientists in France, and perhaps in the world. He was born in Spain in 1787, and began his education there, but in 1807 he went to Paris where he stayed. He was primarily a toxicologist, and his *Traité de Toxicologie Général* went through five editions. A *Traité de Médecine Légale* went through four editions, and a *Traité de Chemie* went through eight. In 1811, Orfila received his doctorate at Paris, his studies and his career apparently having been helped considerably by the interest taken in him by the well known chemist L. N. Vauquelin (1763-1829). He became a physician to the Court of Louis XVIII in 1816, Professor of the Faculty of Medicine in 1819, and a member of the Academy of Medicine in 1820. In 1823, he was made Professor of Chemistry, and in 1830, Dean of the Medical Faculty at Paris. Over the course of his career, he became embroiled in a number of disputes over scientific issues, and it appears that he generally prevailed even when he was wrong. Reading through the papers in this section, it will be clear that F. Raspail disagreed with Orfila about the first blood tests. The two apparently had a number of disagreements over toxicological questions as well. Orfila's rejection of the microscope as a major tool for blood examination and identification and for seminal stain identification (see in Section 2) laid the matter to rest for a number of years. Orfila died in 1853, and an obituary appeared in the *Annales d'Hygiène Publique et de Médecine Légale*, of which he was a founder, in that year.

The case reported by Orfila, Barruel and Chevallier in 1835 is interesting in that the blood indentification procedure developed by Orfila, and the "odor test" for human species identification developed by Barruel (see in Section 3), were applied to case materials. The 1845 paper by Orfila is an assessment of a technique developed by Prof. Persoz at Strasbourg employing hypochlorous acid.

There was a controversy in the early 19th Century about whether microscopical examination or chemical tests should have priority in examining bloodstains, especially in regard to which of these should be regarded as giving more certain results. The same arguments existed in connection with seminal stain examination (Section 2). There was a further dimension to the arguments about the examination of bloodstains. Prévost and Dumas had found in 1821 that the red cells of different species differed in size, and this in addition to the fact that mammalian red cells are anucleate, while those of birds, repiles, etc. have nuclei. Many of those who favored microscopical examination of bloodstains thought that these characteristics could be exploited for species differentiation. The finding of elements of blood (such as red cells) by microscopical examination was taken as incontrovertible evidence of the presence of blood. In Mandl's paper, a strong argument is made for the use of the microscope, and the early history of the subject is discussed. The papers of Robin and Salmon (1857) and of Robin (1858) strongly reinforce the recommendation of the use of microscopy for the assessment of blood and body fluid stains. Charles Robin (1821-1885) was a recognized histologist, and it is to the case report by him and Salmon in 1857 that Masson (1885) attributed the beginning of a new period in the history of medico-legal stain examinations, characterized by the use of the microscope as a primary tool (see in Section 3). A number of chemical tests are discussed by Rose (1853) in Germany. Indications are that he was familiar with the earlier French literature, but he makes no mention of the use of the microscope.

The crystal tests for blood have been considered by a number of workers to be certain proof of the presence of blood. The hematin crystal test grew out of the studies of Prof. Ludwik Teichmann (1823-1895) in 1853. Teichmann's biography may be found in the *Bulletin of the Polish Medical Society* 8 (3) for 1965. A tribute to him, which also discusses his life and work, appeared in *Gazeta Lekarska* 6 (26) for 1886. The other important type of blood crystal that has been used as a basis for medico-legal identification of blood is hemochromogen. These were prepared using pyridine in 1893 by Donogany. The classic paper on the preparation of pyridine hemochromogen crystals as a means of identifying blood for medico-legal purposes is that of Dr. Masao Takayama (1872-1943). This paper does not seem to have been correctly cited anywhere in the European or American literature since its publication in 1912. Takayama went to study with Prof. Kobert in Germany from 1904-1907, and it seems reasonable to suppose that his interest in hemochromogen crystals was kindled in those years. The laboratory was very active in these studies around this time. Dilling's *Atlas* in 1910 (see in Unit II, section 3 of Sourcebook) was the result of work in Kobert's laboratory at Rostock. Takayama spent much of his professional career in the Department of Legal Medicine at Kyushu University in Fukuoka, which he founded. He held important Directorships and Deanships of the medical faculties at Kurume and Nagasaki as well. For a time in 1936, he was Rector of Kyushu University. He is recognized in Japan for work he did on fingerprint images as well as for his work on blood identification and hemochromogen crystals.

The spectrum of hemoglobin and of its derivatives has been very important in the development of hemoglobin anal-

ysis. Spectral tests have been used in the examination of blood stains as well. Hoppe (1862) first reported on the visible spectrum of hemoglobin.

The catalytic tests, which are almost universally employed at present for blood identification, are based on the peroxidase activity of hemoglobin and its derivatives. The procedure was introduced by van Deen in 1862, using guaiacum as substrate. The paper was signed "J. van Deen," but a Dutch biographical source lists him as "Izaak van Deen." He lived from 1805 to 1869. The guaiacum test was widely used until 1904, when Rudolf and Oskar Adler published their now classical paper on catalytic reactions using benzidine, the leuco base of malachite green, toluidine and tolidine as substrates. A variety of other oxidizable organic substrates were tested as well. The luminol test, which is not quite a catalytic test in the same sense as the others, has enjoyed some popularity, and is still used sometimes. This method was orginated by Specht in 1937. Specht lived from 1907 to 1977. During his career, he was in Jena, Halle, and later in Munich. He was associated with the editorial board of the *Archiv für Kriminologie,* and a brief tribute to him may be found in volume 159 of this journal, numbers 3–4 for 1977.

Blood Considered in the Context of Legal Medicine Memoir Read at the Royal Academy of Medicine*

M. J. B. Orfila

/365 Before delving into the section which is to be the particular object of this memoir, I feel it necessary to present succinctly the series of works which I propose to communicate in it. I will present, in a first memoir, the means necessary for differentiating rust, blood stains and stains from lemon juice on iron: this subject, as can be seen, is immediately connected to the medico-legal matter of *wounds.* In a second work, I will present the characteristics of semen stains, compared to those of fat and the matter of various discharges from the vagina and of the urethral canal, in acute and chronic leukorrhea, in gonorrhea, in chronic urethritis, etc. I will prove that it is not difficult to recognize if linen has been stained by semen and this will contribute much to the clearing up of certain questions relative to defloration and rape. The third memoir will treat *asphyxia by submersion:* numerous experiments done on living animals, and about fifty autopsies on drowned corpses, several of which remained in the water for several months, will enable me to carefully trace the medico-legal history of submersion and lead me to the remarkable conclusion that, except in very rare cases where slime, mud, gravel, etc., is encountered in the *last bronchial subdivisions,* and where the cadaver has /366 not been found in a vertical position, head on top, it is impossible to determine if anyone has been submerged alive, unless wounds necessarily immediately mortal are discovered on the corpse, which indicate that the individual could not have thrown himself into the water. The meaning to be drawn from the presence or absence in the airways of an aqueous and sanguinolent froth will be of much less importance than was believed up to now. In the fourth memoir I will be concerned with poisoning from a new point of view. How do venomous substances, mineral and vegetable, behave in rotting animal matter? Up to what time can their presence or that of new compositions they have formed be demonstrated, supposing they have been altered? This question, of paramount concern to physicians and pharmacists charged with certain legal exhumations, will be resolved by experiments in which the principal mineral and vegetable poisons were mixed with animal matter that was left to rot for twenty months, either in containers exposed to air, containing water, or buried in earth: it is unnecessary to mention that, especially in the first case, the putrefaction was at its peak, and that the necessary elements to give a satisfying solution to the problem were consequently present.

[*Section consisting of further discussion on the matter of poisoning and poison detection not translated.*]

Blood Considered in the Medico-Legal Context

Physicians are often required by courts to determine if 367 stains, noted on instruments of iron or steel or on linen, are produced by blood. This matter having been the object of a work published by Lassaigne in 1825, we feel it necessary to announce, in order not to be accused of plagiarism, that we have treated this subject since 1823 in one of our lessons at the Faculty of Medicine. Moreover, if our experiments have some resemblance to those of the distinguished chemist, it will be seen they differ in several respects, and especially, that they consider the question in a much more comprehensive manner. We feel it necessary to examine successively blades of iron or steel and stained fabric.

Blades of Iron or Steel

Stains produced by blood on these instruments can be confused with those produced by lemon juice and rust. It is of consequence, then, to study them comparatively. 368

Characteristics of Dried Blood Stains

Points of the blade on which there was only a small quantity of blood were light red; they presented, by contrast, a dark brown color where blood was deposited in greater quantity. If the portions of this blade, where a layer of blood of appreciable thickness is found are exposed to a temperature of 25° to 30°, they drop off in scales and leave the metal rather shiny. In heating a portion of dried blood in a small glass tube, a volatile ammonia product is obtained which turns the color of litmus paper blue; the paper was arranged on the upper part of the tube prior to heating. When a drop of pure hydrochloric acid is placed on the dried blood stain, the stain doesn't yellow or disappear, nor does the iron become shiny, as happens with a stain produced by lemon juice or rust. In immersing the stained portion of the blade in distilled water, reddish striations quickly appear, running from top to bottom, and the coloring substance is soon found gathered at the bottom of the liquid which remains uncolored with the exception of its lower portion. If, at this time, the blade is withdrawn, the stained parts, thus treated with water, show whitish or slightly reddish white filaments.

* Translation of: "Du Sang, considéré sous le rapport de la médecine légale; Memoire lu à l'Académie royale de Médecine".
in *Journal de Chimie Medicale de Pharmacie et de Toxicologie* 3 (8): 365–374 (1827).

These filaments, formed by the fibrin of blood, could very well not be perceptible if the stain being operated on is of small thickness. The aqueous liquid from which the iron
369 blade was withdrawn was shaken in a glass tube, and acquired a rose or red color, according to whether it involved a greater or less amount of coloring substance. It enjoyed remarkable properties; it doesn't reestablish the color of litmus paper reddened by acid; chlorine used in small amount colors it green without precipitation. If more is added, it discolors the solution without making it lose its transparency, but soon after, it colors the solution opaline, and finishes by forming a deposit of white flakes. Ammonia doesn't visibly change the color, whereas it alters several red vegetable colors, like cochineal, Brazil wood, etc.; nitric acid gives rise to a greyish-white precipitate and the solution is almost discolored. Concentrated sulfuric acid gives rise to a similar precipitate only when used in rather large quantity; potassium ferrocyanide doesn't trouble it in any way; aqueous solution of gall nut provokes a precipitate of the same nuance as that of the liquid. The liquid discolors after filtration, or at least only conserves the yellowish color of dilute gall nut. Submitted to heat, the liquid of which we are speaking coagulates, unless it is very diluted in water, where it simply becomes opaline at first and only coagulates when a notable quantity of water is evaporated by boiling.

If, instead of withdrawing the iron blade stained with blood at the moment when the liquid is colored red in its lower part, it is left for several hours in water, in contact with air, the iron passes into the state of reddish-yellow trioxide, which remains in great part suspended in solution and imparts a yellowish tint to it. Another part of the trioxide, in
370 depositing, mixes with the red coloring substance occupying the bottom of the container and alters the color; but filtration suffices to separate all the trioxide, and the solution then passes to clear, colored in *light rose, deep red,* or *red* and shares all the properties which we have just assigned to water colored by blood. If the water in which the stained instrument was immersed contains only a small amount of coloring substance, or, in other terms, if the stain was not very sensitive, the solution will be troubled once again by gall nut or nitric acid.

Characteristics of the Stain Formed by Lemon Juice (Iron citrate)

When lemon juice is deposited on an iron blade exposed to the air, iron citrate of a reddish-brown, which is possible at first glance to confuse with dried blood, quickly forms. A man was recently suspected of having murdered another. Found on his chimney was a knife which appeared blood stained. This new burden seemed to overcome the accused; then it was learned at the laboratory of the Faculty that these presumed blood stains were only iron citrate produced by the simultaneous action of air and citric acid on a knife that wasn't wiped when, several days beforehand, it was used to cut a lemon. The points of the iron blade on which there was only a small amount of lemon juice were a yellowish red, whereas they present a dark brown color similar to that of dried blood when the juice was used in a stronger proportion. In the latter case, the stain scales, the iron citrate detaches and leaves the metal shiny when the temperature rises to 25° or 30°. If a portion of this citrate is heated in a small glass tube, a volatile acid product is obtained; also, litmus paper placed at the upper part of the tube, and moistened before- 371
hand, quickly turns red. In placing a drop of pure hydrochloric acid on the stain we are discussing, the liquid yellows and the iron becomes shiny in the same instant; iron hydrochlorate is formed. Also, distilled water with which the stain is washed, after treatment with hydrochloric acid, furnishes a precipitate with potassium ferrocyanide and gall nut similar to that produced with a saline solution of iron. In immersing the stained portion of the blade in distilled water, the iron citrate quickly dissolves and colors the liquid yellow. This solution reddens litmus paper, gives a more or less dark violet precipitate with gall nut, a green or red one with alkali, according to whether the iron is in the state of dioxide or trioxide, and a blue one with potassium ferrocyanide. Sometimes, to attain this last hue, it is necessary to add a little chlorine.

Characteristics of Rust Stain (Iron trioxide subcarbonate)

The color of this stain is yellowish-red, ochre yellow, or red. Exposed to a temperature of 25° to 30°, the blade thus rusted does not scale, as happened with stains of blood and lemon. Heated in a glass tube, rust gives off ammonia, as was demonstrated by Vauquelin and Chevallier; also, reddened litmus paper, placed on the upper part of the tube in which the experiment was being done, turned blue. A drop of pure hydrochloric acid placed on the rust turned yellow in an instant; the stain unrusted, and in diluting the acid with distilled water, a yellowish solution behaving like iron salts toward reagents is obtained. Put in distilled water, the rust is not dissolved at all; however, it detaches and remains suspended in part in the water, in part at the bottom of the container; the solution yellows because of the rust in suspension, but it suffices to filter it to decolorize it, which never hap- 372
pens with an iron blade stained with blood or iron citrate. This filtered solution, holding no iron in solution, when examined a few hours after the beginning of the experiment, does not become clouded by alkali, gall nut, or potassium ferrocyanide.

Fabric Stained by Blood

If the layer of blood is of a certain thickness, and the stain is formed by all the materials of blood with the exception of water, a portion of the fabric stained in red brown is cut out and immersed in distilled water. The coloring substance of blood detaches soon after, traverses the liquid from top to bottom in the form of red striations, and gathers at the bottom of the container, whereas the supernatant water is scarcely colored. At the end of a few hours, when the coloring sustance is dissolved, at least the greater parts of it, in place of the stain will be found fibrin of blood, in the form of soft matter of a greyish or a rose white which is easily removed by a finger nail. The more this layer was whitened by the water and the browner the fabric on which the blood was applied, the more apparent will the layer be at first glance. In the case where it is of a hue too dark to be recognized, the cloth is immersed once again in pure distilled water for a few hours, to remove another portion of coloring matter. The solution, at the bottom of which is found gathered this material, is shaken in a glass tube, and shows a reddish color, behaving with heat, acids, chlorine and other
373 reagents as we have already described in our presentation of an iron blade stained with blood.

If the stain, instead of presenting a notable thickness, is the result of simple absorption by the material, as happens when parts of the linen surrounding the portions on which the blood had been applied, are examined, or if it comes from other blood stains which were scrubbed or washed after drying, it will be impossible to confirm the presence of fibrin, for this does not exist in stains resulting from absorption and would have been detached in the case of rubbing or washing. One is limited then to separating the coloring matter by distilled water. The solution is subjected to the same procedures as in the preceding case, and if it has the characteristics already presented, the stain can be affirmed as being formed by the coloring matter of blood, provided that none of the substances with the property of coloring the water red or rose (cochineal, Brazil wood, cartham, madder-wort) furnish a liquid behaving with heat, and all the aforementioned reagents, like aqueous solution of blood.

The preceding experiments were done in turn with human blood and with the blood of beef, sheep, dog and pigeon.

It will not be useless, in ending this work, to note successively the manner in which the major red coloring substances behave with the reagents which we said must be used to identify the coloring principle of blood.

Cochineal. A solution of dilute cochineal is the color of red currant. Ammonia changes it to violet without clouding it. Solution of gall nut doesn't give a precipitate. Sulfuric acid and nitric acid, far from giving a precipitate, render it more 374
transparent and give it a scarlet color. Potassium ferrocyanide does not cloud it, but darkens the color a bit. Chlorine completely discolors it without turning it green or giving a precipitate. If the solution of cochineal were concentrated, chlorine would yellow it and after a certain time produce an abundant flocculent, yellowish deposit.

Brazilwood. Diluted with water, the solution of Brazilwood is an orange red. Ammonia renders it violet without clouding it; gall nut gives no precipitate. Sulfuric and nitric acids change it to a fallow yellow, without making it lose its transparency. Potassium ferrocyanide darkens the color a bit. Chlorine does not cloud it, and changes it to yellow without a change to green.

Red substance of madder-wort dissolved in alcohol. When diluted with water, its color is analogous to that of the coloring substance of blood. Ammonia deepens the color. Solution of gall nut does not trouble it. Sulfuric and nitric acids yellow it and render it cloudy. Chlorine yellows it first, then changes it to green, and finishes by discoloring it without the solution even becoming opaline. It is seen by these experiments that if this substance resembles the coloring principle of blood in some aspects, it nevertheless differs enough so that they cannot be confused.

Red substance of cartham. It is yellowish when diluted in water. Ammonia deepens the color. Solution of gall nut gives a yellow precipitate. Sulfuric and nitric acid cloud it without changing its color. Chlorine discolors it right away without rendering it opaline.

Blood Considered from the Medico-Legal Viewpoint*

M. J. B. Orfila

413 It is noted in the *Journal de Chimie Médicale* of last August "that on the occasion of a memoire of Orfila, Monsieur Dulong observed that one of the most marked characteristics of blood stains, even if very old, is the form of blood cells seen by the microscope; it permits, in addition, differentiation of blood of different classes of animals: dried mammalian blood cells look like a white disc surrounded by a red circle, whereas in the blood of birds the white disc is surrounded by an elliptic globule. This mode of examination is that much more valuable since it requires minimum quantities for its use and it doesn't deprive the substance of any material used in the application of analytical procedures". [Compte rendu de la seance de la Societe philomatique du 14 July[1]].

Such a positive assertion, uttered by a scientist of this caliber, must leave no doubt as to the possibility of recognizing in every case, and with facility, not only if a stain is formed by blood, but again, in certain circumstances, to
414 which class of animals the blood producing it belongs. We don't think that the problem is as easy to resolve by microscope, based on the facts we have observed with the greatest care, and which have been verified by Lebaillif, whose ability and experience no one will contest in any area of microscopical investigation.

1. **Dried human blood on a glass slide.** This blood came from a fingerprint; it had been diluted with about an equal weight of water and placed on the glass slide, where it dried out eight years ago. A very large number of perfectly *spherical* cells, transparent in the center, is seen; many of them were grouped together, forming an aggregate, which didn't hinder distinguishing them clearly. In examining a drop of the same blood, but thicker, with the same microscope it was impossible to perceive *any distinct blood cells.*

2. **Human blood dried on cloth.** Human blood was deposited on a piece of cloth as it poured from the vein. Four months later, a strip of this cloth, stained by a considerable quantity of blood, was left in water for an hour, until the liquid was colored red. Two drops of this solution were placed on a glass slide, at a certain distance from each other, and were examined after complete dessication.

The first drop, thick and wide. Perfectly *spherical* blood cells, transparent in their center, can be seen at many points. In another area, there are, in addition, those whose form is not easy to determine; finally, elsewhere are perceived those which are round and others which are *elliptical.*

The second drop, small, not very wide. It is impossible to find *any blood cells* in the center of this drop; irregular 415
bodies from the cloth on which blood deposited can be seen. In another part near the center two transparent, rather voluminous spherical blood cells can be seen, along with many others equally transparent and much smaller, one form *less regular,* difficult to determine, and others which are somewhat elongated.

3. **Human blood dried on cloth, diluted in water, and viewed in the microscope before dessication.** A portion of the blood which had been used in the preceding experiment was placed on a glass slide after dilution in water and examined while still liquid. A very large number of small, *transparent, ovoid and spherical* corpuscles were seen. In another part, it was difficult to recognize *perfectly* spherical corpuscles. A portion of the same drop which had been deposited on the glass slide, such that there was but a slight layer of blood, showed transparent, *elliptical* corpuscles; others spherical, but smaller in number; others were of an irregular form, and several were elongated, opaque bodies, undoubtedly coming from wool, dust and other refuse soiling the fabric.

4. **Pigeon blood dried for six days on cotton linen.** A portion of this linen containing all the substances of blood was left in a small amount of water until it was sufficiently colored. Three drops of this solution were desposited on a glass slide and were examined after complete dessication.

First drop, rather thick. In the center are some small, transparent, *irregular* bodies; between the center and periphery there are *elliptical, square, spherical and triangular* bodies and, in addition, there are *opaque* particles of black, which are square, triangular, etc; at the periphery, the mass 416
is cracked from the dessication and colored in a more intense red and no transparent *blood cells* can be seen.

Second drop, much less thick. Only some *opaque,* elliptical, square and spherical corpuscles, which are not red blood cells, are perceived in the center. At the periphery can be seen a perfectly *spherical,* transparent blood cell, another elliptical, and several *opaque* corpuscles of different forms.

Third drop, very thick. The center presents several *transparent* corpuscles, of *irregular* form, and others *triangular, trapezoidal,* etc; there are also opaque particles of varied forms. In a point near the central part there is an agglomeration of *transparent* corpuscles, whose form is not easily identifiable. Finally, there are neither blood cells nor corpuscles near the periphery, where the mass is cracked and deeply colored red.

5. **Pigeon blood dried on cloth, diluted in water, and seen under the microscope before dessication.** A portion of the blood which had been used in the preceding experiment was deposited on a glass slide after being diluted with water and examined while still liquid. A piece of elongated, opaque cotton is seen to which a multitude of transparent corpuscles of different forms seem to adhere. At another point in the drop, blood cells are seen in rather a great number, *elliptical* for the most part and isolated; others which appear to have the same form are agglomerated. Elsewhere can be distinguished two *perfectly spherical,* transparent blood cells, *comparable in every way to those of human blood,* and beside them are three more which are elliptical.

6. **Pigeon blood dried on cotton cloth for six days and**
417 **containing only the coloring matter.** A portion of this cloth, adjacent to the area where the blood had been deposited, and containing no solid parts, was treated with water, as previously. When the solution was sufficiently colored, it was placed on a glass slide and examined after dessication.

First drop. In its center are seen a considerable quantity of *dendrites* and a few *rounded* and fringed corpuscles. The periphery presents no foreign bodies and a yellowish-red hue. *Second drop.* With regard to the periphery, there is absolutely no difference between the two drops; as for the center many *dendritic* crystals and no *rounded corpuscles* at all. In the *third drop* numerous ramifications analogous to the preceding and some fringed, irregular corpuscles, apparently elements of crystallization, are seen; along with these are other corpuscles, more rounded, but so tenuous that the form can't be exactly determined; and other corpuscles, of a spheroidal form, very transparent at their center, which have a smaller diameter than human blood cells, of which we had first been speaking, *but of just about the same form.*

It results from the preceding, and from other facts which we will pass over without reference, 1) that, granting that the blood contains a multitude of blood cells serving to characterize it, it is sometimes impossible to determine the presence of these blood cells in blood dried on a glass slide, and even more so on fabric, either because the drop of blood is too thick, or because it contains only the coloring matter (cf. section 6 above), or any other reason; 2) that though it is true, in general, that mammalian blood cells are circular, while those of birds and cold-blooded* animals are elliptical, it is no less certain that in a matter of blood *detached from* 418
cloth, elliptical blood cells can be perceived in mammalian blood, and spherical blood cells, as well as triangular, square, etc. corpuscles in the blood of *birds,* probably resulting from an atom of dust or of the material of the fabric which unites with the blood. It is easy to imagine that a blood cell which had been spherical when seen alone, presents another form when coupled to a foreign corpuscle.

Let us add that it appears, from numerous observations done by Hewson, that in animals in which are found the very representative elliptical corpuscles after a certain time of life, only circular blood cells are found when they are very young (*Hewsoni opera omnia, Tabula prima, Lugduni Batavorium,* an 1785). And isn't it well known, moreover, how difficult it is to do good microscopical observations when not accustomed to them? These diverse considerations lead us to attach less importance to these observations than was believed necessary for resolution of the problems engaging our attention, and to prefer the chemical characteristics we discussed in our memoir on blood.

To justify this conclusion, we feel it necessary to point out that after examination of human blood and pigeon blood detached from fabrics, during several sessions and with several repetitions with excellent microscopes, not only was it difficult to distinguish one from the other, but even sometimes to recognize that it was blood. Let us now consider the quandary in which a physician, who hasn't devoted himself to microscopical research, would find himself. It might be said that we started off wrong, that we haven't fulfilled all the conditions. Very well! But we then request, in our turn, that these diverse conditions, and especially the numerous sources of error which can be committed, be indicated.

* Translation of "Sur le sang, considéré sous le point de vue médico-légal", in *Journal de chimie médicale de pharmacie et de toxicologie* 3 (9). 413–419 (1827).

[1] In this same meeting Adophe Brongniart noted that blood of beef had been differentiated from human blood by microscope by Monsieur Dumas in a medico-legal case . . Brongniart probably confused this fact with another, since Dumas said he knew nothing about it.

* poikilothermic.

New Memoir on Blood Considered in a Medico-legal Context*†

M. J. B. Orfila

105 At the meeting of the 15 of this month, the section of medicine heard the reading of a memoir of Raspail, having as its objective to prove that neither the microscope nor chemical experiments can identify blood stains. This memoir was addressed to the Royal Academy of Medicine by the author, as he claims in the covering letter, only because six months before I spoke to this association on the same subject and concluded, on the contrary, that it was possible to confirm the presence of these stains. Raspail, aware of the importance of the question he was treating, thought it not appropriate to leave any longer in error the numerous French and foreign physicians and pharmacists, who could have accepted my work as a guide. Nor did he economize any means for attaining the proposed goal: not only did he create out of whole cloth blood, which according to him shared all the characteristics I had allotted to human blood, but he also gave the prospect of the possibility of discovering someday at least twenty substances enjoying the same properties. He went further; he claimed that it was only *up to a certain point*, and not in an absolute way, as I have said, that human blood can be distinguished from iron citrate and iron trioxide, madder-wort, Brazil wood, cocchineal, earthame.

106 To listen to Raspail, I would have to esteem myself happy if science conserved a slight memory of my work. The section easily felt that it is scarcely permitted me to keep silent in those circumstances; and more so since I am certain that, since the publication of my memoir, it has been concluded more than once before the courts of the kingdom that certain stains have been formed by blood because they presented the characteristics I had indicated. I will approach this question frankly.

The memoir of Raspail is composed of two distinct parts, one with chemical experiments as their objective, and the other, observations by microscope. I will say first, relative to the latter, that I won't bother with it, since it is in no way contradictory to what I had advanced. I will only recall to Raspail, and to the section, that I read on last August 21, in this context, a note in which I *already* established the insufficiency of the microscope in identification of bloodstains on fabric. (See the *Journal de Chimie Médicale*, issue of September, 1827).

Raspail's chemical experiments tend to establish: 1) the existence of a red substance into which stains similar to those of blood can be made; 2) that no one can be assured that, one day, twenty substances capable of putting in error the reagents which I recommended for the identification of blood won't be discovered. Let's examine each of these points.

A. *There exists a red substance with which stains similar to those of blood can be made.* This substance is none other than eggwhite of hen left for a few hours in a canvas sack filled with powdered madder-wort, slightly moistened with water; the mixture is then exposed to a temperature of 25 to 107
30 degrees centigrade to dry it, and to give it the appearance of a red stain.

I don't doubt that Raspail borrowed from my first memoir on blood the idea for preparing such a substance: "When this substance is diluted with water its color is *analogous* to that of the *coloring substance of blood.* Ammonia deepens this color; a solution of gall-nut doesn't cloud the solution; sulfuric or nitric acid yellow it and render it a bit cloudy; chlorine yellows it first, then turns it green, and finishes by discoloring it, without it becoming even opaline. It is seen by these experiments that this substance resembles, *in some of its aspects,* the coloring principle of blood; it differs enough, however, so as not to be confused with it" (*Journal de Chimie Médicale,* issue of August, 1827).

But it is of little consequence to science that a discovery was realized here or further on; what is of concern is to find out whether the enunciation of a new fact is true or false. In this case, there is no doubt. Raspail is wrong.

Let someone repeat the experiment, as he recommends in his memoir, with a whole egg white and some madder-wort, without the addition of water, or after dilution of the egg-white in three or four times its volume of water; it is left in contact with the madder-wort for a few hours. A substance is obtained which, dried at 25° or 30° centigrade, presents a red color, whose properties I am going to compare to dried blood.

Physical properties. To distinguish these two substances, one can, in the extreme, benefit from the differences in color and transparency existing between blood and the completely 108
dried mixture of albumin and madder-wort. In effect, the hue of this last mixture will never be the same as that of blood, and it can happen, when too little madder-wort is used, that it is so different it becomes useless to turn to other characteristics. But I willingly acknowledge that these physical properties are insufficient for establishing this distinction when the artificial mixture is strongly colored; this will not hold for chemical characteristics.

* Translation of "Nouveau Memoire Sur le Sang, considéré sous la rapport médico-légal"
in *Journal de chimie médicale de pharmacie et de toxicologie* 4 (3): 105–117 (1828).

† Read at the Royal Academy of Medicine January 29, 1828.

Chemical properties. In comparatively treating these two materials with cold distilled water, blood releases its coloring matter to the water and leaves fibrin in the form of more or less colored filaments, depending on how well they were washed; but *in no case* does this fibrin *dissolve* in the liquid. The mixture of dried eggwhite and madder-wort, on the contrary, treated in the same manner, releases both coloring matter and albumin to the water, considering that, after dessication at 25 or 30 degrees centigrade, this is very soluble in this cold liquid, a fact which certainly must have escaped Raspail. I will say, however, that if the egg white has not been diluted with water and was filtered before drying, cold water does not completely dissolve the red stain and that there remain some light filaments, which are only released corpuscles seen swimming in the liquid when egg white is shaken in three or four parts water. But it is impossible to be mistaken; the amount of undissolved substance is scarcely discernibie, unlike a bloodstain treated with water. Moreover, it is easy to recognize the filaments of egg white.

The aqueous solution coming from the action of water on
109 the stains of the two substances presents the following differences:

1) It is orange-red when colored by madder-wort, whereas in the other case, it is brown-red.

2) Heated in a glass tube just to boiling, it coagulates or only becomes opaline, depending on the content of albumin; but if it comes from a mixture of madder-wort and egg white, it furnishes a rose-yellow or red liquid and a roseate coagulum, a part of whose color can be removed by washing with water. Whereas, blood gives a liquid and a coagulum of a greenish-grey without the lightest trace of a red hue. This greenish-grey coagulum can be rapidly dissolved with potassium and the liquid then acquires a red-brown color when seen by refraction. This important difference is known by every chemist and even by the worker dyers using madder-wort; and I was astonished to see Raspail was unfamiliar with it. There's more; if the mixture of madder-wort and egg white were to lose its red color by boiling, as does blood, the operation bearing the name "madder-worting" would not exist. Indeed, in dye workshops, when dyeing cotton red, isn't 400 pints of water boiled with fifty pounds of madder-wort and just about as much beef blood[1]? If, in coagulating, albumin of blood caused madder-wort to lose its red color, there would be no possibility of tinting in this color. Besides,
110 I can assert that, having boiled a mixture of beef blood and decoction of madder-wort, the solution conserves its red color, instead of the greenish-gray color which coagulated blood presents.

3) Nitric and sulfuric acids coagulate solution coming from blood; the clot is rose-grey and the supernatant, when left to deposit, is uncolored and a bit cloudy. The liquid mixture of albumin and madder-wort, treated by these acids, also coagulates, but the clot is straw-yellow and the supernatant is yellowish.

4) Solution of gall nut, made in the cold, coagulates the blood in rose-grey, whereas the alleged blood gives a yellowish-white precipitate.

5) Solutions of alum and stannic chloride only dilute the color of blood, *without changing it.* On the contrary, the mixture of albumin and madder-wort is yellowed by these solutions.

6) Concentrated alcohol gives rise to a *meat-red* coagulum at the end of a few hours, unless the solution of blood is too dilute. The filtered liquid is completely uncolored, whereas alcohol and the alleged blood give a *rose* coagulum, and a solution which, when filtered, is fallow bordering on rose.

7) Ammonia doesn't alter, or scarcely alters the color of blood, whereas that of a mixture of albumin and madder-wort changes appreciably toward violet.

8) Let us add that pure, concentrated hydrochloric acid does not yellow the bloodstain, but it browns the color more; the solid mixture of albumin and madder-wort, on the contrary, passes gradually to yellow by the action of hydro- 111
chloric acid, such that 20 to 25 minutes are sufficient for this hue to be very evident[2].

Will there be objection, by any chance, that the two solutions of which I've been speaking up to now present such clear cut differences only because they were not very dilute, and that the contrary would certainly be observed in examining very small stains? I would reply that in comparatively treating a bloodstain and a colored albumin stain with 16 grains of water, each stain weighing one-fifth of a gram, all the preceding indicated phenomena take place, and the characteristics are so clearcut that there is no doubt that a fifteenth of a grain of dried blood dissolved in 10 grains of water can be identified.

After all these facts, so positive, how can it be that Raspail claimed that a mixture of albumin and madder-wort cannot be distinguished from blood? How is it he was not aware that, in the interest of humanity, to use his expression, I would seek to give the greatest publicity to his so-called discovery and that, at this very time, facts incapable of sustaining the most superficial examination are perhaps being verified everywhere? Here, I believe, is the most plausible explanation of this inconceivable conduct of Raspail: he saw 112
that heat, sulfuric and nitric acids, and solution of gall nut coagulate a mixture of albumin and madder-wort, and because these reagents also coagulate blood, he concluded they were identical!!! A strange way to reason: it would be necessary to conclude that iron and mercury salts are also

[1] See the *Elements of the Art of Dyeing* by Berthollet, Vol. 2, page 158. 1791 edition; and *Elementary Course in Dyeing*, by Vitalis, page 324, 1827 edition.

[2] It is useless to recall that in medico-legal research relative to bloodstains, it must never be forgotten to treat the red solution with chloride and with potassium ferrocyanide, as I pointed out in my first Memoir. If I haven't discussed it here, it is because I wanted to mention only those reagents which can serve to distinguish blood from the substance prepared by Raspail and, in effect, chlorine and potassium ferrocyanide behave with this substance almost like they do with blood.

identical because both are precipitated by hydrosulfates, potassium, sodium, ammonia, potassium ferrocyanide, etc. Certainly not. And just as it suffices to examine the color of the different precipitates to distinguish iron salts from mercury salts, blood can likewise be distinguished from albumin, colored by madder-wort, for the four above-mentioned reagents give rise to coagula of different colors, and the supernatant liquids are also differently colored.

B. The first proposition advanced by Raspail having been completely refuted, I pass to the second, which is: *No one can be assured that one day twenty substances will be discovered capable of placing in error the reagents used by Orfila to identify blood.* "In organic chemistry, where almost all is in chaos, or almost all is mystery", said Raspail, "who would dare to assure me that twenty substances won't be encountered capable of placing my reagents in error by the versatility of their characters and the delicacy of their combinations?" (the Memoir cited). It is easy to see how difficult it is, when led into in the *realm of possibilities,* to assert nothing beforehand. I strongly doubt, however, that any such results will occur. And I sincerely urge Raspail, in the interest of legal medicine, to seek the compositions he foretells. When he has found them, I will agree with him: *Blood cannot be identified by chemical means.* In waiting, I *affirm,*
113 the contrary.

I would be at fault if I didn't take this opportunity to point out the importance of the last question raised by Raspail. Legal medicine offers little else of such great interest.

[*Section dealing with poisons and toxicology not translated*].

I will end this memoir, already too long, with the following
115 conclusions:

1) In claiming that bloodstains on fabric cannot be identified by microscope, Raspail was in accord with the truth, as I had demonstrated before he did.

2) In denouncing chemical experiments as insufficient for identifying these same stains, and notably to distinguish them from stains produced by a mixture of albumin and
116 madder-wort, Raspail has committed one of the most grievous of errors.

3) In presenting this new proposition, that a substance cannot be confirmed as blood because several substances resembling it might later be discovered, Raspail establishes a medico-legal principle it would be dangerous to adopt and which is even rejected by a healthy logic.

I recall to the section that at the last meeting, I urgently requested it to name a committee charged with reporting on the memoir of Raspail; I also asked to be admitted before this committee to prove to it the inaccuracy of the results proposed by the author of the memoir. I again demand a favor which the Academy will undoubtedly be eager to accord me: that Raspail be invited to assist at the work of the commission and that the experiments be done with materials prepared by himself.

I cannot urge too much that those physicians and pharmacists, most often called before the courts to judge questions of legal medicine, *repeat the experiments* of Raspail and compare his would-be blood with real blood. They cannot fail to recognize with me not only that there exist the differences between the two substances I have just pointed out in this work, but also that it is easy to distinguish them in considering only those *characteristics I had already pointed out* in the memoir read to the Academy in July, 1827. I discussed the action of water and of hydrochloric acid on solid blood and that of heat, sulfuric and nitric acids, ammonia and aqueous solution of gall nut on aqueous solu- 117
tion of blood. All one need do is to look to be convinced that these varied reagents act otherwise on blood than on the mixture of albumin and madder-wort. Moreover, it doesn't appear that our famous Vauquelin is disposed toward adopting the new ideas of Raspail; for, having been called upon, together with Barruel, on February 4 of this year, to determine if stains on a hat, smock, pants and shoe were produced by blood or not, he *replied in the affirmative,* as can be assured in reading the report he addressed to Sir Vanin de Courville, the examining magistrate. Vauquelin was acquainted with the experiments Raspail had read to the Society *one month before.* Even more remarkable, is precisely the fact that the conclusion of Vauquelin and Barruel was affirmative only because the material which they examined had the characteristics I had allotted to blood in my first memoir[3]. Will Raspail respond, by any chance, that the illustrious chemist which Europe has placed in the first order of analysts, and whom the courts have so often consulted, has not understood the question?!!!

[3] Vauquelin limited himself to confirming the physical properties of stains, to treat them with water, and to submitting the aqueous solution to heat, chlorine and gall nut. (See the report already cited).

Bloodstains. A Medico-legal Report*

M. J. B. Orfila, J.-P. Barruel and J. B. A. Chevallier

/349 We, the undersigned, . . . charged by the writ of Mr. Gaschon, examining magistrate of the court of first instance of the department of the Seine on the days of July 1st and 5th, 1834, as a consequence of the rogatory commission of June 22, 1834, executed by Mr. de Saisseval, examining magistrate of the Chateau-Thierry district in the affair conducted against men named Jean-Baptiste Boileau, Alexandre Boileau, Jean-Louis Boileau, and Victor Darez, all four accused of voluntary homicide committed in collusion, the 2nd of the said month of June, on the person of Mr. Hochet, a rural constable, with proceeding with the examination of garments and objects attached to this rogatory commission and with the operations necessary to respond to the following questions as much as possible:

First question. Is it possible to determine if the blood
/350 mixed with earth, seized in the woods of Mesnil, is human blood, if it is from the same man as the blood found on the clothing of Hochet, on the clothing of Jean-Baptiste Boileau and on that of Victor Darez?

Second question. Is it possible to determine if the traces noted on the clothing of Jean-Baptiste Boileau, are traces of blood; if this blood, in the case of an affirmative finding, is blood of man or the blood of hare; if it is from the same man as that found on the clothing of Hochet and that mixed with earth from the woods of Mesnil; and, finally, if these blood stains have been on the clothing of Jean Baptiste Boileau for about three weeks or four months or even more?

Third question. Is it possible to determine if stains noted on the clothing of Darez are bloodstains and if this blood, in the case of the affirmative finding, is the blood of man or the blood of sheep; if it is from the same man as that found on the clothing of Hochet and as that found mixed with the earth of Mesnil and, finally, if these blood stains have been on the clothing of Victor Darez for three weeks or for five weeks?

Fourth question. Is it possible to determine if traces noted on a piece of blue cloth, found near the place where the earth of the woods of Mesnil was taken are blood stains; and if in the affirmative, if this blood is from the same man as that existing on the clothing of Hochet, on that of Jean-Baptiste Boileau, on that of Victor Darez and in the earth from the woods of Mesnil?

/351 To conform to the requirements of the writ, the experts met at the chemical laboratory of the Faculty of Medicine, Monday the 7th of July at nine o'clock in the morning to be sworn by Lafontaine, police commissioner attached to the office of judicial deleg tions, and to faithfully fulfill the mission confided to them.

The oath taken, the objects designated by the rogatory commission of Mr. de Saisseval were turned over to them, these consisting of:

1) a sandstone pitcher containing earth, leaves, pebbles and moss taken from the woods of Mesnil;
2) the bloodied garments of Hochet;
3) a piece of blue cloth;
4) a sorry-looking smock, blue on both sides;
5) trousers of blue cloth, patched and torn in several places;
6) a pair of old, large clogs;
7) a shirt of coarse white cloth;
8) a smock of old blue cloth;
9) another smock of newer blue cloth.

The experts having determined that all these objects, contained in large white wooden box bearing the address of the Crown's prosecutor, were furnished with appropriate tickets attached to the objects, took custody of them and adjourned until the following day, July 8, to proceed with the necessary operations and to reply to the questions posed by the rogatory commission delivered by Mr. de Saisseval the 22nd of June, 1834. On the said day of July 8, 1834, the experts met 352
once again in the laboratory of the medical school, where they proceeded in the following manner:

Examination of the clothing of constable Hochet. This clothing was in a packet formed by a piece of home-spun linen which was sewn in such a way that nothing might escape. This packet was closed by a cord whose ends were furnished with the seal of the examining magistrate of the court of Chateau-Thierry. To this packet was attached a tag on which were found the words: *clothing found on constable Hochet.*

The integrity of the official seals having been confirmed, the packet was opened and the clothing extracted. The packet contained: 1) a waistcoat of goathair in stripes and small colored points; this waistcoat which at first had a *water-green* color, had become *yellow* through use. This garment was saturated in blood in almost all its parts, particularly on the back, the neck and toward the pockets.

In one of the pockets of this waistcoat was a knife whose blade was stained by some white matter. Examination of the white matter staining this blade identified it as coming from crumbs of soft bread; indeed, a part of this white matter, separated from the blade burned with the smell of *roasted bread* when placed on live coals. Its volume increased on

* Translation of: "Taches de Sang. Rapport Médico-légal". in *Annales d'Hygiène Publique et de Médecine Légale* 14: 349-370 (1835).

contact with water. Finally, it took on a violet color on treatment with tincture of iodine.

353 On some portions of the waistcoat a flaky white matter was noted. By the manner in which it behaved on being placed on live coals, this matter is comparable to the residue of potato pulp from which the starch was extracted.

2) Grey pants, where three openings, apparently made by a very sharp, cutting instrument, were noted on the waistline on the back to the right of the seam. These pants were saturated with blood on the waistline and near the openings and the surrounding parts. Selvaged suspenders also saturated with blood were attached to the pants.

The same substance, analogous to extracted potato pulp, was noted on the pants by the experts. The presence of this matter has to be explained. It appears that this white matter existed in the place where constable Hochet succumbed or that it can be found in the region where he was carried after his death.

3) A shirt of thick cloth soiled by a large amount of discharged blood; this blood was most appreciable on the back.

On the back part of the shirt, toward the area corresponding to the right kidney, four openings were noted, which were made with a sharp, cutting instrument. The place occupied by these openings is a certain indication they were produced by the same instrument which had pierced the waistline of
354 the trousers. The dimensions of the gashes demonstrates that the instrument was a thin blade.

4) A pocket handkerchief in one of the corners in which is a knot containing two coins; one of silver, a *franc*, the other of alloy, a *sou* with the portrait of Louis XVI.

5) A constable's badge bearing the words: *The law, department of Aisne, Pierre Hochet, rural constable of Lacroix, 1833.* This badge was *attached by an armband of linen.*

6) An old cap of blue cloth with a copper visor. The copper is green beneath.

7) Selvaged suspenders, the half of which are stained with blood. The same flaky white matter previously mentioned is noted on the suspenders.

The presence of blood on the clothing of constable Hochet was sufficiently evident to us. Nevertheless, we considered it necessary to test a portion of material removed from the shirt. This material removed, it was separated into small fragments which were then reunited with pins, then placed in distilled water; after a few moments numerous striations were visible. The water colored in its lower part and gave rise to a brownish-red liquid, comparable in color to old, liquid blood.

After a suitably prolonged maceration, the liquid was separated from the fragments of material and divided into two parts, one of which was again divided and submitted to the following experiments:

355 1) A quantity of this liquid was introduced into a glass tube, closed at one of its ends, and subjected to heating. The liquid soon became cloudy and presented a coagulum of greenish grey. Treatment with potassium dissolved the coagulum and the liquid resulting from the dissolution was greenish-brown as seen by reflection and brownish-red by refraction, particular characteristics indicating that the solution, treated with heat, contained blood.

2) Another portion of this liquid treated with gall nut gave a coagulum of reddish-grey.

3) Another portion of the bloodied water treated with chlorine took on a green color, which disappeared with an excess of chlorine.

4) A portion of the liquid treated with a large excess of alcohol promptly deposited a lumpy precipitate of a splendid roseate-red color. These experiments demonstrated in the most evident way that it was actually dried blood staining this shirt.

A large quantity of this bloodied water, loaded with blood principles, was treated with pure sulfuric acid (of 66°), then stirred with a glass rod. The mixture was hardly completed when a strong odor of human sweat was emitted, an odor difficult to confuse with others.

Examination of the material contained in a sandstone pitcher. The sandstone pitcher, about a pint and a half in 356 volume, was removed from the case containing it. It was closed with a paper acting as a label on which were the words: *sandstone pot containing bloodied earth, moss, leaves and pebbles found in the woods of Mesnil.*

The pot opened, its contents were identified:

1) moss, a small amount of which was stained by a dry, brownish-red substance, which appeared like dried blood.

2) Earth, of which a few small portions were colored by a blackish brown matter, a color attributed to dried blood.

3) Pebbles, two in number, evidently bearing traces of dried blood.

4) Leaves of trees of which a few were soiled by a brown-red substance, comparable to dried blood.

All the substances contained in the pitcher had an extremely strong *musty* odor, or better, of rotten wood, an odor due to the fact that all the substances were moist when placed in the pitcher.

The portions of the different substances, moss, earth, leaves, pebbles, which were stained and had acquired a brown color, were separated from those which had not, and 357 were put aside to undergo the following operations:

The moss was converted into a small bundle and placed in distilled water and left to macerate for a suitable length of time. The water situated in the lower part of the experimental glass acquired a reddish tint. When the maceration was sufficiently prolonged, the moss was removed and the red solution was divided into two parts. One was introduced into a glass tube closed at one of its ends. This solution, which had the same musty odor as the moss, and just as intense, presented the following phenomena when subjected to heating. The liquid changed its color, became cloudy and gave a rather considerable coagulum of a rose-grey color. Treatment of this coagulum with potassium dissolved it, giving a greenish-brown color to the solution seen by

reflection and a red-brown color by refraction. All these characteristics demonstrated that the examined liquid contained blood.

The other portion of the reddish liquid was treated with pure sulfuric acid (of 66°). A peculiar odor developed, but this odor was masked by the odor of rotten wood, which prevented the experts from recognizing the primary odor.

The leaves were then treated with distilled water to which they imparted a red color. The solution resulting from this 358 maceration was examined; it was determined as containing blood. But the small quantity of this liquid, and the odor of rotten wood exhumed by the leaves, as well as the odor particular to the leaves, prevented the experts from experimenting further with the purpose of identifying the odor released by the reaction of sulfuric acid on this solution.

The pebbles were then washed with distilled water which received the coloring substance soiling them. The experiments performed on the colored water with heat demonstrated that this water prevented the experts from treating it with sulfuric acid for the purpose of developing the volatile principle of blood.

Finally, the portions of colored earth were treated with distilled water which colored it red. After division of the solution into two parts, one was suitably treated with heat. It presented all the characteristics indicating the presence of blood, i.e., there was coagulation, formation of a coagulum which redissolved with potassium, giving place to a greenish-brown liquid seen by reflection and reddish-green by refraction.

The other part was treated with sulfuric acid; but it presented a volatile matter having the musty smell or odor of moss.

Examination of a piece of blue cloth. This piece of cloth bore a tag on which could be read: *piece of cloth serving as* 359 *material evidence in the Hochet affair.* Examination of this material demonstrated it was about six feet in length. It appeared to come from an old slip, so worn out that at first glance it appeared covered in flour, which it wasn't, as our experiments demonstrated.

The remains of this garment were patched up several times with patches of different qualities and colors.

Thirty stains, apparently due to blood, were noted on this piece of cloth which presented *these spots strewn on a blue background.* A few of these stains were very large, about two thumbs square. Various remains of dry vegetable matter were attached to this piece of cloth, which were identified as strands of *straw, hay* and *stems of mustard-seed.*

The greater part of these stains, found on the remains of this garment, were removed and placed in distilled water which colored in red-brown. A part of the bloodied water thus obtained was tested with heat and various reagents and presented all the characteristics of liquid blood.

Another part of this water, treated with sulfuric acid, developed a volatile principle which was identified by one of the experts as having the odor of *woman's menstrual discharge,* and by the others as having the odor of human excrement.

Examination of the clogs of Jean-Baptiste Boileau. Exam- 360 ination of the clogs demonstrates they were worn for quite a while. Their interior was filled with earth which had accumulated under the form of mammilate plaques. This earth presented no coloration attributable to blood.

Examination of the exterior identified a stain of blackish color on the inside angle of the heel of the right foot. This stain, which had the form of a diamond, was about one thumb square. Beside this stain were different materials attached to the clog, among which were distinguished *straw, earth, sand,* etc. On the clog of the left foot, various points colored in violet-red were distinguished on the outside in front. Finally, stains made from grass were visible on the bottom of the clogs but none of these stains could be attributed to blood.

The black stain in the form of a diamond found on the heel of the clog of the right foot was removed by scraping with a penknife. The parts scraped off were placed in distilled water; after a rather prolonged stay, the water, which had dissolved the soluble elements, was examined. As a result of this examination it was determined that the stain on the clog was not due to blood. Indeed, this stain, of a yellowish color, emitted a foul odor of excremental matter. Exposed to heat, 361 it didn't cloud nor furnish a coagulum. The odor emanated indicated that this stain was actually due to fecal matter.

The other material, *straw, earth and sand,* which had been found near the stain, and which formed a slight elevation, were removed and examined. By similar means, it was found that, as in the case of the previously examined stain, these materials had been fixed to the clog by the fecal matter.

The stains of a violet red, found on the front part of the clog of the left foot, were removed along with a part of the wood of the clog and placed in distilled water for maceration and left in this liquid.

The resulting solution did not acquire a reddish color. Tested by heat, it furnished no characteristics indicating presence of blood.

Examination of the trousers of Jean-Baptiste Boileau. Scrupulous examination of all parts of this garment revealed to the experts:

1) In the pocket on the right side of the waistline a stain apparently due to blood and whose position indicated it as having been made from the back of a hand.

2) On the lower part of the right leg, in front, three stains apparently made by droplets of blood projected onto the pants. These droplets did not pass through the material. 362

3) On the same lower front part of the right leg, three other stains, apparently due to blood. These stains had a tint different from the first and this is an almost certain indication that they are of an earlier origin than those previously described.

4) A bit below the knee of the left leg a stain, apparently due to dried blood. This stain had all the characteristics of a stain much older than some of those of the right leg.

5) On the lining on the right inside a blood stain could be noted. This stain, like that observed on the pocket, seems to indicate that Jean-Baptiste Boileau was wounded on the back of the head and that he carried the wounded hand in his pants and in his pocket.

6) On the back of the pants, different stains, but which were not due to blood.

About half of the fabric bearing the stain found in the pocket was removed and placed in distilled water. After remaining in this liquid for a certain amount of time, it imparted a roseate tint to it, as a small amount of blood would do. The water, impregnated with soluble elements of the stain, changed color when heated in a glass tube, became cloudy and presented a coagulum which redissolved with potassium with the characteristic phenomena indicating the
363 presence of blood.

One of the three stains, which appeared less old than the others, was also removed and treated in the same manner. It presented a roseate liquid which gave the characteristics indicating the stains were due to blood.

Examination of the smock of Jean-Baptiste Boileau. This smock, of thick blue cloth, was designated under the name of a smock with two sides. However, only the side which could be considered as being the front could be distinguished on this garment.

Examination of this smock revealed to the experts:

1) On one of the sides, designated by an "A" traced on the smock, thirty stains, presenting characteristics of decay. Physical examination of these stains did not permit us to estimate the nature of the substance which had produced them.

2) On the sleeve of the right side when facing the side "A", twenty stains apparently from the same moment as the preceding. No stains were noted on the left sleeve.

3) On the other side of the smock, three stains on the left
364 sleeve and four on the right sleeve. All these stains seemed to have the same origin.

A rather large number of stains were removed from the smock, along with the fabric, and placed in distilled water for more than four hours. The water had not acquired a reddish color at the end of this time, but a yellow color. Heated, this water didn't cloud or present a coagulum, which indicates these stains were not made from blood.

The portions of fabric bearing these stains were removed from the water and left to dry. After drying they were treated with boiling, 40° alcohol. This alcohol colored in green and the filtered alcohol solution became extremely cloudy with water.

After evaporation to the point of dryness of a portion of the alcohol solution by low heat, a material of resinous nature very comparable to glue remained. This matter, which formed the stains noted on the smock of Jean-Baptiste Boileau was green, adhered to fingers, causing them to stick together, had a bitter odor, and emitted aromatic smoke of a peculiar odor on burning. The experts believe these stains to have been produced by glue.

Examination of the (old) smock of Victor Darez. Examination of this smock revealed that it contained stains on various parts which we could not attribute to blood by their color. To be assured of their nature, however, they were 365
removed from the smock and placed in distilled water. These stains did not color the liquid in red, but communicated a yellowish color to it. Submitted to heat, the water did not cloud and furnished no coagulum. It presented none of the characteristics belonging to water impregnated with blood.

After drying the material bearing these stains, and treating it with boiling, 40° alcohol, it gave an alcoholic solution which left a certain amount of fatty matter when evaporated to dryness. It was this fatty matter which formed the stains noted on the smock by attracting dust.

Examination of the new smock of Victor Darez. Examination of this garment revealed it was two-sided. On one of them, marked "A" by the experts, a large number of stains were noted which, with the exception of one, which we surrounded with a square traced in ink, were not made by blood, but by fatty matter. As for the stain enclosed in the square, it appeared to the experts to be due to blood and was preserved, its presence being the only fact which could later prove that this smock was stained by blood.

On the other side of this smock were noted:

1) A large number of stains in various places which were produced by fat.

2) On the right arm, about forty stains which the experts believed due to blood. These stains appeared to have been 366
made by spurting blood; at least, their disposition seemed to imply this. They didn't appear to be very old, to judge by their color, and the shiny appearance which they had preserved.

A portion of the stains we suspected of being made from fat was removed with the fabric and placed in distilled water. But this liquid did not color in red, and the water didn't change color nor become cloudy after being subjected to the action of heat.

Dried and treated with boiling, 40° alcohol, the fabric bearing the stains presented a fatty matter which had caused the stains.

A portion of the stains on the sleeve of the new blouse of Victor Darez, stains which the experts considered as being made by blood, was removed along with the fabric and placed in distilled water, which colored in rose. The water thus colored became cloudy when subjected to heat in a glass tube closed at one of its ends. It then gave a coagulum which redissolved with potassium. At the same time, the solution presented a greenish color in reflection and a reddish color in refraction. These characteristics indicate that the water tested contained dissolved blood coming from the stains.

Examination of the shirt of Victor Darez. The examination of this shirt revealed:

1) On the bottom of the lower part of the front, small 367
blood stains.

2) On the upper, internal part of the right sleeve, toward the middle of the sleeve, four stains, of which two appear to

be of blood.

3) On the back part of the left sleeve, near the wrist and a bit above, five stains apparently due to blood.

Several blood stains were also noted in the inside of both tails of the shirt: 1) on the lower part of the back tail; 2) on the front tail corresponding to the pubis. The form and disposition of these stains seem to indicate that the wearer of the shirt had pursued the act of coitus with a woman during her menses. Tests performed on part of the stains found on the sleeves of the shirt by distilled water and heat demonstrated they were due to blood.

The stains found on the shirttail furnished, with distilled water and heat, results indicating that these stains were also due to blood. The experts did not make any attempts to determine the aroma of the blood for it was found in very small amount on the shirt and, besides, they emitted a very strong odor which would undoubtedly have masked that specific to the blood.

These procedures finished, the experts found it possible to reply to the questions posed only in the following manner:

For the first question:

368 It was impossible to determine if the blood mixed with earth taken from the woods of Mesnil is blood of man or from the same man as that found on the clothing of Hochet, on the clothing of Jean-Baptiste Boileau and on those of Victor Darez for the reasons: 1) the blood was in too little quantity; 2) the blood found on earth and moss taken from the woods of Mesnil had contracted a strong odor of decaying wood which had abolished the odor peculiar to blood no matter from what source it came.

For the second question:

It was possible to identify the blood stains on the trousers of Jean-Baptiste Boileau, but it is impossible to determine if this blood is of man or of hare or if it is from the same man as that found on the clothing of Hochet or that found on the earth taken from the woods of Mesnil. This impossibility is explained by the very small amount of blood found on the trousers of Jean-Baptiste Boileau.

As for the question of determining if the blood found on the trousers was there for three weeks or four months or more, the experts claim that the blood stains observed on these trousers were produced at two times, evidently different from each other.

For the third question:

It was possible for the experts to determine the presence of stains made by blood on the new smock of Victor Darez and on his shirt. But it was impossible for them, because of the small amount of blood, to say if this blood is of man or 369
of sheep, or if this blood is the same as that found on the clothing of Hochet and on the earth from the woods of Mesnil.

As for the question of determining if this blood existed for the past three or five weeks on the clothing of Victor Darez:

It is impossible for the experts to say if the blood stains observed on the clothing of Victor Darez had an existence of three or five weeks. They are convinced that those seen on the upper part of the right sleeve, on the side of the smock marked "B," are of the same date as those observed near the seam attaching the sleeve to the smock. Although the former are less visible, they attribute the difference between them to friction undergone by the former and from which the latter were protected by the stitching of the seam.

For the fourth question:

It was possible for the experts to determine whether the stains found on a piece of blue cloth found near the place where the earth was taken from the woods of Mesnil are due to blood. But the experiments done with sulfuric acid, with the purpose of comparing the volatile principle released from the water which had dissolved the blood found on the clothing of constable Hochet, lead them to believe that the blood which had stained the piece of blue cloth is not the same as that staining the clothing of the constable. Indeed, the volatile principle released from the water impregnated 370
with the blood of Hochet did not resemble that released from the water in which the piece of blue cloth was immersed and was not the same.

Memoir on a New Method for Recognizing Blood Stains*

M. J. B. Orfila

112 About six years ago, M. Persoz, professor of physics at the science faculty of Strasbourg, informed me that, in 1836, he resorted to *hypochlorous* acid for the recognition of blood stains on a smock where urine stains were also found. "This acid," he said, "*destroys* immediately every stain except those formed by rust or blood: the latter turn a blackish brown on contact with the acids. It is that much more important to make use of hypochlorous acid since it *often* happens that blood stains found on these fabrics lose the property of dissolving in water and consequently cannot be determined by this method."

A little while after this communication, I applied jointly with M. Cottereau, the method indicated by M. Persoz, in a medico-legal analysis, on the occasion of a confinement pending trial for murder. It was a matter of determining whether the shirt worn by the murder victim, and a smock and a scythe seized at the home of the accused, were stained by blood or not. After having treated these stained objects with distilled water, and having exhausted the series of char-
113 acteristics I had indicated in 1826 in my memoir on blood stains, we resorted to hypochlorous acid and we determined: 1) that this acid applied on a region of the blue smock where there was no type of stain discolored and whitened this region in an instant; 2) that the dotted stains of brown red, which existed on the smock, resisted the action of the acid and acquired a darker color; 3) that the stains of the scythe blade furnished nothing with distilled water, that they completely dissolved in hydrochloric acid, and were in no way modified by hypochlorous acid.

Prior to this assessment, I was consulted by MM. Magonty and Loust, pharmacists of Bordeaux, who were charged by the Public Prosecutor with determining the nature of certain stains found on the lining of a coat pocket. These gentlemen, having met with a few difficulties in resolving the problem by means known up to the present, asked me to indicate a procedure specific for ridding them of these obstacles. I immediately replied to them that they could use hypochlorous acid, and furnished them with all the information which could guide them in their research. One will see by the letter which I transcribe with what shrewdness these experts operated in this circumstance.

Letter of MM. Magonty and Loust

To Monsieur Orfila, Dean of the Faculty of Medicine of Paris.

Monsieur le doyen,

We would have already thanked you a long time ago for your kindness in replying to us, in showing us a method entirely new to us, a priceless method for determining the presence of blood on fabric, if we had not 114
had to repeat this experiment and commit ourselves to rather meticulous research which was the consequence. Today, now that our work is finished, we are eager to inform you of the cause of our long silence; and in asking you to excuse it, we must express our warm gratitude to you and confide to you the results which we have obtained.

To confirm the facts which you made known to us, not that we doubted their verity, but as an indispensible exercise, we did many practice procedures, operating on fabric stained by us. We assured ourselves that the hypochlorous acid, prepared with chlorine which one hadn't taken the precaution of washing beforehand, gave uncertain results. The stains, in effect, after a half hour of maceration, became very pale without disappearing entirely, however. But the same reagent, washed of hydrochloric acid, behaved as you told us; only, after a prolonged maceration of a few hours, the stain, which at first darkened and browned, became a bit more pale, but did not disappear.

We considered ourselves sufficiently informed, and we undertook to do the legal research which the examining magistrate demanded of us. We were not a little surprised to see the stain disappear in great part; however we noticed *brownish lines* which persisted in the manner of blood stains.

We ask you to recall the nature of the report which we had the honor to submit to you; we had to report on the nature of stains found mainly on the lining of a coat pocket; and we say that the physical characteristics of these stains caused us to believe *that they did not come from a spurt, but from contact with a stained object.*

We had to investigate if there were not a difference 115
between *direct* stains, i.e. those coming from spurt of blood and the immersion of fabric in this fluid, and stains we will call *secondary*, i.e., those produced by contact with a stained body.

There should be, in effect, a chemical difference between direct stains receiving all the elements of blood, and secondary stains produced by them where the beginning of coagulation must have fixed the elements retained by the *clot* on the first fabrics. To assure ourselves of the degree of credibility we should give to this idea, we wanted to perform comparative experiments with direct stains, secondary stains, and those acting as subject for our research. We consequently stained white linen with blood coming from a patient's vein, and a few seconds later we pressed the first linen on a portion of red fabric cut from another pocket of the accused's coat, fabric similar in every point with that of the incriminated pocket. We designated these linens by numbers 1, 2 & 3. We placed three approximately equal pieces of the linen in three glasses containing hypochlorous acid and we were able to determine: 1) that the direct stain (no. 1) behaved as you had informed us; 2) that the secondary stain and that of the pocket (nos. 2 and 3) partially disappeared, both in the same manner, i.e. the threads of the *woof*, more pronounced than those of the *warp*, and which must have absorbed more of the liquid on contact with the stained object, conserved a brownish imprint, whereas the threads of the warp were discolored.

We wanted to investigate further this resemblance of
116 numbers 2 and 3 provided by hypochlorous acid in the first method we had used. We then started the whole operation over in comparing secondary stains with the pocket stains; *we constantly obtained the same results* . . . and from this moment our doubts ceased.

Thus, sir, thanks to your kindness we have been able to use a reagent new to us, of which we have been able to confirm the consistency, and which should henceforth render great service.

It was also demonstrated that there exists a notable difference between direct stains of blood and secondary stains, a difference of which we feel experts in legal medicine should always be aware.

We will terminate this letter as we began it, in asking you, sir, to receive expression of our gratitude and to consider us your very respectful students.

M. Magonty and Loust.
Bordeaux, January 18, 1842.

I thought it necessary to examine the question carefully, to find out exactly if hypochlorous acid offered the advantage associated with it by M. Persoz; for this I attempted a great number of experiments.

Action of liquid hypochlorous acid on blood stains[1]

First Experiment. A white cloth stained by blood flowing
117 from a vein is immersed in liquid hypochlorous acid; at the end of *thirty seconds* the cloth is retrieved and it is seen that the stain is browner than before immersion; *seventeen hours* after being exposed to air it retains the same color.

Second Experiment. A white cloth stained with blood by applying it to a thick stain from a *spurt* is left in liquid hypochlorous acid during thirty seconds. On being removed from the liquid, the stain is a light brownish toward the middle, and almost uncolored at its circumference: exposed to air, this part continues to discolor; however, the points on this circumference where the stain was a bit thicker were light brown. At the end of *seventeen hours* the cloth is dry and presents a *greyish* tint there where the blood stain was first found.

Third Experiment. A white cloth stained like the preceding is immersed in liquid hypochlorous acid; at the end of *three minutes*, the points weakly colored by blood are discolored; after *ten minutes* of contact there remains on the cloth only three small greyish plaques. *Ten minutes* afterwards, two of these plaques are completely discolored; *forty minutes* afterwards the last of these plaques has disappeared.

Fourth Experiment. Linen is stained with poppy oil, then a *thin blood stain* is made on this linen. After *thirty seconds* of immersion of this linen in liquid hypochlorous acid, the stain is red brown, and shows no change on prolonged exposition to air. But if the linen remains immersed in the acid for an hour, the stain disappears entirely.

Fifth Experiment. White linen with blue stripes stained *two years* ago by a small amount of human blood is immersed in liquid hypochlorous acid. Removed at the end of *thirty seconds*, it is almost entirely discolored; whereas the stain still presents a hint of clear red, although it obviously 118
tends to disappear. After *twenty four hours* of exposition to air, all remains in the same state. The same results are obtained with white linen with blue stripes stained *six years* ago with a rather large amount of blood.

White linen covered *two years* ago by a *thin* blood stain is immersed in hypochlorous acid for *thirty seconds;* the stain blanches; after another immersion of a quarter of an hour, only a dirty grey tint is seen on the stained part.

A piece of black sheet presenting a rather thick stain of human blood, *two years* old, is in part discolored after *thirty seconds* of immersion in hypochlorous acid, whereas the blood stain is darker and almost black. After a *twenty-four hour* exposition to air, the sheet is less colored, and the stain conserves its black color.

Sixth Experiment. Pieces of black sheet, grey duck, red calico and white cloth, stained *two years* ago by human blood are left for *two minutes* in liquid hypochlorous acid; with the exception of the red calico, which presents only a thin blood stain, the other fabrics are stained by a rather thick layer of blood. On removal, all the fabrics are discolored, while the blood stains have become a blackish brown. After exposition to air for *twenty-four hours*, all remains the same.

Seventh Experiment. Some coagulated pigeon blood is diluted in a little water and part of the liquid is applied on white linen striped with blue. After a *few seconds* of immersion of the linen in liquid hypochlorous acid, the linen is discolored and the blood stain, red beforehand, has already

* Translation of: "Mémoire sur un nouveau Moyen de Reconnaitre les Taches de Sang."

in *Annales d'Hygiène Publique et de Médecine Légale* 34: 112 129 (1845).

[1] Hypochlorous acid was prepared by the method of M. Ballard, in shaking mercury dioxide diluted in water into *perfectly washed* gaseous chlorine; the liquid was filtered at the end of the reaction, and used in this state, as did M. Persoz.

119 acquired a fallow tint. Six hours after retrieving the cloth from the liquid, the stain was of a hint of fallow, so clear that it was almost entirely discolored.

No change had appeared the following day.

Eighth Experiment. A blood stain, recently made by letting venous blood flow on white linen, was immersed in liquid hypochlorous acid for six hours. At the end of six hours the stain was still a brownish black. After seventeen hours of immersion, the linen reduced to a pulp when pressed by the fingers and only a clear gray plaque was perceptible in the stained area.

Ninth Experiment. White linen, stained *two years* ago by human blood, is left for *six hours* in liquid hypochlorous acid. The stain passes to a deep fallow, then clear fallow, and this nuance is such that it perfectly resembles stains made by alkanet and a fatty matter after *six hours* of contact with hypochlorous acid. All were in the same state the following day.

A piece of black sheet, also stained *two years* ago by human blood, after having remained in the same acid for *six hours,* was retrieved completely discolored; the blood stain had also lost its color, however its discoloration had happened more slowly than that of the sheet.

Tenth Experiment. Pieces of black sheet, of gray duck, of red calico and of white cloth, stained by blood *two years* ago, are immersed in hypochlorous acid. At the end of *two hours,* these materials are completely discolored; but the blood stains are blackish and do not look as if they are disappearing. After sixteen hours of immersion, the red calico presents a very clear *cafe au lait* tint in the area of the stain; the duck is a brownish green with stained points, where whitish debris are seen, probably coming from the action of
120 hypochlorous acid on the blood; the white cloth preserves only two bulging black points of the stain, which are the size of the eye of a needle; finally, the sheet, now a greyish brown, covered in whitish debris, presents three rather thick stains, black in the center, whitish yellow at the circumference.

Eleventh Experiment. A blue-and-white striped material, stained *six years* ago by a rather large amount of human blood, left for *six hours* in liquid hypochlorous acid, is discolored on being removed, and the blood stain is of an excessively clear fallow color.

Twelfth Experiment. An *iron blade* presenting a *thin* blood stain, recently made, is immersed in hypochlorous acid. At the end of *thirty seconds,* there remains only a brown red tint at the stained spot; during the action of the acid, a rather large quantity of gaseous chlorine is released, and red iron oxide is formed. After *an hour* of immersion in the same acid, the blade is covered with a rather thick layer of iron sesquioxide and, if this is removed by a thin trickle of water, the brown-red tint of which I have spoken is perceived at the area formerly stained by blood. The blade is immersed in another portion of hypochlorous acid. After *five hours* of immersion, the iron is once again covered by a thick layer of oxide, but if this is detached by a thin trickle of water, or by lightly rubbing with wet tweezers, small stains of red brown are once again noted here and there: one would say that iron oxide and the portion of blood remaining on these points forms a mixture producing these stains.

Thirteenth Experiment. After *six hours* of contact with hypochlorous acid, an *excessively thin* blood stain, recently made on an iron blade, has completely disappeared, and the metal is thoroughly scoured.

Fourteenth Experiment. An iron blade presenting two *thick* recently made blood stains is left for thirty seconds in 121
hypochlorous acid; chlorine is given off and iron oxide produced; the stains are a reddish brown. At the end of *one hour* they conserve the same color, but detach in parts at a few points and the iron presents its normal sheen. The blade is then immersed in a new bath of hypochlorous acid. After *six hours* of contact, the stains are still brown in the center; their circumference, a dirty red, shows a kind of rim formed by iron sesquioxide. After *fourteen hours* of immersion, one of the stains is a greyish white and encrusted with iron oxide; the other is reddish brown, detaching in plaques; in leaving the blade in hypochlorous acid for *thirty-eight* hours, it is largely covered in iron sesquioxide and the liquid contains a large amount of iron sesquichloride; when this oxide is removed by a thin trickle of water, the stain which had remained is reddish-brown color, still presents the same tint, but is held to the blade only by a few points at its center.

Action of hypochlorous acid on stains produced by various colored materials

Fifteenth Experiment. Material stained in black by fat and *coal* is left in hypochlorous acid for *twenty-four* hours; the stain undergoes no alteration.

Sixteenth Experiment. Blue material recently stained by *alkanet* and fat is promptly discolored, while the stain remains. After *six hours* of contact, the stain, which had been a dark red, acquired a fallow tint, similar to that taken by the blood stain considered in the ninth experiment, p. 119.

Seventeenth Experiment. Material covered for a month by 122
a large and *thick* stain of alkanet and fat is immersed in hypochlorous acid. The material promptly discolors, but the stain, at first a blackish red, has acquired at the end of *two hours* the color of rust. This color has lost none of its intensity after *sixteen hours* of immersion.

Eighteenth Experiment. A portion of the same material presenting a large, *thin* stain, of the same nature as the preceding, discolors almost instantaneously; at the end of *two hours* the stain is a fallow yellow. This tint has lost none of its intensity after sixteen hours of immersion.

Nineteenth Experiment. Blue material on which is found a *thick* stain of alkanet and fat is immersed for a *few seconds* in hypochlorous acid and discolors, while the stain remains red.

Twentieth Experiment. Blue material stained a month ago with a mixture of fat and alkanet is left in the same liquid acid for *thirty seconds.* The material is completely discolored, and the stain, which was *thick* and red brown, shows

a rusty color toward its circumference and blackish at its center. This remains about the same after *sixteen hours* of exposure to air.

Twenty-first Experiment. This experiment is repeated with a large, *thin* stain made a month ago with alkanet and fat; the stain, reddish before immersion, is a fallow color on removal from the liquid. The stain retains a very clear, reddish yellow color after *sixteen hours* exposure to air.

Twenty-second Experiment. White material stained in red by a mixture of madder-wort and poppy oil is left in hypochlorous acid for a *few seconds;* the stain persists.

Twenty-third Experiment. A part of this same material is
123 immersed in hypochlorous acid for *five hours* and the stain covering it just about conserves its color; the following day a portion of the stain is completely discolored; the day after there no longer remain traces of the color.

Twenty-fourth Experiment. After *thirty seconds* of immersion in hypochlorous acid of white material stained by celandine, the thin stains are yellowish and the thick brownish at their center. After *five minutes* of immersion, all the thin stains have disappeared, and rust-colored circular lines, which end up yellow, replace the thick stains.

Twenty-fifth Experiment. White linen colored like the dregs of clear wine by *campanula pyramidalis* is immersed in hypochlorous acid; at the end of *thirty seconds,* the stain disappeared.

Twenty-sixth Experiment. White material colored here and there by clear bister and brown in a few places with *taraxacum dens leonis* is left in hypochlorous acid for *thirty seconds;* the rather thin stains of a dark-brown color are entirely destroyed; the others, four in number and thick, are almost completely discolored; at the end of *thirty minutes,* there remains no trace.

Twenty-seventh Experiment. Stains made on white material with *cichorium intybus* are of a *very clear* bistre; those which are thin entirely disappear after *thirty seconds* of immersion in the acid; the thickest are discolored at the end of *ten to twelve minutes.*

Twenty-eighth Experiment. White material stained a reddish brown by *lactuca virosa* is immersed in hypochlorous acid; at the end of *thirty seconds* the thin stains have disappeared; the thicker ones show a yellow color; the brownest of
124 the thick stains is rust colored. The material is exposed to air for a quarter of an hour, then again immersed in acid; at the end of ten minutes the stains changed to yellow are discolored; ten minutes later, the rusty stain is a lightly yellowish white.

Twenty-ninth Experiment. White material stained a very clear reddish brown by *euphorbia lathyris* is discolored in a few minutes by hypochlorous acid.

The various stains employed in experiments 24, 25, 26, 27, 28 and 29, were done August 21, 1842.

Thirtieth Experiment. White material is stained red with a mixture of fat and *colcothar* (anhydrous iron sesquioxide): the stain undergoes no alteration, even after several days of contact with liquid hypochlorous acid.

Thirty-first Experiment. White material stained like the preceding and not discolored by hypochlorous acid, is put in contact with a mixture of tin protochloride and hydrochloric acid, as recommended by M. Persoz. The stain blanched after a quarter of an hour; *seventeen hours* later, it had almost entirely disappeared for there remained only a few excessively clear red points bordering on yellow.

A stain of *blood* as thick as the preceding had undergone no change, even after several days, with tin protochloride and hydrochloric acid.

Thirty-second Experiment. White material is stained by a mixture of fat and *colcothar;* the stain is then covered with oil. The material is immersed in a solution of *tin protochloride slightly acidified by hydrochloric acid.* After three days of contact, the solution is excessively cloudy, and *the stain persists with no change.*

Thirty-third Experiment. Rusted iron is not discolored in liquid hypochlorous acid even after *six hours* of contact. 125

Thirty-fourth Experiment. Iron, stained by a mixture of fat and madder-wort, is not discolored after *six hours* of contact with liquid hypochlorous acid; but it is the following day.

Thirty-fifth Experiment. Iron, largely stained with a mixture of *colcothar* and fat and not discolored by hypochlorous acid, is put in contact with a mixture of tin protochloride and hydrochloric acid. At the end of twenty-four hours the stain disappears and the iron blade regained its sheen.

Action of water on blood stains

Thirty-sixth Experiment. A piece of black sheet is stained with a mixture of poppy oil and human *blood.* The following day the sheet is put in water; at the end of a few minutes the liquid is rose-colored and it can be confirmed that it has the characteristics of the coloring matter of blood. Stained material likewise provided a rose liquid after *ten minutes* immersion in water, giving an appreciable quantity of coloring matter of blood.

Thirty-seventh Experiment. Thin and thick stains of blood were made on material and on sheets covered with fat beforehand. Other pieces of the same material and same sheet were first stained with blood then covered with a light layer of fat. The following day the various fabrics were put in water, and at the end of a few minutes, it can be shown that they give off an amount of coloring matter to the water, such that this substance can be easily identified by the action of *heat,* chlorine and the other agents which I recommended use in my public memoir of 1826.[2]

Thirty-eighth Experiment. A small piece of white mate- 126
rial, stained *six years* ago by a rather large quantity of

[2] This procedure has already survived the test of time; during 20 years there has not been an assessment of blood stains that has not made use of it; all those who have recently written on legal medicine adopted it without modification, to begin with by M. Devergie who presented it verbatim in his work, without indicating the source from which he had borrowed it and without even mentioning my name.

human blood, is put in contact with a gram of water. At the end of a *quarter of an hour* the liquid yellows and tends to acquire a rose tint similar to that which water gives to a very small amount of blood; the heated liquid foams and coagulates; chlorine and the other agents behave with it as with a solution diluted with coloring matter. If another portion of this material is left in distilled water for *twenty-four hours,* the liquid acquires a color a *bit more intense,* evidently bordering on rose, and it undergoes the same changes with heat as had been obtained after a quarter-hour contact. *The material stays red.*

Action of water on stains produced by various colored materials

Thirty-ninth Experiment. Material stained by *chelidonium majus, campanula pyramidalis, taraxacum dens leonis, cichorium intybus, lactuca virosa,* and *euphorbia lathyris,* was put in contact with distilled water, which colored pale yellow, brown, or blackish brown. These different liquids, heated to boiling, retained their colors and did not coagulate.

Conclusions

1) Of all the methods proposed up to the present for the recognition of blood stains, that consisting of treatment of the stain with water and then working with the solution, as I recommended in 1826, is undoubtedly the best. M. Persoz
127 is evidently mistaken when he claims it *often* happens that blood stains found on fabrics lose their property to dissolve in water and cannot consequently be disclosed with the help of this liquid. The hundreds of assessments performed up to the present, and experiments 36, 37 and 38 reported in this memoir, establish, to the contrary, that in *almost all cases,* blood stains, even very old, made on clear material or coated with fatty bodies, or on iron, give off coloring matter to the water in quantity great enough for blood to be easily recognized. Moreover, numerous experiments, which I undertook in 1826 and results of which I related in this memoir (cf. exp. 39), demonstrate that all coloring substances, without exception, other than blood, applied on materials, produce stains behaving in water *otherwise* than do blood stains.

2) Hypochlorous acid is far from having the advantages indicated by M. Persoz; experiments 1 14 described in this memoir demonstrate that the greater part of blood stains, thin or thick, recent or old, on material or on iron, entirely, or almost entirely, disappear after a stay *a bit prolonged* in hypochlorous acid; that if some of them don't completely disappear, far from being brown red, they leave only a greyish tint. To tell the truth, some of these stains, even though disappearing almost totally, conserve a brown red color in their center.

In accordance with what was said by Mr. Persoz, if the action of hypochlorous acid *is not prolonged* for more than a few seconds, one or two minutes, the blood stains *persist* and *turn brown,* even though dried-out and old; but, on the other hand, stains from a mixture of alkanet and fat or fat 128
and charcoal or madder-wort or poppy oil or with *chelodinium majus,* etc., *behave a bit like blood stains in hypochlorous acid;* it is, therefore, impossible to positively *characterize* the nature of a st ain by the action of this acid *only,* even if the immersion of the stained parts is of short duration (cf. experiments 15 and 24).

3) However, if hypochlorous acid is insufficient for *positively* establishing that a stain is formed by blood, it can be used with some advantage as an *accessory* method, provided it remains in contact with stained material only for one or two minutes at most; if there exist some coloring substances other than blood, which behave somewhat like the latter with this acid, the stains produced by these matters, even though persisting, do not acquire precisely the same tints as blood; besides, the number of coloring substances which hypochlorous acid destroys in less than two minutes is great, while this time is insufficient for the acid to obliterate blood stains.

4) Hypochlorous acid is completely useless for distinguishing *thick* blood stains on material or iron from rust stains or those produced from a mixture of *colcothar* and fat because these stains persist even after *prolonged* action of the acid. But if this is insufficient in this case to resolve the problem, one can successfully turn to the method proposed by M. Persoz, consisting of treatment of *thick* blood stains with a tin protochloride solution acidified by hydrochloric acid; the thick blood stain will resist, while rust stain and that produced by a mixture of colcothar and fat will disap- 129
pear at the end of a few hours, provided that the latter is not covered by a layer of oil.

5) The action of hypochlorous acid on blood stains coming from a spurt of blood or by immersion of material in blood, visibly differs from that exerted on stains one might call *secondary,* i.e., those produced by contact with a body stained *by a spurt;* indeed, these latter resist much less than the others the discoloring action of this acid.

Legal Medicine. Medico-legal Research on Blood*

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Chapter I. The Use of the Microscope in Medico-Legal Research

561 The first physician who tried applying the microscope in a medico-legal assessment is, indisputably, M. Orfila. In 1827, this distinguished professor speaks of it in his work on blood (*J. de Chimie Med.,* v. III; Paris, 1827, p. 413) and on semen (ibid, p. 473). One must regret that his research was not characterized by successful results. We will explain later (Chapt. II, § III) the circumstances which must have hindered M. Orfila from making use of his microscopical observations in recognizing different types of blood; it suffices to point out here that analogous circumstances were encountered in his research on semen. M. Orfila succeeded in identifying animalculi in dried semen on a slide 18 years old; but when he wanted to examine dried semen on linen by microscope, after having dipped the linen in water, he was led to the conclusion that the zoosperms were no longer perceptible.

M. Rattier (*Journ. de Chimie Med.,* March 1837, p. 120) making some observations on linen stains, in a medico-legal context, saturated linen with water. The water washing the linen contained debris of spermatic animalculi and some whole animalculi. It appears, according to M. Rattier, that at the time of the Contrafato trial, Lebaillif was already using the microscope for identification of semen stains; but, for reasons we can hardly understand, his research was guarded with the greatest secrecy.

M. Ollivier (of Angers) was the first to apply the microscope practically in medico-legal expertise. In the month of June 1837, he was charged with determining if there did not exist hairs adhering to an axe seized at the home of an individual accused of homicide, and, if affirmative, to determine the color of the hair. M. Ollivier, with the help of a microscope, recognized that the filaments in question were fur, completely differing from hair, while perfectly resembling the fur of a horse, beef or cow, when comparatively examined; the judicial inquest fully confirmed the correctness of this observation (*Arch. génér de méd.,* Dec. 1838).

In the meeting of the 20 Nov., 1838, M. A. Devergie read a note on the characteristics of hanging in a living man. He noted the presence of spermatozoa in the urethral canal. He 562
claims, in addition, to have noted spermatozoa in seminal stains on linen ten months old. M. Devergie, however, pointed out that procedures specific for separating spermatozoa from the linen on which they were deposited very often altered them, in separating the tail, and of rendering microscopical examination not only difficult, but fruitless. Historical facts, which we have presented in chronological order, clearly demonstrate, it seems to us, that M. Devergie was going a bit far when he said of himself, "happy to be the first to introduce the use of the microscope in medico-legal research". *(Ann. d'Hygiene Publique,* Paris, Jan, 1839, p. 169). He appears to agree on this point himself later on (*Ibid,* April, 1839, p. 478). We do not feel it necessary to occupy ourselves here with the question of priority raised by M.M. Devergie and Bayard, since on the one hand, priority undisputably belongs to M. Rattier, and on the other, only M. Bayard is seriously concerned with microscopical examination of seminal stains, as we will later see. Let us add here that M. Donné, since 1837 (on spermatoza), noted the possibility of recognizing the presence of zoosperms after a more or less prolonged stay in urine. These findings were discovered in a physiological study, which does not hinder at all their application to legal medicine.

M. Gaultier de Claubry was charged, in June 1838, along with MM. Labarraque and Ollivier (of Angers), with a legal assessment, which had as its object the study of a large amount of denatured, adulterated opium; they proved by microscope not only the adulteration, but also discovered, by this means, the different methods of extraction of opium from Smyrna and from Egypt (announced in the previously cited work of M. Ollivier (of Angers), *Arch. gèn. de méd.,* 1838 and published in *Ann. d'Hygiène,* Oct. 1839, p. 374).

M. Bayard (*Ann. d' Hygiène,* July, 1839) did some further research concerning the microscopical examination of dried semen on linen or material of varied color and nature which were undertaken during the month of November, 1838. For the recognition of dried seminal stains on linen, and making use of microscopical observations, M. Bayard pointed out it was necessary to take care not to crumple or to separate the strips being macerated. Filtration of the liquid of maceration and examination of the deposits remaining on the filter reveal the presence of spermatozoa, isolated from mucus,

* Translation of: "Médecine Légale. Recherches Médico-légales sur le Sang".

In *Gazette Medicale de Paris* 10(37): 561 567 (September 3, 1842).

complete, and without tearing of the tails. He could thus recognize semen dried out two months, two years and nearly three years before. The nature and color of fabric stained by semen has no bearing on the microscopical analysis and the recognition of spermatozoa; one finds them as easily in fabric of thread or of cotton as in that of wool or of silk. One can easily confirm the presence of spermatozoa in vaginal mucus taken after coitus.

Let us now permit ourselves a few reflections on the historical account just presented. It is evident that legal medicine could already have drawn much use from the microscope in many questions. Thus, certainly every time it is a question of determining the presence of sperm either on linen, in the vagina, or in urine, etc., one's recourse is necessarily the microscope as the sole specific means of resolving the question. We would be most satisfied to see the use of the microscope sanctioned in medico-legal research, not only in theory, but also in practice, by the approbation of forensic physicians who have not made a special study of the microscope, and who have consequently viewed this question with all the necessary reserve and circumspection. This circumstance prompted us to use the microscope in the resolution of a very important question, where all attempts up to the present have failed.

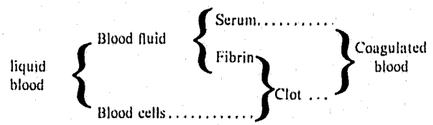
Let us admit that chemical reactions have determined certain stains as coming from dried blood; in the case where one would like to know to what species of vertebrate the blood belongs, one would not be able to decide at our present level of science. It is to this point that we have directed our attention; it is in this question that we have found a new opportunity for the use of the microscope, which, with the help of well-determined characteristics easy to grasp, can distinguish these different types of blood. We well know there will always be those who will rise against the use of the microscope, on the basis, in particular, of the varied illusions to which people unaccustomed to the use of this instrument are vulnerable; but the response to this question is quite simple; if physicians are not accustomed to this instrument, let them become so; their laziness or schedules cannot be an obstacle to the progress of science.

Chapter II. The Differences Which Exist between the Varied Types of Blood

Part I. Physical, chemical and microscopic properties of blood in the different classes of vertebrates

Everyone knows that blood having left the vessels coagulates and separates into two parts, one solid, called the *coagulum* or *clot*, the other fluid, which makes up the *serum*. The clot and serum consequently compose coagulated blood, or as one usually puts it, dead blood. When a more or less considerable portion of coagulated blood is left to itself, it will completely dry out, and there will remain only a fragile, brittle, solid crust of dark red, provided however, the amount of serum wasn't too great. In this case, putrefaction will settle in before dessication. This dry mass comprises all the materials of blood, with the exception of water, which has evaporated; i.e., it is composed of the dried clot and the elements dissolved in serum, forming a dry residue when the water has evaporated. It is easily understood, then, that its size, its extension, etc., depends mainly on that of the clot which forms the most considerable part of the coagulated blood.

We say then that coagulated blood is composed of clot and serum. But circulating blood is quite far from presenting the same elements: examined under the microscope, in the transparent part of animals (tail of tadpole, tongue of frog, gills and fins of fish, intestines of young animals, etc.), it is seen to be composed, in the normal state, of corpuscles swimming in a reddish-yellow fluid. These corpuscles are usually called *blood cells*. What relationship then exists between the cells, the fluid suspending them (which we will call *blood fluid*), the clot and the serum? It has been known for a long time that the clot is composed in great part of fibrin, the same 563
substance obtained as filaments in beating blood with stirrers. Microscopical observation, in addition, demonstrated that a clot contains whole blood cells, not separated from their envelope. Modern experiments, finally, which we have presented elswhere (*Arch. Gen. Med.*, 1840, v. IX, p. 185), demonstrate that the fluid in which these cells swim contains dissolved fibrin. As soon as the blood has left the vessels, the fibrin dissolved in the blood coagulates, enclosing the cells in its mesh, and forms the clot. The blood fluid, deprived of its fibrin and cells, becomes serum. We can present this composition by the following table:



Let us now examine the principal properties either of coagulated, dried blood or of that still in circulation. It is understood that attention will be drawn only to those points relating to the research forming the substance of this paper.

A blood clot is red and soft; it is saturated with serum, which causes the softness. The color is from contained blood cells; the cells are colored red by the coloring matter of blood (hématosine), which dissolves when the clot is placed in water. The blood clot, composed, as we earlier pointed out, of fibrin and blood cells, consequently discolors when made to soften in water. The fibrin stubbornly retains a portion of blood cells; which is why it is necessary to macerate the clot in water, which it is necessary to replenish, until the liquid no longer colors. The finished product represents the fibrin entirely, white and uncolored, in soft, long masses, formed by intertwining filaments, similar to ribbons, whose volume is much less considerable than that of the clot which provided them. In this state, the fibrin is heavier than water, and sinks to the bottom. What we have just explained finds its entire application in the case of chemical examination of

blood stains (Part II). Without further comment on the chemical properties of fibrin, we will only say that, in this state of coagulation, the fibrin is insoluble, in both hot and cold water, and is dissolved by caustic potassium, even when this base is very diluted. According to Berzelius, when fibrin is immersed in a caustic solution dilute enough to contact one's tongue without objection, it gradually transforms into a gel, as it does in concentrated acid, and finishes by completely occupying the solution. If one then encloses this in a closed vessel at a temperature of 50-60°, it dissolves bit by bit and thus produces a weakly yellow solution.

Dissolution of the red coloring material in blood is achieved by leaving the clot in water for a few hours, it being of little consequence whether it is soft or already dried. This dissolution plays a major role in medico-legal research on bloods. The chemical and physical properties of blood by treatment with different reagents have also been carefully studied. This liquid we are discussing, easily obtained by macerating the clot in water, is not only an aqueous solution of coloring matter, but contains, in addition, the elements of serum contained in the clot. The principal constituent of serum is albumin, to which it owes its most salient characteristics. In evaporating the serum, the albumin dries out, but is again soluble in cold water. When heating serum in a glass or porcelain container, at a temperature which gradually rises, it begins to lose its clearness at 65°, and at 75° coagulates into a mass the color of pearl, opaque, with translucent edges, insoluble in cold or boiling water. This appearance of coagulated albumin varies greatly according to the proportion of albumin and water. We saw earlier that albumin forms a solid, opaque mass; when the serum is diluted with a little water, the albumin will only form flakes. Further dilution of this liquid will cause coagulation to give it only a milky or opaline tint, becoming clearer as amount of water increases.

It is clear, then, what should happen when a blood stain is macerated in water. It dissolves the coloring matter of blood, which sinks in the form of reddish streaks to the bottom of the container. In addition it dissolves the dried albumin of serum; when heating this reddish solution, albumin forms flakes, or produces only an opaline tint in proportion to the amount of water used for the maceration. Finally, the insoluble part is fibrin; we have already pointed out its insolubility in hot and in cold water. These are the principal phenomena which take place when leaving portions of dried blood in water and which are of the greatest importance in medico-legal research of blood (Part II).

We are going to examine now the principal microscopic characteristics of blood, apart from any theoretical discussion, with the intention of rendering intelligible our studies on this subject (Part IV).

If a blood droplet is placed on a glass slide, and a very thin second slide is placed on the edge of the droplet, infiltration of the blood gives a transparent layer ready for observation. If it is blood of a mammal, one can see swimming in the fluid round, flat corpuscles, the diameter of which never surpasses one hundredth of a millimeter; they are of a very pale red, almost yellow; they are the *blood cells*. Also evident is a second type of corpuscle, white, mammilated, with a diameter of at least a hundredth of a millimeter; we have called these elements *white fibrinous cells*, or simply *white blood cells*.

The blood cells have swollen borders on both sides, their center is depressed, which gives them the form of a very elongated "8" seen from in front, and when a considerable amount of water is added to the blood droplet, the blood cells, on examination after a certain time, are much more pale, almost entirely uncolored; their edges are scarcely visible. On the contrary, there are no changes in the white blood cells. This loss of color in the blood cells is more pronounced 564
with greater amounts of water and with longer action on the cells. At the end of half an hour, there is no more trace of these corpuscles and one would think them entirely dissolved. However, on adding a little tincture of iodine, the uncolored cells become yellow, and are once again perceptible. The blood cells are not actually dissolved until after one or two days; but, we repeat, they are already so uncolored after a quarter or half an hour that they entirely disappear, because of their great transparency, and it requires long experience with a microscope to distinguish them in the midst of the serous fluid in which they swim.

We have spoken up to the present of the blood of man and mammals; but we've known for a long time that oviparous blood contains cells of an entirely different form; in addition, our observations have demonstrated that animals belonging to the camel family present blood cells similar to those encountered in oviparous blood. These blood cells are elliptical, instead of round as in mammals, and their large diameter almost always surpasses a hundredth of a millimeter. They are also flat and yellowish; but instead of presenting a central depression, they present on the contrary a central elevation, such that, seen from the side, they are bulging. This elevation comes from a central, oblong, granulated nucleus, which becomes more manifest as the cells remain for a longer time between the two slides. On dessication of a very fine layer of oviparous blood, one can see the central nucleus better in isolated cells. (For more detail, we refer the reader to our *Anatomie Microscopique*, 2nd series, 1st edition Paris 1839). In adding water to this type of blood, these cells also discolor, but their nuclei remain very distinct and do not at all disappear by the action of water.

Coagulated fibrin presents an amorphous mass, i.e., deprived of structure, white or gray, soft and elastic.

We have already previously pointed out that a clot consists of coagulated fibrin, which encloses blood cells. What does one see, then, if one macerates a portion of dried clot, for example, a blood stain, during a half hour or an entire hour? It is easily understood that when it's a case of mammal blood, one perceives only an amorphous mass, containing some white cells; the blood cells can no longer be distinguished. If, on the contrary, it is oviparous blood, all the nuclei remain distinctly visible. (cf. Part IV).

Part II. Chemical Procedures for distinguishing Blood from every other Substance

Every chemist concerned with expert medico-legal examinations now agrees that blood can be distinguished from every other substance, and they also agree on the specific means for reaching the results, since M. Orfila published his research on the subject (cf. *Jour. de Chimie Medic.*, Paris, 1827, vol. III, p. 367). The purpose of this paper is not to present those facts already known to science; we refer the reader who would like to acquaint himself with them to treatises on legal medicine. However, it would not be useless to say a few words to better understand our procedures which we are going to present later on (Part IV).

To find out the nature of a stain, it is macerated in cold distilled water, taking care that there is a certain distance between the stain and the bottom of the container. If it is a blood stain, red streaks are seen in no time at all going from top to bottom, and bit by bit, deposit on the bottom part of the liquid, coloring it red. At the same time, the stained parts thus treated by water discolor, and there remains in place of the stain a small greyish layer of whitish or reddish-white filaments. This layer, or the filaments, are formed by fibrin and the insoluble parts of blood cells; the reddish streaks, on the contrary, come from the red-coloring matter of blood, extracted from cells by maceration.

We must then clearly distinguish two essentially different parts: the liquid of maceration and the filaments. As for the liquid, it acquires a rose or reddish color when shaken in a glass tube; gradually heated to a temperature near boiling, it becomes cloudy, immediately changes color, and deposits flakes of coagulated albumin, or becomes only opaline. If the flakes are deposited, i.e., if a coagulum forms, it is greenish-gray without the least trace of rose or red, and the supernatant liquid is uncolored or lightly colored in yellow-green; if the liquid is filtered and treated with potassium, it takes on a green tint, with reflected light, and a red tint with refracted light. If, on the contrary, it is not filtered, and treated with potassium while the coagulated albumin is suspended or deposited as flakes, the result is according to M. Orfila, about the same: the liquid acquires a reddish color by refraction, and greenish color when seen by reflection. There is not a coloring matter belonging to an animal substance which can produce this set of phenomena. As for the filaments, they are soft, a bit elastic, soluble in potassium; and the potassium solution, treated with chloride and a little hydrochloric acid, gives flakes of coagulated animal matter.

The chemist can always, with the help of these characteristics and of certain others, which we do not need to present here, distinguish blood stains from every other type of stain, for example, iron stains formed from lemon juice (iron citrate), rust stains, stains of substances which enjoy the property of coloring water a red or a rose (cochineal, Brasilwood, cartham, madder-wort, etc.). M. Raspail, far from sharing the opinion just aired, says: "it suffices to let a cloth sac filled with powdered madder-wort, slightly moistened with water, sit for a few hours in chicken egg white; then to expose this mixture to a temperature of 25–30°C, finally to dry it, to give it the appearance of a red stain similar to a blood stain". But M. Orfila fully refuted this opinion (*Jour. de Chimie Medic.* vol. IV); it would seem to us that the reaction with lime would be the only one to resolve the question.

There is then no doubt that *legal medicine can distinguish* 565
blood stains from every other substance producing stains of analogous color.

Part III. Examination of Methods Proposed for Distinguished different Types of Blood one from the other

At the reading of a work of M. Orfila at the Royal Academy of Medicine, which was reviewed by the Philomathic Society, at the meeting of July 14, 1827, M. Dulong remarked: "One of the most distinct characteristics of blood stains, even when they are quite old, is the form of cells seen under the microscope; it allows, in addition, distinguishing of the blood of different animal classes; dried mammalian blood cells present a white disc surrounded by a red circle, while in the blood of birds, the white disc is surrounded by an elliptic cell". (In this same meeting, M. Adolphe Brongniart said that beef blood had been able to be distinguished from human blood by microscope by M. Dumas in a medico-legal case; but M. Dumas hastened to point out the incorrectness of this assertion. It is certain M. Brongniart had confused two different facts). M. Orfila hastened to verify the opinion proffered by M. Dulong; but the conclusions he derived from his research, confirmed by Lebaillif, are not favorable toward the use of the microscope.

Indeed, the experiments of M. Orfila show: "1) that, even though blood contains a multitude of cells able to characterize it, it is sometimes impossible to realize the presence of these cells in dried blood on a slide, and even more on fabric, because the blood drop is too thick, contains only coloring material, or some other reason; 2) that, if it is true, in general, that mammal blood cells are circular, while those of the blood of birds and cold-blooded animals are elliptical, it is no less certain that in the study of blood detached from linen, elliptical and spherical cells in mammal blood, as well as triangular, square, etc. corpuscles, in the blood of birds can be seen, which probably results from an atom of dust or from the material of the fabric which united with the blood. It is easy to imagine that a cell which is spherical seen alone, presents another form when united with a foreign body" (*Jour. de Chimie Medic.*, vol. III, p. 413; Paris 1827).

We see, then, that M. Orfila, in contrast to the opinion of M. Dulong, could not distinguish human from pigeon blood when removed from fabric, and "even sometimes that it was blood". We can understand how M. Orfila could arrive at these results when we examine the manner in which he conducted his research. Soon after "a portion of linen containing all the material of blood (of pigeon) had been left in a small amount of water until this was sufficiently colored, three drops of this liquid were deposited on a glass slide until dessication was complete". But what could this liquid contain? Assuredly hardly any blood corpuscles, for the greater part remained attached to the linen (Part IV), and the water dissolved only the coloring matter. At the time that M. Orfila was conducting his research, these properties of the cells had not yet been studied; we should not then be astonished to see this distinguished chemist looking for cells in this liquid and finding only irregular, elliptical, square, spherical, triangular, etc., corpuscles. These corpuscles are either molecules foreign to the constituents of blood, or some blood cells, detached from the fabric, not deformed by dessication, except many of them stuck together, in such a way as to form irregular corpuscles. This is the only way we can understand how M. Orfila would have found elliptical cells in human blood.

At other times, human blood dried on a sheet, dipped in water, and seen in the microscope before dessication, offered "a great number of small, transparent, spherical and ovoid corpuscles; on the other hand, it was difficult to find perfectly spherical corpuscles". Here again, it was scarcely blood cells which presented themselves, since they are almost all dissolved by water.

The result of this research is that the microscope is of no use in the examination of dried blood when only the dissolved portion of the stains is studied, on examination of either the dessicated or liquid state of the solution. As for the rest, we must remember here the influence of a few circumstances not well known at the time when the observations were made, and which would have hindered even the recognition of the presence of blood cells in the drop examined. For example, the drop examined should have been covered with a second glass slide to see it extended as a thin layer, and to see in this way all the suspended particles, whereas an uncovered blood drop presents for observation only the particles suspended on its surface. The alterations undergone by blood cells during a stay in water had not yet been well studied; it was thought, according to Hewson, that certain animals present corpuscles sometimes elliptical, sometimes circular. We presently know that every oviparous animal, coming out of its egg, always shows elliptical corpuscles, and that the circular form is due only to the effect of water on the cells.

It is evident after all we have just said that we had not arrived at a point of being able to distinguish the different types of bloods, and since these experiments have not been undertaken since that time, the result is that science has no microscopical method for distinguishing mammalian from oviparous blood. But before presenting our research on this subject, we will first say a word about a few chemical experiments undertaken with this end in view.

M. Barruel (*Annals d'Hygiene*, vol. I, P. 267) proposes the following method for the distinction of different kinds of blood. Blood is placed in a glass; one third or one half of its volume of sulfuric acid is added, and it is stirred with a glass rod; immediately a volatile aromatic element manifests itself, characteristic for each kind of blood. Immediately upon stirring, it is recommended to blow briskly into the glass, to dispel the primary atmosphere, in which some sulfuric acid can be encountered. M. Barruel claims he can thus distinguish, by the odor alone, between the blood of man and of woman, and consequently the blood of the diverse species of animals. After the publication of these results, one must look to verify them, and many chemists repeated these attempts. Though several physicians have completely confirmed the 566
results of M. Barruel, all agree, however, that the sense of smell is too fallacious, too uncertain, and most often too little developed in different people to dare apply M. Barruel's discovery to judicial assessment, however interesting it might be from the physiological point of view. On the other hand, to perform these experiments, a very considerable amount of blood is necessary, which is very rarely found in these assessments. It is true that M. Barruel asserts that even two weeks after the production of the stain, the species to which the blood belongs can be ascertained; but no one has verified this assertion. M. Morin of Rouen believed that he had discovered a great difference between the coloring matter of man and that of fish; but M. Lecanu showed the error of this opinion. M. Chevallier (*Journ. de Chimie Méd*) could find no chemical means for distinguishing blood stains from stains of bed bugs who has sucked blood and been crushed on fabric; the only difference is that stains of bed bugs, left by themselves for several months, ended by taking on an olive tint.

The result of all the preceding is then that *legal medicine has no method, either microscopical or chemical, to distinguish among these different kinds of blood.*

Part IV. Methods for distinguishing blood of man and mammals from oviparous blood

In a medico-legal investigation, once the nature of the stains has been determined and they are confirmed as coming from dried blood, there sometimes remains another question to be resolved. The accused might contend, admitting to the nature of the incriminating stains, that it is the blood of bird or fish found dried on his linen, knife, or hands. We have seen (Part II) that the forensic physician can, without hesitation or leaving the slightest doubt on this subject, determine the nature of the stains; but, from the facts we have previously presented (Part III), it is evident that the forensic physician has no method for distinguishing the blood of different species one from the other. We thought it necessary to do some research on this subject; we succeeded, not in resolving the entire question, but at least in distinguishing the blood of man and mammals from oviparous blood, i.e. blood of birds, fish, and reptiles. Here is the manner in which we proceed.

It is known that a blood stain macerated for a while in water discolors and that a small grayish layer or whitish-grey filaments of fibrin remain attached to the substance which bore the stains (Part I). It is this discolored fibrin which we examine; indeed, this alone can show the discolored cells, while the colored liquid coming from the maceration of the stain contains only the red coloring matter,

dissolved albumin, and sometimes some detached blood cells. We are sure then that the microscopical examination of this liquid is of no use, and that it is necessary to submit the discolored fibrin to examination.

Here is the way to proceed to obtain the discolored portion of the stain adapted to microscopical examination; first, prepare a glass slide as for any other microscopical examination; place a drop of distilled water on the slide; then detach with any type of point whatever, most conveniently a cataract needle, a few particles of the stain; it is best to choose the edges of the stain, for here dried blood forms the thinnest layer, and consequently presents the most favorable conditions for microscopical examination. The particles detached in this way will have more or less the size of the eye of a needle; there will even be some which are smaller. It is always good to have at least four or five.

Once these particles of stain are procured, they must be transported into the drop of water placed on the slide. This is most easily done by slightly wetting the point serving to scratch the stain with some distilled water. All the particles will adhere to the point, which is then dipped into the drop of water on the slide, all the particles being made to drop by careful, light taps on the point. One must avoid rubbing the point against the slide, for this operation might alter the sharpness of the results. There will now be five or six particles, very small and very thin, floating freely in the drop of water: these are, so to speak, many microscopic blood stains. Now leave them for a while in the water to discolor them; one can easily see much less time is necessary to produce this discoloration than would be the case for a large stain. Indeed, after a quarter or half an hour, the particles are already discolored. To accelerate the dissolving of the coloring matter a bit, incline the slide in different directions. This will produce movements of the drop of water, which accelerates the discoloration.

When it is noted that the small particles have paled quite a bit, i.e., the coloring matter is dissolved, the examination proceeds in the following way: first, the amount of water in which the discolored particles are suspended is decreased by inclining the slide to pour off a part of the drop of water. A very thin second slide, one which usually serves as a cover for the object to be examined in microscopical observations, is placed with caution on the particles suspended in the water. Any considerable pressure must be carefully avoided. Those accustomed to microscopical observation will soon come to know the amount of water necessary to render the observation clear and distinct. There must not remain too much of the drop serving to dissolve the coloring matter, because the water will easily cover the second slide; nor must there remain too little, because the presence of air bubbles will render the particles too opaque. These are the precautions to take, by which one masters the technique in repeating it a few times.

We now have the discolored particles placed in a drop of water between two slides. The whole is placed on the stage of the microscope, and the particles submitted to examination. There is hardly need to add that the general rules, as in any microscopical observation, must be heeded, for example, as concerns light, etc. (On this subject, refer to our *Traité du Microscope*, Paris, 1839). In examining these particles, attention should be directed especially to their transparent edges: it is here the elements soon to be questioned can be distinguished most clearly. The central part is most often not sufficiently discolored: the examination is thus more difficult. Now here is what is observed in these discolored particles, which, as we know, are formed by fibrin and blood cells deprived of their coloring matter.

Particles of blood stains of mammals will present an amorphous layer, i.e., without any organization, in which here and there can be seen a few white cells. Of those blood cells which are completely discolored, there will appear no trace. When, on the contrary, the discolored particles belong to stains produced by oviparous blood, a very great number of oblong nuclei, crowded together, will be perceived in a very fine layer of coagulated fibrin, whereas the external contour of the cells will be no longer visible.

In this manner, one will have a very easy method for determining the species of blood producing the stain. But, the blood of man and that of mammals presenting cells of the same structure, it is easily understood that neither the blood of man and that of any other mammal, nor of one mammal from another can be differentiated by microscope. On the contrary, it will be very easy to establish if the stains in question belong to the blood of man or mammal, or to oviparous blood, i.e., fish, bird, or reptile. The blood of camels and all animals belonging to this family presents the same characteristics as oviparous blood; this is a result of observations we made in 1839, and which have been confirmed in a report made to the Academy of Sciences by MM. Milne-Edwards and Isid. Geoffroy-St.-Hilaire. This circumstance merits note, due to our possessions in Africa.

We reject the use of the microscope to distinguish the different species of blood of mammals: however, adherent fur can sometimes give very important information. It will thus not be very difficult to recognize fur of a rabbit, a steer, etc. or to differentiate them from hair. (See our *Anatomie Microscopique*, 1st series, 4th edition, Paris, 1840). The microscope might also determine, if necessary, whether the stain in question is actually composed of blood. In the case where chemical analysis has not decided this question, we reserve for another occasion the presentation of further details on this subject; however, we do not find it useless to add the following facts. Any mineral substance which can imitate blood stains will not discolor, and will show under microscopical examination a mixture of amorphous, red or opaque particles, without any trace of cells, which break under pressure. Fibrin, on the contrary, is white or grey, and elastic. Vegetable substances not presenting a grey layer like fibrin, the colored liquid obtained on their dissolution is amorphous, or presents a few vegetable parts which differ according to the plant examined. But we hasten to drop this subject, because chemistry has always previously determined the nature of the stain in these cases. When it is a matter of stains of bed bugs, one can discover parts of crushed bed bug by microscope in macerating the stain.

One might perhaps think it more advantageous to dip the entire stain in water, and then take a small portion of the greyish layer to submit to microscopical examination. But this opinion is erroneous: indeed, the entire stain macerated in water swells considerably, and the thin, transparent edges of these stains are thus lost to microscopical examination. Let us not here another circumstance which can sometimes be useful, but which we believe of no actual importance in the question occupying us. If the stain is not entirely discolored, the contours of the imperfectly colored blood cells are perceived in the fibrin layer; one can easily render these contours visible again by dipping the discolored layer in a weak tincture of iodine, or, even better, in a solution of sugar syrup (one in five parts distilled water) to which has been added a little tincture of iodine to color it slightly. The sugar solution does not alter the form of the cells; but we recommend these last procedures only to those already accustomed to the microscope because the thickness of the stain most often hinders distinct perception of the cell forms. Let us remark, finally, that the smallest stain can serve in a large number of microscopical examinations.

We have previously said that one will have "in this way a very easy method to determine the species of blood producing the stain". However, it would be useful to present a few explanations in regard to this subject. When it is a matter of putting this research into practice, when one would like to make use of the results we have obtained in a medico-legal case, it must not be thought that just anyone can make these observations. A man is not a microscopist just because he owns a microscope: he must also be accustomed to it. In every case, we recommend to the physician the greatest reserve in the expression of his opinion. When he has confirmed the presence of nuclei, he can unhesitatingly pronounce the stain to have been produced by oviparous blood for two reasons: 1) because mammalian blood never presents a similar appearance; 2) because this judgment can only be in favor of the accused. But when the presence of nuclei cannot be determined, we believe it much wiser to announce a negative result, i.e., the physician would do better to claim that he could not confirm that the blood was oviparous; at least his opinion will not overwhelm the accused. On the other hand, he will avoid the error of wanting to make a judgment by a negative result, by the absence of nuclei, although from the scientific point of view the absence of nuclei is a characteristic as positive as their presence. We say in conclusion that, in practice, this research must only serve in favor of the accused, and that it appears to merit that much more attention from forensic physicians as it presents the sole scientific means for helping an innocent defendant.

Memoir on the Examination by Microscope of Blood Stains on a Blue Cotton Smock in a Homicide Case*

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and

Dr. A. Salmon

Surgeon of the Hotel-Dieu of Chartres

368 Examination of blood stains presents one of the most delicate problems in legal medicine. When limited to the use of chemical reagents in performing this examination, as happens in the greater part of assessments which come to attention in this context, the results are always incomplete or more or less approximate, which limits the operations to the use of only immediate principles, such as *albumin* and *fibrin,* and not the direct, constitutive elements of blood, i.e. *white blood cells* and *red blood cells.*

The use of the microscope, combined with that of reagents, offers then, in the research we are discussing, a security not found in any other procedure.

The details which we will present, on the occasion of an assessment with which we had been charged, will furnish very clear proof.

We will limit ourselves in this work to a description of the facts relating to the anatomic elements characterizing blood. But it is useful to point out that the guarantees of certainty and precision offered in the study of stains of various fluids by the means we have employed can be seen even more clearly with regard to animal and vegetable tissues. Their
369 anatomic elements, invisible to the naked eye, but easily recognizable by microscope and less alterable than those of liquids, permit the differentiation of very small portions of these tissues at all ages of intra- and extra uterine life. Thus debris from envelopes of the embryo, the placenta, the decidual membrane, and blood clots from the matrix can easily be recognized; hair of man and fur from animals of varied ages, fatty tissue, nervous tissue, etc., can be clearly determined. It is known that it is precisely these cases where chemical methods are found to be ineffective.[1]

§ I. Preliminary Questions

". . . Given that this soiled smock, seized eight days after the crime, had not been washed since the crime, and that it preserved several blood stains by the very admission of the suspect, who recognized them.

Given that these blood stains are seen not only on the sleeves and on the front of the smock, but even on the back; that some of these stains, notably those on the sleeves and the lower part of the smock, appear to have been rubbed with either water or earth.

Given that Doiteau [the suspect] attempted to explain the stains by saying they came from blood spurting from a duck 370/
killed in his presence . . .

Given that, even though this fact is inexact, and that the duck concerned had been killed out of the presence of the suspect, it is nonetheless important to determine if, *firstly,* the blood stains found on the smock are, or are not, blood stains from a duck, and if in any case the blood would have been able to spurt in quantity great enough to explain the numerous stains on the smock, on the sleeves, the front, and even the shoulders and back; if, *secondly,* this blood by its nature, color, adherence, form and the multiplicity of the droplets, is not rather the blood of a septuagenarian woman violently struck on the head with cutting and contusive instruments.

Given that in comparable circumstances the microscope is used today by science as a means of verification with the greatest success, etc.

In these circumstances, let us request, etc. . . .

Here are the questions concerning the smock the two doctors were asked to treat in their capacity as sworn experts:

1) Are the stains on the smock, and particularly the dark stains bordering on red and on yellow, of blood? (Do not limit your testimony to saying that they contain albuminous elements of blood, but say in no unequivocal terms if it is actually of blood, what is called blood.)

2) Apart from stains which appear to be blood stains to the naked eye by their color and form, are there not other stains on the smock of the same nature, but less colored as if someone had attempted to rub them off or to dilute them a little while after their formation by any type of rubbing or washing?

3) Are the blood stains in an amount large enough, and in places so multiple and diverse on the smock, that they could

* Translation of: "Mémoire Concernant l'Examen, a l'Aide du Microscope, de Taches de Sang sur une Blouse de Coton Bleu dans un Cas d'Assassinat". In *Annales d'Hygiène Publique et de Médecine Légale* 8 (2nd series): 368–397 (1857).

/371 not be explained by splashes of blood of fowl slaughtered in the presence of a man clothed in this smock and seated facing the fowl?

4) Are the elements of blood, of which the microscope permits recognition, elements of blood coming from a living duck who would struggle at the moment of having his throat cut?

5) Would not the elements of blood be, on the contrary, elements of blood belonging to the human species, belonging in particular (if science can go up to this point) to the septuagenarian woman violently struck on the head?

6) Finally, if these stains are of human blood, could not they have been produced, according to their placement on various parts of the smock, in the course of a homicide where a single man armed with a cleaver, a bill-hook, and notably a spade, had delivered fifteen blows to the head of his victim.

7) The appointed physician-experts will, in addition, present in their report the guarantee and certainty of the precision offered by the method of examination used by them in the execution of their assessment."

§ II. Examination of the blood stains by the naked eye and by magnifying glass.

To reply to the questions posed to us we proceeded as follows:

After having counted and measured the blood stains, we verified they were from ¼ of a millimeter to 3.5 millimeters in width, all of them recognizable as such by their dull reddish-brown tint in daylight and shiny black by lampligit. They reflected this light with that peculiar glint, known to be one of the characteristics of blood observed under these conditions. But it is also known that this way of reflecting the
/372 light of a candle, or of a lamp, is specific to stains of egg white, of gelatin, of gum and probably all stains of liquids rich in albuminous principles. However, the coloring in red-brown or blackish, together with the glint of light, present a specific character istic for direction as to the means of investigation which might be used.

All these stains, examined by magnifying glass, demonstrated a small crust projecting slightly above the material of the fabric itself; each crust was brilliant under certain incidences of light, of a dull brown, on the contrary, when inclined otherwise. The thickness of the small crusts was so slight it was impossible to appreciate by the naked eye: about 1 to 2 tenths of a millimeter.

The small dimensions of the stains, and the thinness of the crusts forming them, soon indicated to us the impossibility of resorting to procedures based on examination of the coloring substance of blood and its albumin for determination of their nature.

But the existence of the small crust elevated above the material of the smock became one of the principal conditions permitting us to arrive at the certain determination of the fundamental parts of blood on each of the stains successively, despite their very small size. Stains of three and one half millimeters could even be halved by cutting the material in such a way as to submit each half to a comparable examination by slightly different procedures.

§ III. Examination by microscope of stains on which had been determined by naked eye some of the characteristics implicating the stains to be of blood.

On a certain number of stains, of which we have just presented the external characteristics, after having halved the largest by cutting the material bearing it, we proceeded as follows to determine their nature, their intimate 373/
composition.

After division of the fabric supporting two of the previously indicated stains into the form of strips, they were steeped for six hours in pure water. For this preliminary operation, only the lower extremity of the strip bearing the stain was immersed in the liquid, such that two or three millimeters were left outside the water and applied to the wall of the capsule containing the liquid by the upper extremity of the strip. The fluid soon rose by capillarity up to the stain and gradually swelled the substance forming it.[2]

Once the swelling is complete, we removed the slightly swollen substance, by scraping the material a bit with a scalpel. We placed this substance in a drop of the same water placed first on the bottom slide of the microscope. After having dissociated the swollen substance in this drop of liquid with needles, it became a bit redder than in the dry state and we covered the preparation with one of the *thin slides,* or glass slides, used in every examination by microscope. This done, the preparation was placed under the objective of the microscope at a magnification of 514 actual *diameters* which demonstrated the following:

In the liquid of the preparation were seen more or less large fragments of the substance of the small crusts belonging to the stains. They were swollen by the liquid. These fragments were irregular, some greyish and others a bit 374
colored by the previous particles. In addition, around the fragments, the liquid in which they were immersed was colored a red tint, similar to that given by the coloring substance of blood dissolved in liquid. The portion of the liquid thus colored formed a red zone, more or less wide, around each of the fragments of substance placed under the microscope.

Finally, either in the liquid of the preparation, or in the thickness of the fragments of the substance of the stains, were seen thin, microscopic filaments, twisted around each other, offering all the characteristics of filaments of cotton: all were uniformly of a slightly dark blue indigo, which contrasted with the red tint of the liquid of clear blood.

§ IV. Examination of the fibrin of blood in stains formed by this liquid

In adding some water under the microscope to the previously indicated fragments of the matter from the crusts, and even before this addition, we could determine very clearly that these fragments, swollen on contact with the liquid used, were formed principally of fibrin and secondarily by

white blood cells.

The facts we are going to discuss below were also clearly evident:

1) Either in using pure water to swell the stains present on the last two strips of the four we had removed from the smock;

2) Or in scraping the small crust visible by magnifying glass on each stain and dropping this in small fragments, or powder, onto a drop of pure water placed on the *bottom slide* of the microscope.

In proceeding thusly, water discolors the stains, or the
375 matter removed by scraping; it renders the substance greyish, swelling it a bit; the water colors slightly red in accepting some of the coloring matter of red blood cells, whose uncolored elements it also dissolves after sufficiently prolonged action, without leaving any visible particle, such as nuclei or granulations.

After dissociation of the discolored fragments of the matter of the stains with needles, examination by microscope shows that they are formed principally by a transparent substance, scarcely greyish and finely granulated. In addition, the fragments of this matter placed under the microscope clearly demonstrated a fibrillary appearance of thin, rectilinear or delicately curved, interwoven filaments, some free and floating on the periphery of the fragments being examined.

After treatment with acetic acid, this fibrillary substance became extremely pale, gradually swelled, lost its characteristic fibrillary property and the fine granulations with which it had been strewn. It was thus observed to pass from the striated, finely granulated state specific to it, to the state of a homogeneous, transparent, gelatinous substance.

It is known that these attributes belong specifically to the fibrin of blood, and that taken on the whole, they result in an aspect entirely characteristic and constantly found by anatomists in this important principle of blood.

Thus the web of the small crusts or stains submitted for our examination was formed entirely of fibrin just as the web of a blood clot in blood-letting (which represents one of the crusts on a large scale) is formed entirely of fibrin, retaining in its thickness the two other characteristic solid parts of
376 blood, namely, the white blood cells and the red blood cells.

§ V. Examination of the characteristics of white blood cells retained in the fibrin of the cells.

On each of the fragments of the substance of the crusts placed under the microscope, formed by fibrin and cleared by water of the red blood cells it had carried along in coagulating, were recognized several white blood cells.

In the thickness of the fibrinous web just described were found transparent, greyish, round, finely granulated cells, of a width of 8 to 10 thousandths of a millimeter. In the center of several of them were also noted one or two small nuclei, greyish, irregularly spherical or oval, of a width of 3 to 4 thousandths of a millimeter. Such cells, in the middle of a web of fibrin, could come only from blood. In adding acetic acid to these corpuscles, we demonstrated characteristics exclusive to such cells.

This acid, as its action gradually exerted itself, rendered the body of each cell transparent, slightly swelled it, and gave its contour a regular though paler aspect; at the same time, this reagent cleared away the fine granulations of each cell and showed their nuclei more clearly. These were soon presented in numbers of two or three, or even four, toward the center of each cell. They were disposed sometimes side by side, sometimes in triangle or horseshoe arrangements, or were superimposed. The reagent rendered the edges of these nuclei darker, easier to see, as can be noted on fresh blood comparably examined; it also rendered them a bit more irregular than the action of water alone.

The white blood cells, whose fundamental characteristics we identified, were sometimes isolated, scattered about in the fibrinous web of the stain, sometimes contiguous, united in groups of four or five or even in numbers two or three
377 times greater side by side.

§ VI. Special examination of red blood cells retained in the fibrin of stains or adhering to filaments of the fabric of the smock.

Knowing that solutions capable of preserving red blood cells intact are obtained by mixture or dissolution of various fluids and salts and that the cells regain their natural suppleness in these solutions after having been dessicated, we decided to make use of them, since experience had long since taught us their advantages.

These liquids were furnished to us by Mr. Bourgogne, manufacturer of microscope preparations, who, after many long attempts, managed to fulfill all the necessary conditions. This capable artist keeps the secret of the composition to himself, a secret we have not tried to uncover, each expert being easily able to procure this reagent for himself, in asking for it under the designation *liquid* 4_u.

Here is the procedure by which, with the help of this liquid, we easily found the red blood cells characteristic of blood in the stains submitted for our examination.

We worked with three stains, each of which furnished us with two preparations. One of the stains was a half-millimeter, and the two others each a millimeter in width.

The *first series of preparations* was done by scraping the superficial crust of each stain and letting the scrapings fall into a drop of liquid. Covering this with a small slide, we let the small brownish fragments of the crust lie in the solution for twelve hours. There is no precaution to be taken during this maceration other than to protect the preparation from
378 dust, for the liquid employed, being slightly hygrometric, will not evaporate.

At the end of this time, we saw that the small fragments of the scrapings of the crust immersed in the liquid were swollen, had become more transparent and redder than they had been at the beginning of the operation. They regained the characteristics of color, transparency, consistency and elasticity specific to the small groups which form during the

accumulation under the microscope of red blood cells of fresh human blood.

By the delicate maneuver of sliding the glass slides back and forth over each other, a maneuver to which one becomes accustomed in using the microscope, we succeeded without too much difficulty in detaching a rather large number of cells forming the group. We could then study the characteristics with as much ease as on fresh blood.

Each isolated blood cell had just about regained its circular, flat, biconcave form. Some still preserved a bit of the polygonal form which the reciprocal pressure in the accumulation had given them; others were concave on one side, as in the fresh state, but convex on the opposite side, as can be seen in blood cells placed in sodium or potassium sulfate solution. All were of 6 to 7 thousandths of a millimeter in width, rarely any bigger; this being the normal diameter of blood cells. All had regained their yellowish red tint specific to this species of blood element. Finally, in adding one or two drops of acetic acid to each preparation, the blood cells paled and gradually dissolved as in samples of fresh blood.

It was possible in the previous examination to arrive more quickly at the swelling and dissociation of the red blood cells
379 of the fragments of crust of a stain by adding a bit of water to the liquid used. Indeed, this liquid is prepared for preserving the elements of fresh blood; its action on those that have been dried is thus slow and even incomplete. But the addition of a small amount of water, slipped between the two glass slides of the microscope preparation, renders its action more prompt without removing any of the characteristic attributes of the isolated blood cells.

The *second series of preparations*, made with the three preceding stains, was done as follows. After scraping of the small crust on their surface, there remains beneath a small stain, paler, without projecting above the surface of the fabric, reproducing the size and form of the crust. Then, after cutting the surface of the fabric thus stained, with either scissors or a very sharp bistoury and dissociating the whole in the liquid used in the first series of preparations, the dissociated filaments were left immersed in it. In examining the cotton threads dissociated in the liquid after the same lapse of time, the characteristics of form and volume distinguishing the cotton filaments already pointed out above were evident. Also present was the blue indigo tint with which they were impregnated by the dye. But, in addition, we determined that many of them, in part or all of their length, were covered by a single layer of red blood cells or by small reddish agglomerations formed by blood cells of this type, accumulated and adhering to each other.

It was even easier here than in the first series of preparations to isolate red blood cells, to detach them from the surface of cotton filaments with the same maneuvers of pressure and sliding the glass slides. The characteristics of flattened, biconcave form, the volume, color and reactions on contact with acetic acid were all easily determined.

380 On the greater part of the filaments, moreover, it was possible to identify red blood cells before they were detached. They were perceived as having become slightly polygonal by reciprocal contact, but retaining their normal colors and dimensions, forming a layer on the surface of the cotton filaments.

The blood cells presented themselves to the observer sometimes face on, sometimes sideways, at other times they were seen adhering to the cotton filament in half their length, while the other half being free, projected outward, showing its circular, flattened form.

Thus, here again, no doubt remained: it was evidently red blood cells before us, an element found absolutely only in blood and it was the blood cells of mammalian blood and not of duck or any other species of bird.

This conclusion was further verified by the reaction to the addition of excess water, or a small quantity of acetic acid, to the preparation placed under the microscope. The red blood cells adhering to the cotton filaments disappeared under our eyes by the effect of these agents. Those which had been detached and the more or less irregular, voluminous accumulations formed by these blood cells existing here and there between the filaments, could be seen, as in the normal state, at first to swell, while paling at the same time, then gradually to fade by dissolution and soon to disappear altogether.

§ VII. Examination on the same blood stain: 1) of fibrin and white blood cells; 2) of red blood cells

With two other blood stains, one circular, of a width of one millimeter, the other oval, of the same width as the preceding, but two millimeters long, we proceeded as follows:

The small crust, red brown, served to make the first of a 381
series of two preparations. For this, it was placed for some time in pure water, after having been removed by scraping. It was left there until almost completely discolored, and was then dissociated with the needles designed for this purpose (see § III). Examined by microscope under a magnification of 514 diameters, all the characteristics of fibrin on one hand, and those of white blood cells on the other, as we have described above (see § IV and § V), could be recognized in the discolored fragments.

Having proceeded with these fragments of red-brown crust as for those of other stains, it was not astonishing to find the same blood cells retained between the thin fibrils of the web of fibrin.

Having placed some of the fragments of the red-brown crusts in the preservative liquid used above, they preserved their color and became slightly swollen. But by addition of excess water in one of the preparations and of a small quantity of acetic acid in the other (which was slipped between the two glass slides of the preparation according to standard procedure), these fragments were seen to swell a bit more, become pale, then completely dissolve before our eyes. Those treated with water left only a thin web of fibrin surrounded by a halo of liquid, weakly colored in yellowish red by the coloring matter of blood, which the water held in solution.

The small russet stain remaining beneath the crust removed from the surface of each of the two stains, and having

the same form as them, provided another series of two preparations; but these preparations served specifically to see the red blood cells and in order not to destroy them to observe
382 the fibrin and white blood cells, we proceeded otherwise than in the first. We used here *preserving liquid* 4_a mentioned in the preceding paragraph (see § VI). Having removed the surface of the stained fabric with a sharp bistoury, we dissociated it in this liquid and examined its filaments under the microscope, after leaving them in this same liquid for a few hours, either pure or diluted with about a tenth of its volume of water.

The red blood cells adhering to the surface of the cotton filaments were thus rendered perceptible (see § VI, the description of the *second* series of preparations, p. 379).

By the same procedure of sliding the glass slides, we could isolate the red blood cells from the filaments, so as to determine their characteristics of form, again generally circular and flat, of biconcave disposition, of the volume, the color and reactions already indicated. It is important to note that the central spot of the red blood cells of man, indicating the central depression of the two faces, in a word their biconcave disposition, is less pronounced in blood cells which have been dried and then softened than in fresh blood cells. In a word, these anatomic elements, after softening and isolation in blood stains, do not regain a biconcave form as pronounced as they show in the fresh state. But this property does not hinder their easy recognition, when one has already observed human blood in its various conditions.

It is thus evident that, with a single stain, the existence or absence of the three most characteristic constitutive elements of blood can be determined by microscope. The superficial crust should serve to determine the characteristics of fibrin and white blood cells; whereas we maintain that the subjacent threads of fabric, between which the blood serum has infiltrated, carrying with it red blood cells,
383 should be reserved specifically for demonstrating the existence of red blood cells.

§ VIII. Examination of the characteristics of blood cells in stains formed by the blood of a duck

In response to the questions posed to us by the rogatory commission of the examining magistrate, we let the blood flowing from the carotid arteries of a duck we had decapitated fall in a shower of droplets onto a blue cotton smock. Having left these stains in a dry place for two weeks at the normal temperature for this month of January, 1857, we proceeded with our examination, adopting exactly the same methods as for the examination of the blood stains on the smock of the suspect Doiteau.

After treatment of the small detached crusts of the blood stains of duck with the preserving liquid (4_a, see § VI), a certain number of oval-flat blood cells could be isolated, at least twice as large as those of man, and bearing in their center a small, oval, elongated nucleus, not less characteristic of the blood cells of fowl than the elongated, oval form of the cells themselves. This small nucleus soon became very evident, with clear, well delineated edges, under the influence of excess water and of acetic acid which consistently produces the effect of dissolving the reddish body of the cell, and leaving the greyish nucleus intact without any specific color.

After treatment of the small crusts detached from the blood stains from a duck with pure water, they gradually discolored, becoming greyish. For a relatively long time, they remained surrounded by a layer or halo of liquid colored in pale blood-red by the coloring matter of the blood cells, extracted by the water from the mass of accumulations of blood cells submitted to experimentation. Once the discoloration is practically complete, it was determined that no 384
evident fibrinous web remained in place of each fragment, as was the case in the blood stains suspected of having come from the body of a woman.

There remained only a considerable number of oval, greyish nuclei, without any coloring specific for blood cells of duck. These nuclei were 5 to 6 thousandths of a millimeter long, half of this diameter wide and thick. They were all very close to each other, the greater part remaining agglutinated by a small amount of uncolored substance, in which the fibrillary aspect specific to fibrin could be confirmed only with difficulty. Acetic acid soon rendered the nuclei darker, their edges blacker; at the same time tightening them and rendering them a bit less regular, the usual action of this reagent on fresh blood cells of birds.

It was impossible to identify white blood cells in this mass of nuclei remaining after the action of water and of acetic acid on these fragments of crust removed from the blood stains of duck.

Thus: 1) the oval form and the doubled volume of the blood cells here examined compared to those encountered on the stains of the smock of the indicted; 2) the absence of nuclei in the latter, the usual case in man, and the presence of oval nuclei in the blood cells of all birds, does not permit us to acknowledge that the blood stains on the smock submitted for our examination are formed from the blood of duck or any other fowl. These characteristics permit the easy identification of the nature of the blood of man in one case and of the blood of bird in the other, with no possible confusion, since the flat, circular form with the absence of nuclei is constant in blood cells of man after birth, whereas the flat oval form with a central oval nucleus is constant for every 385
blood cell in birds.

In addition, the differences derived from the almost complete absence of the fibrillary fibrinous web in the blood of birds, as compared with that of man, in which this web is abundant; the small number or absence of white blood cells in the blood of the first as compared to their notable quantity in the fibrillary web derived from human blood stains: here are distinctive characters which, although of second order, have a value not to be neglected by the expert.

It is useless insisting on an understanding that these observations should be considered as the most delicate of those which a legal physician might be called on to make, in that

all these characteristics are obvious only after repeated observations of blood of diverse animals in diverse conditions, and to persons accustomed to judging the value and precision of the nature of these facts.

§ IX. Examination of other stains of the smock, less colored, presumed to be of the same nature of the first, i.e., presumed formed by blood and attempted to be rubbed out or diluted a little while after their formation by some type of rubbing or washing.

We examined microscopically either the powder, or the filaments of the fabric of the smock, detached from the large stains of the sleeves, the front and the back of the smock, stains of a reddish-brown, almost the color of rust, or analogous to those which might be formed by blood which was wiped, half-washed or rubbed with earth.

It was immediately recognizable that the isolated, free microscopic fragments, as well as those still adhering to the filaments of blue cotton, were composed of small, irregular,
386 polyhedric, angular grains of multiple facets, unstructured in their reciprocal disposition. Some of these grains were without any specific coloring, greyish or uncolored in the center, more or less brilliant, with thick, blackish contours.

Their diameter varied from 5 to 70 thousandths of a millimeter and more. Water was without effect on them; acetic acid, added to the preparation, scarcely attacked them, releasing a few bubbles of gas from their substance. Only hydrochloric acid dissolved them rather rapidly with release of a certain quantity of gas.

Others of these irregular grains, a bit less in number, presented the same irregularities of form, but were of a rather brilliant red-brown tint, which can be noted by microscope in various oxides and especially iron carbonates. These irregular, red-brown fragments had a diameter varying between 4 and 35 thousandths of a millimeter. Water had no effect on them, acetic acid, added to the preparation had an effect, but very little, only at the end of several hours. Such that, in this report, no more than in the preceding, there is nothing comparable to the fragments from the crusts of blood stains. These irregular grains were, on the contrary, quite rapidly attacked by hydrochloric acid in the same way and in the same time as the uncolored grains mentioned above with which they were mixed.

The characteristics just mentioned being those that the microscope exhibits with the majority of terrestrial powders, and having none of those which this instrument, with the aid of chemical reagents, exhibits in blood, it was necessary to investigate their nature and their composition with the help of the appropriate reagents furnished by science.

To achieve this, we proceeded as follows:

First, thirteen stains of a red brown were removed with a
387 sharp bistoury. The irregular ones were spread out; the others were small, round, 1 to 3 millimeters wide, superimposed on the large, less-colored, russet-like stains on the lower part of the right sleeve. We didn't touch the left sleeve.

The same operation was performed on four small stains, 1 to 4 millimeters wide, of a brown-red, on the back of the smock, which were not used in our previous operations, and which were also evidently superimposed on the large, less-colored, russet-like stains of this part of the smock. We left the front of the smock intact. The matter thus removed, and submitted to examination by microscope and with the reagents already employed, showed successively the characteristics of fibrin and white blood cells retained in its thickness, and red blood cells, a constitution similar to that of stains that the same examination indicated as being formed from blood. The paler stain remaining after removal of the small, obviously red crusts previously indicated, while having the form and dimensions of the others, no longer showed blood cells adhering to the blue cotton threads, as had been seen on the analogous stains taken from the cleaner portions of the smock. We found here only fragments or irregular grains of powder, some greyish, without any specific color, the others of a deep red, such as those described in the parts of the large, less-colored, russet-like stains bearing none of the small stains of a dark red-brown.

This done, the large, *russet-like stains less colored* than the small one, as many of them on the lower part of the right sleeve as on the back of the smock, were carefully scraped with a scalpel. A large porcelain capsule received the powder falling from them. This was then collected in the capsule by washing the capsule with hot distilled water. The murky, clouded water thus obtained was left to cool until the follow- 388
ing day. As a result it separated into three parts:

1) The *first part,* floating on the surface of the liquid in the tube, was flocculent and blue. Submitted to examination by microscope, it was shown in be composed of filaments of cotton tinted in blue, accompanied by irregular particles presenting the aspect of the grains of the powder described at the beginning of this section and not being affected by water, Moverover, these particles were present in such a small amount that it was useless, in the presence of the facts which follow, to do a special analysis. We then rid ourselves of this flocculent magma of cotton filaments tinted in blue.

2) Separated from the liquid, and deposited on the bottom of the tube, was a finely granulated powder forming a thick layer of 8 millimeters in our tube, which was 15 millimeters wide. Decantation isolated the powder from the water from which it had separated by gradually depositing, because of its more considerable specific gravity. Examination by microscope showed it to be formed entirely of irregular corpuscles, some grey, without any specific color, others of a red brown of oxide or iron carbonate, such as those discussed at the beginning of this section. A few, adhered to the microscopic filaments of cotton, which had been carried along to the bottom of the water in depositing.

This powdery deposit was placed aside for submission to an analysis, the details of which will be developed later on.

3) Finally, the liquid interposed between the blue, flocculent magma of cotton filaments floating on the surface and the powder deposit described earlier was examined separately after decantation. This liquid was uncolored, but of

a cloudy tint of a bluish grey. The microscope soon showed
389 that this cloudiness was due to very short, broken, cotton filaments suspended in the water and also tinted in blue. This liquid was submitted to boiling, which caused neither any new cloudiness, nor coagulation, nor clarification. The liquid, not having changed its aspects, was filtered and then became perfectly clear. Heated again, it remained as such. This liquid was also put aside, to undergo a special analysis, the results of which will be presented in the following section.

From this preliminary examination, we have already been led to several conclusions which it is important to point out at this moment for they guided us in the use of the methods of analysis remaining to be presented, and which served only to confirm these conclusions.

1) Introduction of the powder derived from the large, russet-like stains into water did not change the aspect of this powder nor color the distilled water in red or wine rose. The examination by microscope, which hadn't shown any elements of blood in these powdered particles, was thereby confirmed; for the powder derived in infinitely less quantity from the scraping of blood stains was enough to noticeably color an equal amount of distilled water.

2) The absence of coagulation in the liquid submitted to 100° temperature, before and after filtration, showed that it held no albuminous substances in solution.

Consequently, this chemical examination already demontrated, as had the microscope, that it was a matter of mineral powders only, of an origin outside the human body, and not of blood stains which were half-washed or submitted
390 to rubbing or incomplete wiping.

§ X. Concise exposé on the chemical analysis of powder coming from the stains, which had been considered by their russet-like tint as possibly formed by blood which had been washed or wiped.

The elements which distilled water had removed from the powder of the stains were first determined.

For this, it was divided into three parts in as many different tubes.

Silver nitrate gave in the first an appreciable white, flocculent precipitate which dissolved in ammonia, indicating the presence of a small amount of soluble chlorides.

Barium chloride produced in the second tube a very abundant white precipitate, which did not dissolve after acidification of the liquid with a little sulfuric acid. This reaction showed there existed an appreciable quantity of soluble sulfates.

In the third portion of the liquid, a very light blue color was obtained by addition of potassium cyanide, indicating traces of iron peroxide salts. This tint became a bit dark only after evaporation by heat reduced by half the small quantity of liquid tested.

Water, acidified by hydrochloric acid, was poured on the powdered deposit already discussed, and set aside for a future analysis. The whole mass was seen to dissolve in the space of about an hour, producing the release of small bubbles of gas. The small amount of matter precluded its collection; but everything pointed to the conclusion of carbonates decomposed by hydrochloric acid, displacing carbonic acid.

The resulting solution offered a very light bluish tint; the 391
liquid thus obtained was still appreciably acidic for it obviously reddened litmus paper. It was divided into three equal portions in separate tubes.

In the first, addition of a small amount of barium chloride gave no precipitate; but the addition of ammonia to the point of neutralization produced an abundant white precipitate of barium phosphate. A white flocculent precipitate was also produced when excess lead acetate was added to another portion of this liquid. These characteristics indicated a certain amount of phosphoric acid, most of which had combined with lime, to form irregular microscopic grains of mineral powder observed by microscope and deposited in distilled water. The following reactions attempt to prove this:

In the second portion of liquid, excess potassium oxalate was added, which immediately produced an abundant granular precipitate, rapidly collecting at the bottom of the tube, and formed by oxalate of lime.

Finally, in the third portion of the liquid, addition of yellow potassium ferrocyanide developed a very pronounced Prussian blue color.

As in examination by microscope, these reactions demonstrated 1) that the powdered matter derived from the russet-like stains, less colored than those presenting red blood cells and fibrin, were not composed of elements of blood nor by other substance of animal origin.

2) that this powder was composed of cotton filaments in small quantity, but principally by mineral substances, such as are generally found in the greater part of terrestrial powders.

3) that these latter substances were irregular grains, some
392 greyish, with no specific color, formed mainly by phosphate and lime carbonate, with traces of soluble sulfates and chlorides, and probably also lime sulfate.

4) that the remaining irregular grains, less abundant than the preceding, but giving the powder its tint of russet-like grey, were undoubtedly composed of iron oxide and carbonate, whose presence the reagents disclosed in a quantity much more considerable than that contained in any animal substances.

5) that consequently, the less-colored stains, apparently rubbed or diluted by some kind of rubbing or washing, were not produced by spread-out blood, but by powder or mud, soiling the smock of the accused, before perpetration of the crime.

In addition, this conclusion is confirmed by the fact that on the stains identified as being formed by mud, the existence of superimposed blood stains could be demonstrated, presenting the same characteristics as those found on stains scattered on the surface of the unsoiled parts of Doiteau's smock.

§ XI. Response to the questions posed by the examining magistrate[1]

Firstly, recognition that the greater, russet-like stains, the lightest in color, are not composed of blood, but by terrestrial matter mixed with particles of iron oxide and carbonate forming rust, permits the response:

Yes, blood could have spurted from the carotid arteries of a decapitated duck in great enough quantity to form or explain the formation of the numerous but small stains actually composed of blood on the sleeves, the front, and even the shoulder and back of the smock.

393 *Secondly*, and *a fortiori*, these multiple droplets, whatever their form, could come from the arteries of the soft tissue of the head of a sixty-eight-year old woman, when these arteries are torn or cut by violent blows delivered to the head with cutting and contusing instruments: and this is strengthened all the more since examination of the indicated blood stains peremptorily demonstrated that, by their constitutive elements, their nature was that of drops of human blood, having none of the characters found in the blood of ducks.

The undersigned sworn experts can then resolve the questions posed in regard to this smock as follows:

1) Yes, the dark stains of the smock, bordering on red brown, are of blood. They are formed by blood, without the water which renders it fluid, because this water was released by evaporation after the blood left the vessels.

Indeed, only in blood are found the red blood cells we succeeded in isolating from these stains; only in blood are fibrin and white blood cells, which were identified in the thickness of the web it formed, and the red blood cells which were isolated grouped together.

Only the microscope could have decided this question, because these stains were too small for it to be possible to demonstrate the existence of blood albumin. Besides, it is known that albumin or analogous albuminous principles with the characteristics found on large blood stains can be encountered not only in a large number of animal but also in colored and uncolored plant sap. On the contrary, only blood offers together, simultaneously, fibrin, flat, circular red blood cells without nuclei, and spherical white blood cells
394 furnished with one to three nuclei after the action of water or of acetic acid.

2) No, apart from the stains which appear to the naked eye to be blood stains by their form and color, there are no other stains on the smock of the same nature, but less colored, which would have been incompletely effaced or diluted a little while after their formation by some sort of rubbing or washing.

Indeed, examination of the substance of these large stains, less colored, russet-like or bordering on yellow, demonstrated they contained none of the elements of blood. This same examination by microscope, completed by chemical analysis of the substance retrieved from the indicated stains by scraping, demonstrated they were formed by irregular grains of a mineral nature. Some were composed of insoluble calcium salts, such as phosphates and primarily, carbonates, with traces of soluble chlorides and sulfates. The rest, a deep red brown under the microscope, were composed of iron oxide and carbonate, elements composing rust, often encountered in mud or other soiling matter. But on these mud stains, or at least russet-like stains, were found superimposed blood stains, presenting all the characteristics to the naked eye and by microscope indicated above found in the stains of the aspect scattered on the unsoiled parts of the smock.

3) The stains which were actually of blood, without its water, were not in great enough quantity to be able to conclude that splashes of blood from a fowl could not have produced them. Blood spurting from the carotid arteries of a decapitated fowl, could, if this fowl were not on the ground, rise high enough so that some one facing the person killing the fowl could receive blood in places as multiple and diverse as those on the smock submitted to us.

4) But the elements of blood composing these stains 395
are not those of the blood of duck. These elements have, on the contrary, all the characteristics of elements composing the blood of man. They do not have the flat, oval form, nor the volume, nor the central, ovoid nucleus found in red blood cells characteristic of fresh or dried blood of ducks and other fowl.

5) The elements of blood forming the stains of the smock are elements of blood belonging to the human species. Fibrin is found, with its fibrillary aspect, its reactions on contact with acetic acid, etc. White blood cells are found with the volume, form, granulations, nuclei and chemical reactions found in the white blood cells of the blood of man. Red blood cells are found with the volume, the flat, circular, biconcave form, the rosy yellow color specific to those of man viewed by their transparency in the microscope and dissolving like them in water and acetic acid without leaving a trace of nuclei.

But it is impossible to say more with our present level of knowledge; it is impossible to determine with this blood the sex or age of the individual from whom it originated.

6) Yes. Finally, these blood stains, for they are stains of human blood deprived of its water after dessication, scattered and small as they are, could manifestly have been produced, there, where they are, present on various parts of the smock, by the spattering of blood from veins opened by a violent blow. Or, better, from the spurt which arteries give off before death during a murder where one man, armed with a cleaver, a bill-hook or a spade, would have delivered fifteen blows, or even less, to the head of his victim.

7) The undersigned medical experts, in presenting their
methods in their report, demonstrate, by the detail into which 396
they have gone, that the means of verification employed by them in the execution of their assessment offers safeguards, a security and a precision superior to means employed up to the present. Indeed, the microscope alone permits seeing, not the albuminous or ferruginous elements of blood, but its very composing elements, which are most characteristic, permit-

[1] See § I, pp. 369-371.

ting the claim that a liquid is blood and not any other liquid. Only the microscope permits the determination with a single stain, be it only a millimeter or more in diameter, of the existence and all the attributes of the three solid parts, the most characteristic, of blood, namely: fibrin, red blood cells and white blood cells.

Finally, the safeguards and security of precision of the procedures they employed are emphasized with the very strong evidence of the following fact. This fact is that only examination by microscope can determine if the indicated stains are formed by blood of duck or of man, because, as we have just pointed out, it shows the very elements which float in blood, giving it its color and its other characteristics. These elements, then, differ between man and birds, reptiles and fish by their form, volume and intimate structure, of which only the microscope permits the determination. And it shows them, wherever these elements might have been deposited, unless putrefaction has set in.

Besides its application with an equal precision on small stains, even on one small stain, and on large stains, this method offers still other advantages over those generally employed up to the present. Indeed, these latter are based only on examination of the coloring substance of blood, on that of the iron it contains and on that of albuminous matters of blood soluble in water. These elements are identical, with no possible differentiation, with regard to color, reactions, 397
etc., in the blood of man, birds and other red-blooded animals; such that the questions posed to us, relative to the nature of the stains, concerning the determination as to whether they are formed by the blood of man or of duck, remained absolutely insoluble without the use of the method of verification we had adopted, either as a method in itself, or in using the older procedures as a simple adjuvant to the microscope.

References

1. Ch. Robin: Sur la distinction, à l'aide du microscope, de la matière cerebrale, de l'albumine, du fromage et du jaune d'oeuf; observations publiées à la suite d'un mémoire de M. Orfila, intitulé: Recherches médico-légales sur la matière cérébrale desséchée, tenteés, à l'occasion de l'assassinat de Louvet, par Gontier (*Ann. d'Hyg. et de méd. lég.*, v. 44, p. 190, avec une planche gravée).
2. This procedure must also be followed when examining stains of semen, of vaginal, nasal or urethral mucus and of meconium or fecal matter. See. Ch. Robin and A. Tardieu, *Mémoire sur l'examen microscopique des taches formées par le méconium et l'enduit foetal*, vol. VII of the 2nd series of this collection, 1857.

Memoir on the Medico-legal Comparison of Stains of Menstrual Blood and Other Types of Blood Stains*

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421 **§ I. Preliminary observations on the question to be resolved in this work.** In the preceding memoir, we made known under what circumstances we were committed by Mr. Edward Choppin, examining magistrate of the borough of Chartres (Eure-et-Loir), to compare stains of mentrual blood with other types of blood stains.

It was a question here of making a positive determination of whether blood stains indicated by the accused as coming from menstrual blood were actually of this origin; whether they were not formed rather by human blood coming from
422 wounds produced by telling blows having death in mind; or finally, whether there were not stains of both types of blood simultaneously on the same sheets and shirts.

It has been impossible up to now to resolve questions of this kind. Procedures available to legal medicine for the differentiation of blood stains of a menstrual period from other types of blood stains are limited to but one, that of Barruel. This procedure consists of pouring concentrated sulfuric acid on the blood, and its origin is recognized by the odor *sui generis* which is released, an odor different between one animal and another, different also between the blood of man and the blood of woman. But this procedure has been rightly considered, by its author and by forensic physicians, as liable to give only presumptions on the origin of blood stains and not proof leading to legitimate conclusions. We could not find any work mentioning its use in a legal assessment. We thus believe it has never served to resolve a question of the type for which we had to find the solution.

As we had succeeded experimentally in determining in a precise manner the characters distinguishing the types of stains we have been discussing from each other, we considered it useful to publish the results of our research. Our experiments were performed as a result of the questions which had been posed to us. As these are summarized in a way by the description of the procedures which we definitively adopted for the study of the evidence submitted for our examination by the examining magistrate of Chartres, we will limit our presentation to the steps we followed and the results we obtained. We will, however, do a preliminary presentation of peculiarities relative to menstrual blood under normal conditions with which it is necessary to be familiar to understand the value of our medico-legal ex- 423
periments. As is known, this matter has already been the subject of very precise observations by microscope by Pouchet.[1]

§ II. Examination of the anatomic constitution of menstrual blood. At the beginning of a menstrual period, when the mucus which flows from the vulva or just moistens the labia begins to stain the linen in reddish brown, it is found by microscope to be constituted as follows: In a more or less viscous, finely granulated liquid are seen some not very regular, prismatic epithelial cells and some nuclei of nuclear epithelium resembling that of the uterus, undoubtedly coming from the mucus of the uterine body and cervix. But especially found here is cuboidal epithelium of the vagina, of finely, uniformly granulated cells, the greater part of which are furnished with nuclei, sometimes nucleoli, and which we don't have to describe here, as they should be known in all their conditions to anyone working in medico-legal research. Leukocytes (*pus or mucus corpuscles*) are also seen in greater or lesser quantity, according to the subject, especially numerous in women in whom catamenial congestion determines the appearance of *leukorrhea* or purulent mucus. Finally, a certain quantity of red blood cells are found, to which the reddish hue of the liquid is due.

After twelve to twenty-four hours of this weak discharge, which may or may not be followed by an interruption which can last an entire day, the uterine hemorrhage gains its full intensjty.

The liquid flowing from the vulva, collected from the vulva with a curette or spatula, is much more fluid than before, because of the amount of blood serum, mixed in with the 424
mucus. However, the same elements we have been discussing are always found here; the red blood cells nevertheless outnumber the epithelial cells and leukocytes. The delicate fibrillary web formed by coagulated fibrin is not produced in the blood, though it is observed in drops of blood, a bit voluminous, drawn from the finger for examination by microscope, a fact resulting from the influence of mucus on blood plasma and particularly on fibrin. It is not uncommon, however, to see red blood cells united in stacks between the epithelial cells or the bits of epithelial cells which accompany them, as they do in blood drawn from a finger or in a bloodletting. However, a certain number of molecular granulations are always found in the muco-serous liquid in which

* Translation of: "Mémoire sur la Comparaison Médico-légale des Taches de Sang Menstruel et des Autres Espèces de Taches de Sang." In *Annales d'Hygiène Publique et de Médecine Légale* 10 (2nd series): 421-434 (1858).

[1] Pouchet: *Theorie positive de l'ovulation spontanee.* Paris, 1847, in-8, p. 241-244 et atlas, pl. XII et XIII.

these elements are floating, such as those encountered in most mucus, that of the uterus in particular, and which is lacking in blood serum.

After a duration of two or three days this blood flow becomes thicker, regains a mucus consistency, and at the same time loses the pure red color it presented. It takes on a reddish or reddish-brown hue. The nature, consistency and viscosity of the liquid holding the anatomic elements in suspension, becomes apparent. The molecular granulations are once again abundant, as well as mucus corpuscles and the different varieties of epithelium, as much nuclear and prismatic as cuboidal; the latter, however, are always more numerous. These cuboidal cells, as those of the vagina in any other condition, are for the most part provided with nuclei; but a few of them are lacking in nuclei. Many are wrinkled, and sometimes others are grouped together in pieces or lamellae of overlapping cells, sometimes large enough to be
425 perceptible to the naked eye. In bloody mucus flowing at the end of menstruation, it is not uncommon to also find flakes or filaments or homogeneous or striated uterine mucus, including within their thickness ovoid nuclear epithelia similar to those described above. At this period, the red blood cells no longer gather in stacks and are small in number. In all stages of menstruation, moreover, the number of mucus cells varies widely from one subject to another and some show almost none at all.

§ III. Examination by naked eye of the stains whose nature we were to determine. The shirt under examination is an old women's shirt, patched at the right shoulder, and mended in several places; it is marked in front on the bosom with the letter "M" in partly discolored blue thread.

Numerous blood stains exist on this shirt which is reddish in front and in back, inside and outside, from the waistline to the lower border. However, in considering attentively the disposition of these stains, it soon appears that, despite their number, almost all of them have a common origin in the interior of the fabric of the article of clothing, in that part of the shirt corresponding to the position of the genital organs of a woman.

Whence, three types of stains:

Stains of the first type. Thick stains starching the entire thickness of the shirt, with a possibility of coming as much from the outside of the garment as from the inside and of a very deep red color. These stains are found especially on the back of the shirt, where they occupy an extremely considerable space of about 18 centimeters. In this part, the stains are not isolated, and despite the vertical pleats of the shirt where the color is a bit less dark, they evidently form one bloody sheet, in the middle of which some dried clots are encountered towards the inside. Stains of this type, but a bit
426 less extensive, can be noted also: 1) in the front of the shirt toward the middle; 2) on the lower border in front; 3) on the lower border in back, but these last stains, instead of presenting a bloody sheet extending about the same distance in all directions, are, on the contrary, very long with no width, as if they resulted from imbibition of a thin trickle of blood.

Stains of the second type. Thick stains, evidently produced by bloody imbibition from the inside to the outside, also starching the material of the garment, and of a deep red color. These stains exist only on the inside of the shirt. They are more numerous in back than in front. They occupy, on the limit of the preceding stains in back, a rather considerable space, but with a lesser surface, and present a large number of gaps. Many of those stains have an irregularly rounded form, and seem to have been produced by dried drops of blood.

Stains of the third type. Stains not starching the material of the shirt, existing only on the inside of the shirt, without imbibition of blood, of a somewhat deep pale-rose color. Just about all these stains are very long from top to bottom, and measure scarcely 2 to 3 centimeters in width. They are individual, separated from one another by not very large vertical spaces in which the fabric of the shirt takes on a soiled yellow color. They occupy especially the inside back of the shirt, a part of the lower border on the back, and in front they merge with some yellowish stains apparently due partly to imbibition by the material of urine or vaginal mucus.

On the bed sheet were found not very numerous, rounded blood stains, successively placed one after the other similar to those described above under the designation of *stains of* 427
the first type. It is useless then to reproduce a detailed description here as we have already written it.

§ IV. Examination by microscope of blood stains claimed to have been formed by menstrual blood. To determine the nature of stains we had to study here, we proceeded in the same manner as if it had been a matter of pure blood stains. We won't review the methods employed for this, for they are absolutely the same as we had presented in a work previous to this one. (Ch. Robin and Salmon, *Mémoire concernant l'examen de taches de sang a l'aide du microscope, Annales d'hygiene et de medicine legale,* Paris, 1857, vol. VII, §§ III and VI; and in Briand, Chaude and Gaultier de Claubry, *Manuel complet de médecine légale,* Paris, 1856, 6th edition, pp. 705, 707, 805 and 807.)

After the strips of fabric bearing the stains, which were softened by the procedure just mentioned, were scraped with a scalpel and placed under the microscope, numerous filaments of hemp were perceived. These were surrounded by small masses of red hue, on the edges of which red blood cells adhering to each other, a bit deformed, but still flat, could be recognized. Some were isolated and showed their biconcave form or were concave on one side and convex on the other. These blood cells had become pale, had swollen on contact with water and dissolved after addition of acetic acid. In similar strips softened in pure water, microscopic filaments of hemp were surrounded here and there by small red masses or magmae, finely granulated, in which the fibrillary disposition of fibrin, which are shown by clots of stains of pure blood treated with pure water, could not be recognized. The peculiar red hue of these masses, however, permitted the confir- 428
mation of the presence of red blood cells which had been

softened, partially dissolved, and rendered individually unrecognizable by the water.

Numerous irregular granulations of various hues, some of which were soluble in acetic acid and presented other characteristics specific to microscopic granulations of dust in general were seen between the filaments of hemp or even adhering to their surface.

It is now important to point out that, here and there in the preparation or against the filaments of hemp, were found some cuboidal epithelial cells, some seen face on, polyhedric and regular; others, seen from the side, presented one of their edges. Many were wrinkled and shriveled as often happens; but it was possible to flatten a rather large number of them by pressure of the glass slides. Many of these cuboidal epithelial cells were grouped in epithelial strips or plaques formed by cells overlapping each other. Certain of these plaques or lamellae were pleated or folded over, which rendered the determination of their nature by examination of the constitutive cells a bit more difficult. They can, however, be flattened by suitable movements of the slides, and naturally, flattened cells, easy to study, are also found. Despite their former dessication, these cells can be determined as being finely and uniformly granulated as are those of the vaginal wall and uterine cervix. These greyish rounded granulations, grouped together, are absent in cells of cutaneous epidermis, as is known, or are much less numerous and less regular.

Other than these cells, a few ovoid nuclei of nuclear epithelium are found, finely granulated, about 9 thousandths of a millimeter long, 6 to 7 thousandths wide, similar to those of the uterine mucus.

429 Addition of acetic acid rendered still easier the identification of these characteristics of nuclei and epithelial cells. This reagent causes the disappearance of blood cells or the masses they form, as well as a part of the granules of dust which mask the cells somewhat. At the same time, it swells and pales the cell and brings the nuclei as well as the fine granulations surrounding them into relief.

It also renders the epithelial plaques, formed by overlapping cells, easier to study, making the overlapping more easily recognizable, as well as the nuclei of the cells which cover these overlapping cells. It renders strikingly evident the analogy of these epithelial strips with those obtained by lightly scraping the mucosae, as that of the vagina, for example.

After the action of acetic acid, in addition to the finely granulated, nucleated cells of which we have just spoken, a few cells without nuclei were noted, much less numerous, entirely, or almost entirely, lacking in granulations. They preserved on their surface the folds or projecting lines corresponding to lines of juxtaposition of overlapping cells to which they were adherent, peculiarities found particularly in desquamated cutaneous epidermal cells. These last cells were, at the same time, smaller than those provided with nuclei and finely granulated as are those of the vagina and other mucosae.

If it is imagined that the stains observed here were formed on a shirt in contact with the trunk and thighs for a long time, it will be found quite natural to see desquamated cutaneous epidermal cells retained by mucusy bloody stains of the fabric, and mixed with cells of these stains formed of blood cells and vaginal epithelium, or at least similar to that of vaginal mucus. All these characteristics are easy to determine for anyone used to the comparison of epithelia. 430

The stains whose constitution we have just studied were the least colored of all. They were the color almost of rust or of a reddish brown, staining the linen in the manner of a liquid penetrating by imbibition.

We then studied in the same way stains mixed with the preceding, or neighboring them, presenting an irregular contour, thicker, and truly red.

Now, we found absolutely the same elements as in the less colored stains. The blood cells were much more numerous, but the fibrillary web specific to fibrin could not be discovered in either one or the other. Cuboidal epithelial cells were found in rather considerable quantity, and were especially easy to recognize after addition of acetic acid, as well as ovoid nucleated epithelia, but these were less abundant.

On none of these stains, before or after addition of acetic acid, could be found *mucus corpuscles* or *white blood cells* presenting characteristics distinct enough for a description or a determination of their presence with certainty. These blood stains scarcely soaked through the thickness of the fabric, i.e. scarcely colored the outside surface of the shirt. An anatomic element was nonetheless sought, the presence of which might be useful to determine. A few epithelial cells without nuclei were found here and there with very fine lines projecting from their surface and presenting the other characteristics noted as specific to cutaneous epidermal cells fallen by natural exfoliation. These cells were present in very little number; only about 2% were found on the other sur- 431
face, on the same portion of the stain which was in contact with the external genitals.

Some rare nucleated cells, similar to those of epidermis, were also found on the unstained parts of the shirt, in the intervals between the stains. But here none of the cells presented finely granulated nuclei analogous to those found in the vagina or the labia minora, whose characteristics were established above.

From the facts observed above, from the presence of elements of blood mixed with those of mucus of the genital tract, i.e., with elements of the mucosal epithelium, we can conclude that these blood stains just studied were actually formed by blood of a menstrual period; for similar characteristics are found in normal menstrual blood. The latter, as is known, is an intimate mixture of blood proper, with mucus furnished by the genital tract and by the vagina in particular. Now, this mucus, holding epithelial cells principally and leukocytes in a more or less large quantity in suspension, causes these elements to be added to those of blood which does not normally contain them in vessels. It is easy to identify them, either in fresh blood or in dried stains, and the

nature of these stains thus reveals their origin as well as the organs from which the blood has escaped.

§ V. Examination of small, round, thick blood stains found on a bed sheet. After treatment and examination of these stains in the same manner as the preceding, the presence of red blood cells, white blood cells and fibrin specific to blood stains proper could be determined. These characteristics are so identical to those we have described in analogous stains in our previous memoir on this subject that it is useless to
432 reproduce their description here. (Cf. Robin and Salmon, *loc. cit.* §§ IV, V, VI, VII and Briand, Chaude, and Gaultier de Claubry, *loc. cit.*, pages 806, 807 and 808).

But it is important to note that these stains were completely deprived of epithelium, and despite intensive research, no cells whatever could be found, analogous or not to those found so easily in stains actually formed by menstrual blood.

It could be that, in cases where the menstrual period flows abundantly, exfoliated uterine and vaginal epithelium having been carried along, blood accumulated in the vagina during sleep thus falls in thick drops containing only the anatomic elements of blood. Consequently, we cannot conclude anything from the facts observed above in the case at hand, if not that the drops in question are constituted by blood; but it is impossible to determine their origin from the elements constituting them.

It is important to remark that stains thus constituted, found mixed with stains, some of which are deeply colored, others paler, such as those identified in the preceding paragraph as *actually formed by menstrual blood,* do not invalidate this determination by their presence. A mixture of bloody stains containing only elements of blood and of stains containing, in addition, those of the mucus of the genital tract, does not prove that these stains come from elsewhere than uterine capillaries. Indeed, although observations done up to the present have always showed elements of mucus mixed with menstrual blood, even at the moment of their highest flow, it is understandable that this flow could be quite substantial for a number of hours and produce stains
433 of pure blood, preceded and followed by the formation of stains containing epithelia of mucus usually found during the total duration of menstruation.

It could also be that blood coming from a wound could form stains on a fabric already stained by menstrual blood. The microscope and the preliminary methods of investigation, which we have previously reviewed, give no less precise results and in each of them find no less the anatomic elements which characterize them, such as we have described. The case excepted where there would be a mixture or superposition of ordinary blood to menstrual blood, these tests can always clarify the investigation in a precise manner.

Menstrual blood, always being somewhat mixed with mucus, it is not astonishing that the stains (as menstrual blood itself) can be distinguished from those formed by pure blood. It is by the continuously exfoliated epithelia which it carries with it, that the mucus is recognizable in the microscope. The origin of any mucus can be determined by the characteristics of the variety of epithelium specific to the mucosa from which it originates. It is by the mixture of uterine and vaginal epithelial cells with blood cells that menstrual blood, and the stains it forms, differ from blood of a wound, and it is by the presence of epithelia that the nature can be determined. Only the esophagus can yield to the blood originating from it an epithelium analogous to that of the vagina, for blood flowing into the stomach is rapidly altered and the characteristics of this alteration can be easily recognized.

§ VI. Conclusions. The preceding research permits the following conclusions:

1) On examination by microscope, menstrual blood differs from blood drawn from vessels by the mixture of blood cells with epithelial cells and leukocytes (termed *mucus corpuscles*). The former come from the epithelium of the utero-
434 vaginal mucosae and the latter from the surface of these mucosae.

2) The stains constituted by menstrual blood present elements not found in those formed by blood coming directly from vessels. These elements are those which the mucus of the genital tract, carried along with the blood, holds in suspension, i.e. principally the above-mentioned epithelial cells and mucus leukocytes.

3) Stains formed by menstrual blood can be distinguished from those produced by blood flowing directly from blood vessels by comparison of the two types of stains in the microscope, which will demonstrate the preceding characteristics.

On the Certain Recognition of Blood and Bloodstains in Forensic Investigations*

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295 Blood can occasionally suffer a great alteration in its characteristics, when it has come into prolonged contact with certain substances, so that one is unable to recognize it by a chemical method when using the usual reagents. This is of importance in legal examinations as I have become convinced by my own experiments.

When blood is in a dried state, unadulterated with other substances, and is submitted to testing, it causes no difficulties, even if the amount of dried blood is extremely small. One treats the blood continuously and for a long time with cold, distilled water, pouring off carefully from time to time
296 the water from the undissolved fibrous substance, until that substance is relatively free from blood coloration as a result of the treatment with water. One can clearly recognize the residual fibrous material as such when viewing it through the microscope, especially if one compares it to another sample which has been freshly produced from a small amount of dried human blood by treating it with water with this purpose in mind.

One tests the watery solution of blood pigment with reagents, using for these experiments only the first, relatively concentrated solution, since the following solutions, which served as wash water, contain too little of the loosened blood pigment. The reagents which one uses are well known and are described in the textbooks of forensic medicine. One heats a portion of the liquid to the boiling point, by which a greater or lesser degree of clotting takes place in the liquid, according to the quantity of dissolved blood pigment. If the solution is very diluted, often only an opalescence forms. The color of the clotting is a dirty red. It quickly dissolves in a heated solution of potassium hydroxide; the color of this solution is more or less green. It has the characteristic, however, that, if the liquid is of a certain dilution which is not too strong, it appears green only in direct light; in reflected light it appears red. One can best make these observations in a reagent glass.

If one adds to another portion of the blood solution plenty of chlorinated water so that the solution smells after being shaken, it will lose its coloration, and white flakes will sepa-
297 rate out, flakes which normally float on the surface. If nitric acid is added to a third part of the blood-pigment solution, a grey white precipitation forms, and tincture of gallnut produces a faintly violet colored precipitation in the fourth portion of the solution.

All of these reactions cannot be set up if one has only a very small amount of dissolved blood pigment at his disposal; if, for example, only an insignificant blood stain has been treated with water. In this case it is advisable to boil the small quantity of concentrated or undiluted solution and to treat the boiled solution with potassium hydroxide. If one has obtained the results presented above, this alkaline liquid can be added to a large amount of concentrated chlorinated water whereby white flakes separate out. Or one can choose to use only a half of the alkaline solution for this test so that he can saturate the other half with nitric acid in order to obtain the precipitate mentioned above.

The handbooks describe thoroughly how the solutions of blood pigment can be easily and positively distinguished from solutions of other dyes of organic origin by a chemical method, and also how blood stains can be easily recognized when they are found on undyed linen or wool material. One can easily remove the blood pigment by treating it with cold water so that the fibrous material remains on the cloth; it can then be carefully scraped off and observed with the microscope.

If, however, blood stains are found on dyed cloth, or especially on cloth that consists of an organic nitrogenous 298
substance such as wool or silk, these stains can be identified only with difficulty, if they are not present in a significant amount so that the dried blood can be carefully scraped from the cloth. This process goes very well, even in cases of small amounts, if one proceeds with caution. Sometime ago, I had the opportunity to prove this to myself when I had to submit bloodstains to testing, stains which were present only as very fine drops, which had been squirted onto a black cloth shirt. I was able to convince myself of their existence only by examining the area where they were supposed to be located with a good magnifying glass; this process was more successful in lamp light than in sunlight. These drops were scraped off with great caution. In doing this, many of the cloth fibers were naturally also scraped off. The scrapings, which were present only in a very insignificant quantity, were put into a small white porcelain bowl, and cold water was then poured over them. After a rather long period of digestion in the cold water, the liquid took on a reddish hue. The solution was poured off from the undissolved cloth fibers. On account of

* Translation of: "Ueber die sichere Erkennung von Blut und von Blutflecken bei gerichtlichen Untersuchungen".

in *Vierteljahrschrift für gerichtliche und öffentliche Medizin* 4: 295–310 (1853).

the presence of the cloth fibers, one could not in fact examine microscopically the undissolved fibrin. In the reddish solution, however, a clot was produced by heating–a clot which broke down when boiled in potassium hydroxide into a greenish solution, which clearly displayed the dichroism mentioned above, and which also produced by means of reagents the reactions described above.

299 Of very special significance, however, is the examination of blood, when it is dried on metallic iron. In such an examination, I encountered special difficulties which I find are not mentioned in early experiments of a similar type. It seems to me that describing these difficulties is of some importance. importance.

Vauquelin[1] first made the observation that when iron rust formed on objects of metallic iron inside inhabited houses, it contained ammonia. Chevallier,[2] who confirmed this observation, also found ammonia in the iron oxide which is found in nature. Moreover, Boussingault[3] discovered this alkali even in an iron hydroxide which had not come into contact with the air when it was located. Austin had earlier discovered that ammonia formed when iron oxidized by coming into contact with air and water.

Therefore, if one suspects that traces of dried blood could be on a metal cutting instrument whose surface is coated with rust, one must not think he has found a confirmation of this suspicion when ammonia is found upon heating the scrapings of iron rust.

If, after gentle heating in a dry test tube, the ammonia separates from the iron rust, which one has scraped from the
300 iron instrument, then, even if only small amounts of blood are present, strong heating must produce the well known, strong smelling odor of burning charcoal which arises when albumin substances are carbonized. A brown, malodorous, empyreumatic oil will be revealed on the unheated part of the test tube. The supposition can be confirmed with greater certainty, if one mixes a small quantity of gently heated iron rust with approximately the same amount of potassium, or better of sodium, in a very small glass tube, which has been closed at one end beforehand. After cooling, the mixture is treated with water; the filtered solution is adulterated with a very small amount of iron solution which contains both oxide and protoxide. Then it is soaked with some hydrochloric acid. A greater or lesser quantity of ferric ferrocyanide is left undissolved, if blood was present there. The color of the ferric ferrocyanide is green only if the amount of iron solution added was too much.

These phenomena will surely appear when blood is present in the iron rust, even when the blood is present in the very smallest quantities. These phenomena, however, do not necessarily arise from the presence of blood since they are caused by any nitrogenous, organic substance. If, on the other hand, the iron rust formed only because of oxidation of iron by damp air, these phenomena will certainly not occur.

Moreover, bloodstains, which have dried on smooth, metallic iron, can be easily distinguished from rust spots.
301 The former are dark brown and are only a bright red if the blood has spread itself very thinly in the drying process. Blood spots distinguish themselves especially by the fact that they are easily loosened from the iron after the blood has dried, and they leave the metal relatively clean. Rust spots, on the other hand, are solidly anchored to the iron and can be removed only with difficulty. Thus, when a knife, spotted with blood stains, is stored for a long time as a *corpus delicti,* it is easily possible that, after a certain time, no blood traces can be found on it because the dried blood can easily be removed from the knife by the slightest rubbing and thus be lost.

I have been able to convince myself of these facts through my own experiments in a forensic examination. A knife, which had very probably been used to commit murder, was handed over to me for examination. This murder took place during the summer in a corn field. After the murder, the knife was left lying in the field and was discovered only some time later.

The blade of the knife was heavily coated with rust since it had lain on the moist earth. As a result it was possible to observe the metal surface of the knife only in a few places.

The rust spots looked exactly like rust as it forms on metallic iron under the influence of dampness and air. After the rust had been scraped off and heated in the test tube, it produced ammonia which caused a moistened, red litmus
302 paper to turn a strong blue; but when strongly heated no burnt odor was produced, and no traces of empyreumatic oil were noticable. If the heated rust was fused with sodium, no ferric ferrocyanide could be obtained by the method mentioned above.

The knife in question was such that its blade could have been covered, but it was sent to me with an uncovered blade and was most likely found in this state, either embedded in the ground or lying on it, so that blood adhering to its surface must have been washed away by the rain; the iron rust, forming on the blade, could not contain any of that blood.

The inside of the knife's surface was filled with a dark, almost black substance which, immediately after having been scraped from the knife, was still somewhat soft but subsequently hardened to a fragile mass. When a very small portion of the material was heated in a small test tube, it behaved just like dried blood; a strong burnt charcoal odor developed, a strong smelling empyreumatic oil formed, and from the heated residue, a significant quantity of ferric ferrocyanide could be produced by treatment with sodium.

When water was used to treat a larger quantity of the black material, it did not extract from this material any blood pigment even after prolonged contact. The digestion was continued for a very long time and was even supported by gentle heating; the temperature, however, could not be so high that the albumin-like substances, which were somewhat dissolved, could be coagulated by the process. Despite this
303 operation the water remained completely uncolored. After filtration the use of reagents showed that only a very small trace of albumin-like substances had been absorbed.

If, on the other hand, the black substance was treated with

water and then cooked with some potassium hydroxide solution, this immediately turned a greenish color; the filtered solution displayed the dichroism mentioned above and reacted with the reagents just as a solution of blood pigment in potassium solution. When the substance was mixed with hydrochloric acid, after treatment with water and with potassium solution, this dissolved a significant amount of iron oxide, which, when the solution was saturated with ammonia, gave a voluminous precipitate.

The black mass from the sheath of the knife thus consisted mainly of dried blood and iron oxide, which had formed as rust on account of the moisture on the metallic iron with which the inner shell of the knife was covered.

Through the presence of a great amount of ferric hydroxide, the dried blood had lost one of its essential characteristics, its solubility in cold water. In fact, according to the many comparative experiments which I set up in the last stages of my work, the wet ferric hydroxide precipitates completely the blood pigment from its solution and hinders the dissolution of the pigment in water.

A further investigation of the contents of the inner surface of the knife in question showed that this was the case. Besides the black material, a very small piece of wood was found squeezed in approximately where the point of the
304 blade can strike in order to prevent this blade from striking against the iron of the inner sheath. Dried blood had adhered to this piece of wood, especially on the one end of this piece, which probably had not come into contact with the metal rust. By viewing it with the magnifying glass, one could clearly recognize it as dried blood.

This small piece of wood was anchored to a linen thread at its end where the least blood was found. It was then placed in water in a thin test tube so that it could not float on the top of the water but was submerged for the most part. After some time one could perceive very clearly that red stripes sank from the wood, and indeed from the places where the dried blood was located, toward the bottom of the glass, while a flaky, voluminous material remained fastened to the wood, a material which took on a brighter color the longer the water acted on it. After a time a larger portion of this material separated from the wood and fell to the bottom of the glass. After two days the wood sliver was removed from the glass, and the flaky material which still remained fastened to the wood was submitted to microscopical inspection. It proved to be identical with the fibrin, which had recently been produced from dried human blood by a similarly treating it with water for a comparison with preserved blood. The reddish liquid from which the fibrin had separated was poured off. Even though it was a dilute solution, and contained only a little of the dissolved blood pigment, it displayed
305 unambiguously the presence of the blood pigment when treated with the reagents.

In view of the characteristic of ferric hydroxide to bind the blood pigment, and in view of one of blood's most important characteristics, its solubility in water, it deserves consideration in forensic examinations. I have, therefore, set up a series of tests concerning this aspect, the results of which I want to communicate here in summary.

If freshly precipitated ferric hydroxide is dissolved in the cold, together with a diluted solution of blood pigment, and the mixture is shaken often, after twenty-four hours the filtered solution contains no blood pigment, but cooking the iron hydroxide with potassium hydroxide releases blood pigment which can easily be discovered in the solution with reagents.

If, instead of wet iron hydroxide, red hot iron oxide is treated with a solution of blood pigment, it removes a great deal less of the hemoglobin. After 24 hours the filtered fluid is still colored, but by cooking with potassium solution, a considerable quantity of blood pigment can be extracted from the iron oxide residue.

The sooner the iron hydroxide is used after its precipitation, the quicker it removes the color from a solution of blood pigment; it is, however, difficult in these cases to filter the fluid because at first the iron oxide passes mechanically through the filter system. But if one has filtered the liquid until clear, he can find no trace of blood pigment in it.

A solution of blood pigment is adulterated with a sufficient quantity of ferrous chloride, and then from this mixture iron
306 oxide is precipitated by using ammonia. After this liquid was filtered, it was colorless and contained no blood pigment. It is understood that, in these experiments, the blood pigment is present in such small quantities that the precipitation of iron oxide by means of ammonia is in no way hindered.

If, on the other hand, the solution of blood pigment is mixed with a solution of sulfuric acid ammonium oxide ferrous oxide (iron ammonia alum), and subsequently with ammonia, the filtered liquid is then not completely clear of coloration, even when the quantity of iron salt is rather considerable. If ammonium chloride is also added to the solution, and then ammonia, the filtered fluid is then completely free of coloration and contains no blood pigment.

If one lets blood pigment dry in an iron container, completely coated with rust, at the usual air temperature, and then moistens the dried mass with water, and again lets this dry, one obtains, after a moderate length of time spent in repeating this drying operation several times, a dry mass which, when treated with cold water, does not transfer to the water any red coloration or any of the blood pigment. If the residue, after being treated with cold water, is cooked with potassium hydroxide, one obtains, after filtration, a deeply colored solution in which the presence of blood pigment can easily be demonstrated by using reagents. The undissolved iron oxide in the potassium solution shows, after dissolving in hydrochloric acid, that it contains iron protoxide when tested with potassium ferricyanide.

If one uses a container with a smooth surface instead of a
307 rusty iron one, and if one lets blood pigment dry in this container at normal temperature, it takes much longer before the blood pigment looses its solubility in water as a result of the build-up of iron hydroxide. One has to repeat the moistening and drying process many more times in order

to render the blood pigment insoluble in water. One finally obtains a brown, almost black mass which can be easily pulverized. The substance no longer gives off any blood pigment in the water, and it reacts exactly as the material which was contained on the inside surface of the knife mentioned above.

Aluminum hydroxide behaves with a solution of blood pigment in a similar fashion as does ferric hydroxide. In a freshly precipitated state, it absorbs the pigment, and the filtered solution is colorless, containing no blood pigment. It appears that a greater quantity of aluminum hydroxide than of ferric hydroxide is required in order to remove the blood pigment from a solution of a certain amount of this pigment.

Ferric hydroxide and aluminum hydroxide were not capable of removing so completely hen egg albumin from a solution of it in water, as they could do in the case of blood pigment.

If a diluted solution of blood pigment was mixed with pulverized clay in the cold and was frequently shaken, it took a long time, even a month or longer, to render the fluid colorless. In this process the blood pigment began to decay and developed the well known odor of decaying cheese. The
308 filtered liquid then contained ammonium chloride in small quantities but no blood pigment. The clay, however, which had been a white color, changed its color into a somewhat dusky hue in a few places. When heated, it colored a potassium hydroxide solution a greenish color. With reagents it was easily possible to discover the presence of blood pigment in this solution.

If, on the other hand, a very concentrated solution of blood pigment in a very small amount of water, is left for a long time in contact with pulverized clay, the pigment could not be absorbed by the clay. It began to decay, and after several months the red color of the pigment has been preserved. Only when the whole solution was diluted with a lot of water and thoroughly shaken, was it possible after some time to remove the blood pigment from the fluid.

Clay also displays the characteristic that it removes blood pigment from water, although to a far lesser degree than does wet ferric hydroxide. In any case the condition is to be considered in forensic examinations.

These observations are in apparent opposition to those in an experiment conducted by Lassaigne. He used a blood stain caused by about ¼ deciliter of animal blood poured onto a surface of fine sand (*pave tendre en grès*, clay soil?) in order to see after how long a period it was still possible to identify the characteristics of blood on such a surface. He allowed this piece of earth to be exposed to rain and to light in the open air for one month. After this lapse of time the color was pale and greenish, somewhat inclining toward red.
309 The piece was pulverized and was thoroughly leached in cold water for twelve hours; it took on a red-brown color as a result of this treatment, and it showed the presence of blood pigment when acted on by reagents.

The residue, which no longer gave off anything soluble in the water, also had a greenish color; after several experiments, it also showed the presence of blood pigment. It was mixed only with a diluted ammonia liquid, and Lassaigne did not heat it with a potassium hydroxide solution, by which procedure the presence of the blood pigment would have proven itself even more clearly.

Lassaigne concluded from his experiments that, by using the normal reagents, one could still recognize blood stains absorbed by earth even after a month. Obviously, the results would have been different if the blood had not been applied in such a concentrated state but in a greater dilution, and if it had been left spread onto it for a period of time longer than a month. Though he did not use completely satisfactory reagents, Lassaigne himself was thus convinced that the earth still contains blood pigment after complete leaching with cold water.

The discovery of blood pigment is more difficult if the blood pigment solution has soaked into earth which consists of humus-rich garden soil. For a period of several months I dissolved a diluted solution of blood pigment with earth from a flower pot. After this period the filtered liquid was colorless and, when evaporated on platinum, it left only a very little residue, which, however, contained no blood pigment. The soil, leached with water and then cooked with a potassium 310
hydroxide solution, produced a deeply colored liquid which displayed, after filtration, a dark brown hue but did not display the dichroism which is characteristic of the blood-pigment solution in a potassium solution. In this solution, brown precipitates formed after supersaturation with acids, precipitates which displayed the same peculiarities as did those produced by acids in the filtered liquid; these were also the same as those which the garden soil gave off when treated with potassium solution, even when such earth was not treated with blood pigment. In order to recognize the presence of blood pigment in such a potassium solution, which at the same time contains humus in a dissolved state, it is best to mix the potassium solution with a great quantity of chlorinated water, by which process white flakes form in it, as they do in a solution of pure blood pigment (or of other protein-like bodies), while the humus, dissolved in potassium, does not produce these flakes under the influence of chlorinated water.

If, on the other hand, a concentrated solution of blood pigment is mixed th garden soil at a cold temperature, the pigment will not so easily be absorbed by the soil. Thus, even after several months, water, when mixed with this compound, still produces a red solution which contains blood pigment so that, after completely washing the earth with cold water, it would still have contained blood pigment which would have dissolved in a hot potassium solution along with the humus.

References

1. *Annales de Chimie et de Physique*. Vol. 24, p. 99
2. *Ann. de Chim. et de Phys.*. Vol. 34, p. 109
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Concerning the Crystallization of Organic Components of Blood*

L. Teichmann

375 "When water is added to one drop of blood, starting to dessicate slightly as a consequence of spontaneous evaporation, and when evaporation is again induced under a cover slip, crystals formed from the blood will be obtained."

With these words, Funke specified the conditions under which, in his opinion, crystals are obtainable from spleen vein blood (see this journal. N.C., Vol. I, page 185). He later used the above method (*ibidem*. Vol. II, page 289) and succeeded in obtaining crystals from other blood types. The same preparation method was applied with some modifications by Kunde, who believed that the presence of fibrous material constitutes a definite obstacle and that a certain amount of serum is needed (this journal N.F., Vol. II. p. 274). Apart from the disadvantage that the mechanism of the crystallization could not be clarified, the aforementioned method has the additional shortcoming that it is never certain whether crystals will be obtainable. At first, I used the technique for a prolonged period of time as well. I noticed initially that it is impossible to obtain crystals from fresh blood samples. I then attempted to add various volumes of water to the blood without previous blood evaporation; when
376 4 to 5 or more parts of water were added to one part of blood, then after letting the liquid stand for a sufficiently long period, and when slow evaporation became evident by the dark red to violet color of the liquid, crystals could be obtained on each occasion. With this method I verified that the blood of all animals investigated by me in all types of blood containers underwent crystallization. The frog blood was the only exception; on the other hand, I detected crystals in human blood as well as in the blood of dogs, steers, hogs, rabbits, pigeons and fish.

I used a small cork prop to support the cover slip from one side, so as to retain larger quantities of the considerably diluted blood; regular crystals then always formed at the three non-supported sides. With this system, I never obtained crystallization of the whole blood, nor was it possible to prepare crystals without a cover slip; the first-mentioned phenomenon led to the supposition that another substance is present, besides the crystallizing component, which does not participate in the crystallization process. The possible presence of such a substance could also explain why no crystallization occurred with free access to air. The *a priori* probability existed that the fibrin- and albumin material of the blood plasma does not undergo crystallization and does not promote the crystallization of any other substance. Kunde, as mentioned earlier, stated that when crystallization is to be obtained, the blood should not contain any fibrin, nor should its serum content be too low. The crystallization is in fact accelerated when the fibrin and part of the serum are eliminated from the blood. Due to its albumin content, serum, like all viscous substances, such as for example liquefied glue, gum arabic, etc., is quite an unsuitable medium for crystallization. Serum evaporation is too irregular and results in a desiccated crust on the surface, which will later show irregular cracks when water evaporation from the interior of the mass continues. When the liquid contains a large amount of albumin, but not much water and common salt, for example, crystalline efflorescences or small lumps are obtained during evaporation at varying degrees of temperature. But fine, large and regular crystals, like those forming when pure water is added at the same temperatures, will never form. Kunde's contention (page 274) that water 377
can be used *as efficiently* as serum, should be corrected, in that water is much more suitable than serum.

Since the blood corpuscles contain the crystallizable substance, the latter had to be isolated first, as free as possible from pigment and serum. The currently used and simplest method consists in the filtration of the blood with a sodium sulphate solution. This method could not be applied in our case, since the admixture of another likewise crystallizable substance in large volumes would have been unsuitable for this purpose. I therefore selected the following method: freshly drawn blood was left standing for 48 hours; the clotted blood was then placed on blotting paper, or was rinsed with water; filtration through a linen cloth and later through paper followed. The filtrate, mixed with water, yielded crystals on each occasion. Finer crystals formed proportionately to the successful elimination of fibrous material and serum from the blood corpuscles. Crystallization now took place without a cover slip as well, but the crystals were less perfect in that case. The admixture of water, as in all crystallizations, is useful merely because it reduces the viscosity of the solvent, and prolongs the duration of the time needed for the separation of the dissolved substances from the liquid. The crystallization of the blood corpuscle content can be achieved without water as well; but the resulting crystals will be incomplete and numerous blood corpuscles desiccate jointly with their entire content. The added water evacuates the blood corpuscles and facilitates crystallization in general. It also offers an opportunity for the formation of larger individual crystals.

When we now survey the conditions under which Funke

* Translation of: "Ueber die Krystallisation der organischen Bestandtheile des Blutes",

in *Zeitschrift für Rationelle Medizin* 3: 375–388 (1853).

and Kunde obtained crystals, it becomes evident that—definitely without intending to do so—they complied with the preconditions which we found to be essential. Initially, Funke prepared crystals from the spleen vein blood of the horse. The blood was forwarded to him from Dresden to Leipzig (Vol. I. p. 177), and at least several hours after the animal expired. While he believed that the blood was fresh, the above-mentioned period was nevertheless sufficient for partial blood coagulation. It is quite irrelevant whether or
378 not the blood originated from the spleen vein; he would have obtained crystals after the same length of time from the blood of other vessels as well. On another occasion (Vol. I. p. 101), he prepared crystals from the blood of dead fish, after finding thick blood coagulations in their abdominal cavity. In that case he obtained more crystals with less difficulty, which he ascribed to a specific crystallization capacity of fish blood, without taking the most significant factor into consideration, i.e. that the blood was already coagulated when collected. After detecting crystals in human blood, (Vol. II. p. 288 and following pages) he attributed this finding to the fact that the container in which he had stored the blood contained a few drops of water; however, he overlooked an important fact: he had investigated the blood only 30 hours after the blood-letting, i.e. after the separation of the fibrin. He examined cat blood 2 days after its collection, saying himself that it was coagulated. He states regarding the hog blood investigation that the phenomena occurred more readily when the blood was left standing for a day. Kunde was likewise able to obtain crystals after eliminating the fibrin, and a small quantity of the serum from the blood.

When a few drops of the blood corpuscle-water mixture, prepared as indicated, are placed on a glass slide, letting them evaporate without a cover slip, crystals will form, in particular when plenty of water was added and the evaporation is slow. However, enough viscous serum components are still present to form a surface crust, in addition to the crystals. The crystals obtained with the above method are unsuitable for microchemical studies because reagents are unable to act directly on the same.

I used the two methods specified below to avoid this disadvantage and to obtain as perfect and best isolated crystals as possible:

1) I placed supporting props under the cover slip on four sides;

2) I allowed the liquid to evaporate under a watch glass after dampening the edges of it with blood which, when desiccated, forms an adequate seal, and releases the vapor slowly through the individual pores.

379 I achieved my objective with the application of both methods: the crystallization was perfect, as shown by the large number and size of the crystals, and the crystals were isolated.

As for temperature: blood crystallization requires the same preconditions as the crystallization of common salts. Crystallization improves proportionately to the slowness of rate of the evaporation, and vice versa. When rapid crystallization is wanted, the temperature can be raised, but it should not be too high, so as to avoid the coagulation of the albumin. When blood, containing the full amount of serum and fibrin is to be crystallized, a considerable volume of water must be added and the temperature should be as low as possible.

After determining the conditions needed for crystallization in the blood, I attempted to form crystals from frog blood. I was unable to obtain a quantity of frog blood sufficient for the elimination of fibrin and serum; but crystals nevertheless formed when I mixed the blood with large volumes of water and undertook evaporation at very low temperatures. These crystals differed from those found in other blood types; their number was relatively much lower, but increased when more water was added, followed by evaporation. Finally, the number of the crystals increased to such an extent that these could not be mistaken for crystals originating from any of the salts contained in the blood.

As for the shape of the crystals: it varied significantly. Since I experimented principally with dog blood, I am unable to state how crystals formed from dog blood compared to those formed from the blood of other animals. However, [crystal] form is certainly quite accidental and depends on secondary effects. Not even the crystals from the blood of the same vessel of the dog show identical shapes when they are prepared with different methods. Crystals obtained under a cover glass form needles, rods and plates; those obtained under a watch glass show, in addition to the above shapes, rhomboid and tetragonal plates; the rhomboids even appear 380
at dissimilar angles. Prismatic crystals, pointed at both ends, constantly form from frog blood; I do not contend, however, that their shape will always be the same. Tetrahedrons were recently detected in the blood of guinea pigs. Lehmann, in his recent study of this type of blood, found that besides tetrahedrons, octahedrons and other forms occur as well (*Chem. Pharmac. Centralblatt*, 1853, p. 98).

Crystals obtained under the cover slip show various shades of red or violet; the color originates from hematin. When a drop of red blood (fresh) is evaporated, red crystals result. On the other hand, when a drop of blood which has turned violet after prolonged storage is evaporated, violet crystals are obtained as well. On one occasion, the liquid under the cover slip, supported by props on four sides, evaporated suddenly; the residue consisted of entirely achromatic crystals, forming fine parallel threads. When crystals form under a watch glass, they are either achromatic or pale yellow. Only the largest crystals are red; these are mostly composite forms. Crystals obtained from frog blood, regardless of whether large or small, are always achromatic. Their behavior in this respect differs entirely from the properties of crystals prepared from other types of blood; they appear as light-colored solids in the red fluid. I now had to face the questions of whether colored and colorless crystals in various shades occur simultaneously and of whether the intensity of the color depends on the size of the stained sections, i.e., whether the crystals which seem achromatic are perhaps

merely very fine plates of colored crystals, showing hardly any coloration due to their fineness. These questions are difficult to answer. I can only say that, when I compared the various shapes on a slide, the color grade of the crystals did not seem proportional at all locations to their profile diameter. Professor Henle, to whom I showed the preparations, likewise expressed the opinion that achromatic crystals do exist. This would lead to the conclusions that the crystallizable material of the blood corpuscles, as such, is achromatic
381 and includes hematin only accidentally when separating from the blood.

My findings coincide with those of Funke, Kunde and Lehmann regarding the behavior of the crystallizable substance, and of the crystals when exposed to open air and to certain reagents, as well as regarding their decomposability, weathering capacity, etc. The crystallizing mass (the blood corpuscles) can be stored for several months in fluid or dried conditions, refrigerated or at room temperature, without airtight sealing. The fluid yields the aforementioned crystals when evaporated under the watch glass (as far as I was able to verify) after 4 months; putrefaction and the amount of the developing infusoria (ciliates) do not interfere with the crystallization process in any way. The desiccated mass can be liquefied and crystallized at any time. Accordingly, the desiccated mass does not decompose at all, while the liquid substance does not decompose readily.

Admittedly, crystals prepared according to the method customary earlier seem to disappear gradually, insofar as they are covered by the non-crystallized mass, which dries later. These crystals, however, are merely concealed. When a little water is added and when the glass cover is touched, the crystals seem to float underneath the cover; when a large volume of water is added the crystals dissolve, but reappear later following slow water evaporation. Such errors are avoidable when the crystals are prepared according to the two methods described by me. In that case, more reliable observation becomes possible because, as stated, most of the crystals are exposed. The usual temperature changes do not affect the prepared crystals, i.e., no weathering occurs, as mentioned by Funke (Vol. II, p. 290). In Lehmann's opinion (*Chemisch-Pharmac. Centralblatt*, 1853, No. 7, p. 99) the crystals do not decompose readily; this is confirmed by the fact that the crystals or the mass can be liquefied and recrystallized, as reported earlier by Funke (Vol. I., p. 191 and Vol. II, p. 290).

When chemical reagents are used, it should be taken into consideration that crystals, even those which seem to be pure, might nevertheless be covered with a more or less thick
382 layer of an albumin-like substance which inhibits solvent access and mars the results.

The crystals are water-soluble. The degree of their solubility is not precisely determinable at this time: depending on the serum volume which was added during crystallization, the added water contains more or less albumin. Therefore more water must always be added than the volume required for the dissolution of the crystals alone.

When strong (approximately 89°) alcohol is added, the crystals shrink, their sharp outline and their plane surfaces vanish; most crystals dissolve slowly in diluted alcohol. According to Funke (Vol. I. p. 189), crystals prepared from watery alcohol are merely "crystallographic malformations". Kunde (Vol. II, p. 275) states that the crystals are never quite regular, and according to Lehmann (*Centralblatt*) the planes of crystals treated with alcohol (the alcohol concentration is not indicated) are no longer quite level and the crystals retain their shape only to a more or less limited extent.

The crystals are not soluble in ether, and retain their shape in it. When water is subsequently added, the crystals remain insoluble; but their consistency changes: they become gelatinous. They dissolve readily in caustic ammonia. Concentrated potassium hydroxide does not dissolve the crystals but they are soluble in acetic, hydrochloric and nitric acids.

According to reports so far, and based on Lehmann's recent investigation results, it can no longer be doubted that the crystalline substances, whose reactions are described here, originate from the blood corpuscles and are organic components of them. It is still not quite certain which of the blood corpuscle components is crystallizable. Our earlier statements on the preparation of achromatic crystals indicate that it is, to say the least, highly questionable whether such crystals can be defined as *hematin crystals*. It is in fact doubtful whether hematin is involved in blood crystallization at all. Another substance, globulin, if one wants to give it that name, constitutes the residue in blood bubbles after the elimination of hematin. Both substances are obtainable in 383
pure condition by means of water extraction from the membranes. However, the medium used to separate them from one another in the watery solution could modify one and possibly both substances; in particular when coagulated globulin forms, crystallization investigations are no longer feasible. I, therefore, used the freshly desiccated blood corpuscle mass. Concentrated hydrochloric acid dissolves the mass (diluted hydrochloric acid has no effect on it); the dissolution occurs more readily at higher temperatures, while gas (carbonic acid) is released. The resulting liquid is clear, purplish-red and precipitates when water is added. At room temperature the admixture of water to the liquid mass will merely cause clouding. When treated with nitric acid, fine gas bubbles will cover the mass, which dissolves slightly at low temperatures and will dissolve completely when the temperature is raised. In the latter case, the liquid stains yellowish-red; when water is added, a dirty yellow precipitate will form.

When sulphuric acid is used, the mass inflates at low temperatures and most of it will dissolve after a prolonged period of time; however, when the temperature is increased, the mass dissolves quite easily and completely, forming a dark brown, clear fluid, while gas is released. Added water causes minor clouding.

When these acid solutions are neutralized with potassium

or ammonia, a yellowish-brown precipitate forms which is once more dissolved into a clear red fluid in excess precipitation medium.

In a concentrated potassium solution the mass is loosened and floats on the surface. Light red drops of various size are visible under the microscope. The process, however, occurs at higher temperatures only; no modification whatsoever is determinable at lower temperatures. The mass dissolves in a diluted potassium solution and a clear red fluid is obtained.

Ammonia dissolves most of the mass slowly at higher as well as at lower temperatures.

The residues of these solutions, regardless whether the evaporation took place at room temperature or at higher temperatures, show nothing worthy of note under the microscope.

384 In acetic acid the mass dissolves at moderate temperatures into a clear, red fluid; however, it will cloud when the temperature is raised and the color changes to a dirty brown. The same phenomena are evident when the mass is treated with oxalic acid, tartaric acid, citric or lactic acid and probably also when treated with other organic acids.

A drop of the above-mentioned fluid, or a minimal amount of the desiccated blood corpuscle mass, yields crystals when dried at 20 to 50° R under a cover slip with any of the aforementioned acids, in particular with acetic acid. The resulting crystals show the following characteristics:

Their color is always yellow, brick-red, brown or black. They form rhomboid columns, either regular or with slightly blunted angles; twin crystals or stars occur frequently, but these are very fine, resembling the needles, rods or grains of the black pigment.

These crystals are not sensitive to the effect of air; they are insoluble in water, ether, alcohol, acetic acid, hydrochloric acid and nitric acid when these substances are added directly; the crystals dissolve entirely when boiled with nitric acid. They will also dissolve in a diluted potassium solution; when the solution is concentrated, the crystals turn black, inflate and their sharp outlines disappear. Concentrated sulphuric acid dissolves them and they are even more readily soluble in ammonia.

Before dissolving the crystals, they should be subjected to careful, isolated examination, since those coated with albumin are difficult to dissolve or do not dissolve at all. Care should also be taken to avoid mistaking the impression (mold) left by dissolved crystals for decolorized crystals.

A yellow ring always forms around these crystals during their dissolution; in particular after treatment with sulfuric acid, color changes will occur.

The crystals described differ significantly from Virchow's *hematoidin* crystals (*Archiv f. Path. Anat.*, Vol. 1., p. 383-445), but some similarities do exist. The main difference is that the crystals described here dissolve completely in the reagents mentioned, without leaving any residual structure of insoluble substance behind. I propose the name *hemin* (*Hämin*) for the substance obtained in crystalline form by
385 me, to distinguish it from the water-soluble hematin of fresh blood and Virchow's *hematoidin*.

I obtained hemin crystals from all blood types which I examined, including human blood, and the blood of the dog, rabbit, steer, hog, pigeon and frog; no dissimilarities whatsoever were determinable and I do not doubt that the crystals can be prepared from any red blood type.

They can also be obtained on a large scale with the following method: blood—regardless whether or not fibrin and serum were eliminated—is evaporated until dessicated; excess concentrated acetic acid is added and the liquid is left standing at the approximate temperature of 30°. After some time, complete dissolution takes place and the crystals remain at the bottom (of the container).

The preparation according to the specified method seems easy; but it involves a certain difficulty, since microscopic crystals can seldom be separated from the liquid by filtration. The crystals could, of course, be allowed to precipitate and could be suspended in water after distillation of the liquid. But this procedure is deficient, in particular when the quantitative determination of crystals in the blood is intended. I obtained the largest and best-shaped crystals when mixing dried, finely pulverized blood with a large volume of acetic acid under constant agitation, so as to avoid the formation of clots; the liquid was then left standing at a temperature of approximately 30°R. The difficulties increased when the desiccated blood is processed with other organic acids, such as oxalic, tartaric, citric or lactic acid. I undertook only occasional tests with these acids and always obtained very fine rods and grains, but the crystals were never as large and perfect as those obtained after treatment with acetic acid.

Regrettably, I have had no opportunity so far to undertake a thorough investigation of crystals present in old extravasations and melanotic tumors. The presence of crystallized hemin in the organism is, in my opinion, highly probable, for the following reasons: water is eliminated by resorption from coagulating blood; the needed heat prevails, and provided that any organic acid is present as well, the preconditions for 386
the formation of crystals do exist.

I searched unsuccessfully for the crystals in lymph and serum first described by Funke but could detect no trace of them with any method. I found hemin crystals in the lymph and serum, but always in small amounts only. I therefore suspect that they might originate from a few blood corpuscles which were accidentally introduced into both liquids. Provided that color is not essential, the first crystals could consist of globulin (?) or this substance could at least contribute primarily to their formation, since no other substance occurs in the blood corpuscles in sufficient quantity for the formation of numerous crystals. The hemin crystals seem to originate from hemin alone, since the albumin-type substances remain dissolved after treatment of the desiccated blood corpuscles with acetic acid. But it would not be justified to speculate here on the nature of the last-mentioned crystals, since they can be prepared on a large scale and can be subjected to accurate chemical analysis.

As mentioned above, hemin crystals appear in the shape of fine black rods and grains, very similar to melanin; large quantities of these crystals develop, in particular, when a small amount of acid is added to a large amount of desiccated blood. This leads to the question of whether a correlation between these crystals and melanin exists. The aforementioned reagents, however, affect small and large crystals alike while melanin—referring to its characteristic reactions only—is decomposed during boiling by concentrated nitric acid and dissolves incompletely in diluted alkali after prolonged digestion. According to these differing properties, the fine crystals should definitely not be identified with melanin, despite the fact that they are evidently similar to the latter. Therefore the question arises whether crystals formed under different conditions could behave as does melanin, or whether they adopt the characteristics of melanin after a specific treatment.

387 I can answer the first question only insofar as I have found that the application of the above-mentioned organic acids to desiccated blood always resulted in the formation of more or less perfect crystals which showed identical behavior when treated with chemical reagents. I determined the following concerning the second question: these crystals, when prepared in adequate quantities, appear black with a blue tint to the naked eye. But when they are placed on a glass slide heated with a red-hot iron, the blue stain suddenly disappears, the crystals turn black as a consequence of carbonization; when boiled with concentrated nitric acid, the black color turns yellow; the crystals then dissolve into a yellow fluid in which sporadic yellow grains and drops of various sizes still float. Digestion with aqueous potash did not dissolve the crystals.

The degree of solubility of the crystals in these reagents is reversed proportionately to the degree of the carbonization.

The presence of black crystals in the organism was confirmed by numerous findings; the possibility of slow carbonization cannot be excluded.

The ash of the crystals is pink; the ash of melanin is whitish-yellow, occasionally red (see references in Virchow, *Archiv f. Path. Anatom.*, Vol. 1., p. 434). Under the microscope, the first-mentioned ash shows large, but rudimentary crystals, while the last-mentioned ash forms non-measurable dots. Could the comparatively significant difference between volumes be responsible for color differences in this case?

When the black crystals verified by MacKenzie, Guillot, Virchow and others in melanotic tumors are present in addition to black pigment, it is reasonable to assume that the crystals developed simultaneously with the black pigment. The reduced solidity of the tumors might offer an opportunity for the coagulation of molecules into voluminous masses. When grains of various size, fine rods, irregular and finally, regular crystals are prepared in a retort under the same chemical conditions, all show the same behavior. These 388
crystals undoubtedly formed while inhibited by mechanical effects; their development stopped while they were still incomplete. Nor can a discrimination be made between the black grains and rods found in melanotic tumors, lungs, etc., and sporadically forming crystals, since all these formations behave identically under the effect of chemical reagents.

According to the experiments performed by Virchow and others, the pigment in the mass, as its color darkens, dissolves less easily in potassium hydroxide. Black pigment and black crystals are completely insoluble. When their solubility is compared with the solubility of the hemin crystals obtained by me—regardless whether the crystals form rhomboid columns, rods or grains—it becomes evident that the behavior of both is entirely identical when treated with potassium hydroxide. Non-carbonized crystals dissolve readily, while the carbonized crystals are quite insoluble. Various degrees of solubility can occur between these two extremes.

The last-mentioned difference, found between carbonized crystals and ocular melanin, cannot be defined as significant; since the black color of the crystals is slate-like, while the color of melanin is brown, the difference is explained by the presence of various intermediate pigmentation stages, not found in crystals. The color of the ocular pigment does in fact approximate the color of synthetically prepared pigment.

I therefore feel justified in reaching the conclusion that the black pigment represents underdeveloped crystals, oxidized to various degrees.

I must leave the further study of the subject's chemical aspect to professionals. I wish, however, to refer to the practical usefulness of the knowledge of hemin crystals, namely that they serve in legal cases for the reliable and easy verification of minimal blood volumes, for example in suspected (blood) stains.

Finally, it is my welcome duty to thank Professor Henle for his advice in the course of this study, and for making the facilities of the Anatomy Institute available.

Preparation of Hemochromogen Crystals*

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629 Hemochromogen or reduced hematin is prepared by means of different processes which are, however, quite difficult. Hoppe-Seyler† prepared hemochromogen by heating to 85°-100° the hemoglobin solution and excluding oxygen with strong alkalis; he also used carbon monoxide hemoglobin for this purpose. Recently, Trasaburochraki‡ pre-
630 pared hemochromogen from sulphur-methemoglobin by means of sodium hydroxide and ammonium sulfide.

In my process I first mix a drop of defibrinated blood with a drop of pyridine on the microscopic slide; I then cover the mixture with a cover slip and examine it by means of a spectroscope and a microscope. The blood corpuscles disappear and the drop becomes a lively brownish-red. In the spectrum, two very beautiful absorption bands appear; one sharply outlined band between Fraunhofer lines D and E, and a brighter, but less well-defined, band between lines E and b. In a thicker layer these two bands merge together into one; in a diluted solution, only the first of the two lines is perceptible.

When I reduced the blood with ammonium sulfide, or even without it, there very soon appeared small, light or dark brownish-red, star-shaped or sheaf-shaped hemochromogen crystals which formed groups. I was unable to examine these crystals spectroscopically because of their small size. There can, however, be no doubt that these are hemochromogen crystals, since they always occur *in the hemochromogen* which forms the basic substance, and also because the crystals disappear if the hemochromogen changes into hematin. The preparation of hemochromogen crystals is also successful with old, dried blood if one pretreats the blood with sodium hydroxide.

These doubly refractive crystals are not stable because, when air penetrates, the red hemochromogen changes into brown hematin, at first around the edges, and then disappears completely after several days; the spectrum then corresponds to that of alkali hematin. If, however, one seals the edges of the cover slip with Canada balsam, then the hemochromogen crystals can be preserved for a longer period.

One can also demonstrate the changing of hemochromogen into hematin by the following method; One can prepare hemochromogen by means of pyridine from defibrinated blood, diluted with water in a test tube. If one transfers a half of the solution into a second test tube and shakes this up with the air, one can see how the red hemochromogen solution loses its red color in a few minutes, and how the hemochromogen changes into brown hematin. The spectroscopic diagnosis also changes in a corresponding fashion.

My method, consequently, permits one to prepare hemochromogen in an easier and faster way than was formerly possible; it is at the same time suited to establish more easily, and perhaps with greater certainty, the presence of blood in dry powder than is possible by means of hemin crystals.

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* Translation of: "Darstellung von Hämochromogenkrystallen".

in *Centralblatt fur Physiologie* 6 (21): 629 630 (1893).

† *Zeitschrift f. physiol. Chem.* XIII.

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A Method for Identifying Blood by Hemochromogen Crystallization*

Masao Takayama, M.D.

615 Clinically and medico-legally blood is identified by the demonstration of:

A. Blood corpuscles, especially erythrocytes, and

B. Blood pigment and its derivatives.

Blood pigment and its derivatives are in turn identified by the following three methods:

1. testing catalase and oxygen-combining activities,
2. examining crystals, and
3. studying spectra.

Among these three methods, the first one that tests catalase and oxygen-combining activities is applied mainly in clinical cases for identifying blood because of its simple
616 procedures and extreme sensitivity. Every year its improvements or modifications are published by clinicians. Unfortunately, however, the reactions demonstrated by this method are not specific to blood pigment and its derivatives; as you are well aware, various organic and inorganic substances give the same reactions. Therefore, we regard this test method as preliminary or auxiliary to the identification of blood: a positive result does not necessarily indicate the presence of blood pigment, but a negative result does prove its absence.

The second and the third methods, the examination of crystals and spectra, are indispensable to forensic medicine for the identificaiton of blood. The positive results absolutely prove blood pigment and its derivatives.

The blood pigment and its derivatives that form crystals are: hemoglobin, oxyhemoglobin, methemoglobin, carboxyhemoglobin, cyanohemoglobin, sulfhemoglobin, hemochromogen, carboxyhemochromogen, cyanohemochromogen, hematin halide, hematin combined with organic and inorganic acids, and hematoporphyrin hydrochloride. Two of these crystals, hematin halide, namely hemin, and hemochromogen, are used for identifying blood, and today I am going to talk about the hemochromogen crystals.

Ever since Teichmann discovered hemin crystals (hence also called Teichmann's crystals) in 1853, the hemin test method has occupied an important place among methods for identifying blood. Hemochromogen was crystallized for the first time in 1889 by Hoppe-Seyler. He crystallized it by dissolving hemoglobin in an aqueous solution of sodium hydroxide and heating it to 100°C in the total absence of oxygen. In 1893 in a physiology laboratory in Budapest, Donogany accidentally synthesized hemochromogen crystals by adding pyridine alone, or pyridine together with ammonium sulfide, to defibrinated or dried blood. These findings remained largely unnoticed by scholars, although R. Kobert's nephew, H. U. Kobert, and Angelo de Dominicis duplicated Donogany's experiments in 1901 and 1902, and later Cevidalli, de Dominicis, and Lecha-Marzo published works on this subject. However, the method finally drew 617
general attention after Bürker reported in 1909 that it was possible to identify hemoglobin and its derivatives by hemochromogen crystallization. Thanks to the experiments by such workers as Puppe and Kurbitz, Kalmus, Mita, Lochte, Methling, Dilling, Hummel, and Heine, this method has been recognized as the most important among the methods of blood identification.

The reasons that the methods of blood identification by hemochromogen crystallization has come to occupy such an important position are:

1. The crystallizing ability of hemochromogen is equal or superior to that of hemin.

2. Because hemochromogen crystals are large and their color varies from copper red to deep ruby red, and detecting them is easier than detecting small, brown hemin crystals. For example, combined with a No. 2 or 4 ocular (3× or 6×), a No. 7 objective (60×) is needed for detecting hemin crystals, while No. 3 (10×) is adequate for hemochromogen crystals.

3. Regardless of the success or failure of hemochromogen crystallization, the hemochromogen produced can easily be identified by the spectrum using a microspectroscope or a hand spectroscope (remove the ocular of a microscope and insert a hand spectroscope to examine). According to de Dominicis the hemochromogen spectrum shows the first and the second absorption bands at the dilution of 1:5,000, while 1 cm thickness of oxyhemoglobin solution gives the first and the second absorption bands at 1:5,000 dilution, and the first band only at 1:10,000 dilution. Thus the light absorbing sensitivity of hemochromogen is almost identical to that of oxyhemoglobin.

The disadvantage of this method is that the slide preparation of hemochromogen crystals cannot be preserved long. If the edge of the cover glass is sealed with Canada balsam or masking lac, however, the slides keep comparatively well. Besides, since the purpose of forming the crystals is to iden- 618
tify blood, I do not think it matters much if the slides keep well or not.

1. How are hemochromogen crystals made? Generally speaking the reagents used in this method are:

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* Translation of: Original Article [Japanese Title Not Transliterated].

in *Kokka Igakkai Zasshi*, No. 306, Pages 463 481 (cumulative) Pages 15 33 (issue) (1912).

(a) as dissolving agent of dry blood—distilled water, concentrated aqueous solutions of sodium and potassium hydroxide, 10% ammonia water, concentrated potassium chloride solution, 2% alcoholic or aqueous solution of iodine, bromine water, or chlorine water,

(b) pyridine, piperidine, or their derivatives (α- and β-picoline, collidine, parvuline, coniine, nicotine, methylpiperidine, ethylpiperidine, etc.)

(c) as reducing agent—ammonium sulfide (Donogany), saturated aqueous solution of hydrazine sulfate (de Dominicis), saturated solution of sodium antimony sulfate (de Dominicis), 10% hydrazine hydrate solution (Mita), or sodium sulfate.

Among these reagents pyridine is indispensable. My guess is that the dissolving and the reducing agents transform blood pigment into hematin and then reduce the latter to hemochromogen, which crystallizes in the presence of pyridine. Since pyridine obviously does not have a reducing action, it has been disputed whether pyridine is the one that produces hemochromogen: von Zeineck, Kalmus, and Kurbitz maintain that hemochromogen cannot be produced with pyridine alone, while Donogany, Kobert and Dilling claim that it is possible to make hemochromogen and its crystals from fresh blood using only pyridine if oxygen is completely excluded. The slides exhibited will convince you that hemochromogen crystals are certainly produced using pyridine alone. In any case, it is an indisputable fact that pyridine plays a major role among the reagents used in this method.

19 2. While the procedures of this method vary somewhat according to researchers, the representative four are:

(a) Donogany's procedure—blood + a drop of pyridine + a drop of ammonium sulfide (or pyridine alone; or concentrated sodium hydroxide solution + pyridine; or concentrated sodium hydroxide solution + pyridine + ammonium sulfide).

(b) Angelo de Dominicis's procedure—blood + a drop of pyridine + a drop of saturated hydrazine sulfate solution or a drop of solution containing 5% sodium hydroxide and hydrazine sulfate (Puppe and Kurbitz mixed blood with pyridine and a saturated aqueous solution of hydrazine sulfate, then heated the mixture to gradually evaporate, and made a permanent slide by sealing it with Canada balsam; Heine recommended a mixed solution containing 2 parts of pyridine and 3 parts of concentrated aqueous solution of hydrazine sulfate.)

(c) Lecha-Marzo's procedure—blood + 2% alcoholic or aqueous solution of iodine, chlorine water, or bromine water, heat the mixture and then add pyridine + ammonium sulfide (although Lecha-Marzo called the crystals thus produced hematin iodide, hematin chloride, and hematin bromide, Kürbitz's research made it clear that they were hemochromogen crystals).

(d) Mita's procedure—blood + a drop of 10% ammonia water + a drop of pyridine + a drop of 10% hydrazine hydrate solution or saturated aqueous solution of hydrazine sulfate.

Though with all these procedures hemochromgen crystals are produced without heating, the researchers are agreed that careful gentle heating increases the efficiency of crystallization.

Such is the summary of the present state of the method for identifying blood, namely blood pigment, by means of hemochromogen crystallization. Today at this meeting I should like to present a new reagent that I have added to these test procedures based on hemochromogen crystallization. My reagent differs from those of others in that I used glucose as the reducing agent. Why did I use glucose? It happened fortuitously. As you know there is a method called Heller's for testing blood in urine. In this method urine is made 20
strongly alkaline by adding sodium hydroxide solution and then it is boiled to precipitate blood together with phosphates. The blood pigment in this brownish red precipitate is called hemoglobin or hematin by different workers, or even hemochromogen by Arnold. One day in order to determine which was the case, I added blood to a urine sample which happened to be on my desk and proceeded with this method. Unexpectedly the blood pigment turned deep ruby red and remained dissolved without precipitating. The pigment had the spectrum of hemochromogen. Puzzled, I tested the urine and found that it was diabetic. From this experience I learned that, in the presence of sodium hydroxide, glucose reduces blood pigment and changes it into hemochromogen. Therefore, I deduced that if blood is first mixed with a solution containing glucose and sodium hydroxide and then with pyridine, and the mixture is heated, the blood pigment would even more easily be changed into hemochromogen, and on a slide its crystals would be formed. I carried out this experiment, which turned out to be successful as I expected.

I repeated the experiment in order to find out what proportion of glucose to sodium hydroxide solution to pyridine would make a favorable reagent for crystallizing hemochromogen. The favorable reagent was found to contain 0.5% glucose, 1% sodium hydroxide, and 10–20% pyridine. Namely:

glucose	0.5
sodium hydroxide	1.0
distilled water	90.0–80.0
pyridine	10.0–20.0
or	
10% glucose solution	5.0
10% sodium hydroxide solution	10.0
distilled water	75.0–65.0
pyridine	10.0–20.0

When the amount of pyridine in the reagent is small, 21
hemochromogen forms small but regular, long, diamond-shaped crystals, which are similar to hemin crystals; when the amount is large, the crystals are very large but irregular, most of them being needle-shaped. The needles cluster in the form of a cross, a tassel, or a chrysanthemum flower. Therefore, taking the value halfway between 10 and 20%, I regard the one containing 15% pyridine as the most favorable. (Let us call this the first glucose reagent.)

The procedure for crystallizing hemochromogen with this reagent is extremely simple: a test object is placed on a slide glass, broken into fine pieces with a glass rod, mixed with a drop of the reagent, covered with a cover glass , and heated carefully until gas bubbles appear in the liquid. For heating, an alcohol lamp or the small flame of a gas lamp that is obtained after turning off the valve may be used at a considerable distance. If the object is, or contains, blood, the blood pigment which is brown at first gradually becomes red and, under the microscope, begins to crystallize two or three minutes after the slide is cooled. After 10 to 20 minutes almost all the blood pigment in the preparation has turned into crystals. I just said that the object is broken to fine pieces, but this is a matter of degree: either too large or too small pieces are undesirable. According to my experience hemochromogen crystals start to grow from the periphery of comparatively large granules and reach the centers to form aggregates; if the granules are too small, blood pigment is leached and sometimes fails to crystallize though it may remain in solution. Thus the favorable size of fragments must be determined by trial and error. I should say a mistake of leaving them too large is preferable to that of making them too small. The same can be said in using ammonium sulfide or hydrazine reagents. When a piece of cloth or paper stained with blood must be tested as it is for reasons such as the quantity of blood being too small or the separation of blood being impossible, if the material is thin like thin silk, Japanese Mino paper, and newspaper, a small piece should be cut out, placed on a slide glass, treated with a drop of the reagent, covered with a cover glass carefully so as not to
22 introduce air bubbles, and heated. If a thick material such as cotton cloth, Chinese silk crepe, or flannel is to be tested, the tissue should be teased with needle tips following the addition of the reagent, covered with a cover glass, heated, and examined under the microscope after the slide has cooled. The iris diaphragm opening of the microscope should be fairly large. Even when the object has a blue, yellow, or red color, hemochromogen crystals are easily detected; only in cases of indigo-dyed fabrics the test fails if the quantity of adhered blood is small.

According to test-tube experiments in which blood was converted into hemochromogen with glucose, sodium hydroxide solution, and pyridine, the heating needed for this method is 80°C. There is no need to heat the slides very much; when over-heated, the preparation may boil, and the dissolved blood pigment could be lost by boiling over. Although great care is needed not to over-heat, reduction will be incomplete and crystals not formed if under-heated. As in using any other methods, some preliminary practice is necessary in this case to learn how to heat the slides.

Since considerable care is thus necessary in heating, I thought that an increased amount of glucose in the reagent might make heating unnecessary for crystallization because of an increased reducing action. I prepared 30% glucose solution, added sodium hydroxide solution and pyridine to it, and made some experiments with this reagent. As I had conjectured, hemochromogen crystals were produced just by adding this reagent, without heating. Then in what proportion should the ingredients be mixed to give the best result? I tried various proportions and confirmed that the following mixture always gives good results. (Let us call this the second glucose reagent.)

30% aqueous solution of glucose (glucose 3.0, distilled water 7.0)	10.0
10% sodium hydroxide solution	3.0
Pyridine	3.0

Strangely, this reagent works better when aged one or two days to take up an orange-yellow or light brown color than when freshly made. With a fresh reagent, the crystallization of hemochromogen takes from 20 or 30 minutes to several hours after the addition of the reagent, depending on the room temperature. With a one day old, orange-yellow reagent, however, blood becomes red and crystallization begins as soon as the reagent is added and a cover glass placed. Within 10 minutes to one hour almost all blood pigment changes into crystals. The effective period of the reagent with high glucose content is short, however, being about two weeks after preparation. As its color becomes dark brown, the action becomes weaker; after one month the crystallization of hemochromogen takes two or three hours, and fewer crystals are formed. If a slide prepared with a fresh reagent is heated, crystallization is of course aided, but numerous colorless fine granules are also produced to obscure the field of view. Thus although this reagent produces hemochromogen crystals well without heat treatment, it is inconvenient in that it must be prepared at least one day in advance because otherwise crystallization takes a long time to begin. To remedy this inconvenience, I heated slightly a freshly prepared reagent in a test tube until it turned very light yellow, let it cool, and left it until it became light orange-yellow. Such a reagent reacts with blood stains as quickly as the aged reagent does: blood stains become immediately red and numerous crystals appear. The only difference is that the crystals are smaller than those produced with either a fresh reagent or a one- or two-day old reagent. For artificial aging it is better to heat the reagent moderately because over-heating gives it a strong color and reduces its effectiveness.

As I mentioned earlier, I hypothesized that my glucose reagents produced hemochromogen crystals from blood pigment by the following process: by the action of sodium hydroxide solution, blood pigment becomes alkaline hematin, which is reduced by glucose and at the same time is crystallized by pyridine. To test this hypothesis I prepared the 24
following reagents:

	No. 1	No. 2	No. 3	No. 4	No. 5
10% glucose solution	0.5	0.5			
10% NaOH solution	1.0	1.0	1.0	1.0	
distilled water	7.5	8.5	7.0	9.0	8.0
pyridine	2.0		2.0		2.0

Of these reagents No. 2 and No. 5 changed blood pigment into hemochromogen but failed to crystallize it; in contrast No. 3, which contained sodium hydroxide solution and pyridine, produced hemochromogen crystals without heating almost as well as No. 1, which was made of glucose, sodium hydroxide solution, and pyridine. Thus, contrary to my expectation, without added glucose, sodium hydroxide solution and pyridine used together produce hemochromogen crystals perfectly well, as Donogany had claimed. Is glucose then unnecessary in my reagents? To answer this question I prepared the reagents listed below and proceeded with the experiment on the following day.

	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7	No. 8
30% glucose sol.	10	10	-					
10% NaOH sol.	3	3	3	3	3	3	3	-
pyridine	3	-	3	3	3	3	-	3
distilled water		3	10				13	13
10% hydrazine hydrate sol.		-	-	10				
saturated hydrazine sulfate sol.					10			
simple syrup						10		

The results of the experiment were as follows: two or three hours after No. 3 and No. 6 reagents were added to dry blood, the periphery of the preparation looked brownish green while in the inner part the centers of blood aggregates became red and gradually produced hemochromogen crystals. On the other hand, the reactions with No. 1, 4, and 5 reagents, which contained glucose, hydrazine hydrate, and hydrazine sulfate, were almost instantaneous—as soon as the reagents were added, dry blood turned deep ruby red and crystals were formed. From this it is clear that the efficiency of crystallization is increased with the addition of even a small amount of reducing agent, glucose in my case. Thus 25
although heating with sodium hydroxide solution and pyridine produces hemochromogen crystals, the presence of glucose no doubt facilitates the reaction, as I had expected.

The next question is: which is to be recommended as the reagent for hemochromogen crystallization, the first one with a small amount of glucose or the second one with a large amount? Both reagents produce hemochromogen crystals well, and their crystallizing action is strong. The difference is that one needs heating while the other does not. In cases in which it is possible to separate dry blood from cloth or paper for testing, the second glucose reagent with a large amount of glucose works better. However, if it is impossible to separate blood, and bloodstained cloth or paper must be tested as it is for the identification of blood pigment, the first glucose reagent with a small amount of glucose is more favorable. When used as I explained in the part on the procedure, it produces hemochromogen crystals very well. With the second glucose reagent, although hemochromogen crystals are produced if the test objects of such kind are about one year old, the results are generally not very good. As I have already mentioned, the first glucose reagent produces the crystals well also from dried blood separated from blood-stained materials. Therefore, in spite of the trouble of heating and the danger of losing dissolved blood pigment from the covered area when carelessly heated—this can be avoided by exercise of care, I recommend the first glucose reagent which contains a small amount of glucose. In contrast to the second glucose reagent that is effective for only about two weeks, the first glucose reagent can produce the crystals for more than a year, as long as the bottle is tightly stoppered. From this point, too, the first glucose reagent is superior to the second one. The periphery of the slide preparations made with an old first glucose reagent looks green, suggesting that the glucose has changed and lost its reducing power because of its small amount. Therefore, in practice a fairly fresh reagent should be used.

Pyridine in my glucose reagents may be substituted with piperidine (20–30%); the reagents still produce the crystals as Cevidalli and Dilling claimed. According to my observations, however, the crystals thus produced are small and somewhat difficult to detect. So I believe that pyridine suits our purpose better than piperidine. I also tried substituting glucose with other sugars. Lactose, fructose, galactose, and honey all function well in the place of glucose. Potassium 26
hydroxide can substitute for sodium hydroxide, but the latter seems somewhat better. However, if sodium hydroxide solution is replaced by ammonia water, my reagents lose their action. According to my experience, even among hydrazine hydrate and hydrazine sulfate reagents, those containing sodium hydroxide solution give better results than those without it.

Last year (1911) Leers reported that when 50% solution of hydrazine hydrate was added to hemin crystal preparations, hemin crystals were reduced to become deep ruby red. He called the product reduced hemin crystals. Heine wrote in the recently arrived *Viertelj. f. ger. M.* (vol. 43, p. 268) that the mixture of two parts pyridine and three parts concentrated aqueous solution of hydrazine sulfate had the same action as 50% hydrazine hydrate did. I treated hemin crystal preparations with the following reagents according to Leers' and Heine's procedures: my glucose reagents, 10% ammonia + 10% hydrazine hydrate, and a similar mixed solution containing saturated aqueous solution of hydrazine sulfate in the place of hydrazine hydrate (Mita), 1 part of ammonium sulfide + 1 part of pyridine (Donogany), 10 parts of (sodium hydroxide 5.0 + hydrazine sulfate 5.0 + water 100.0) + 5 parts of pyridine + 10 parts of water (modified de Dominicis), and pyridine + sodium hydroxide

solution (control reagent). The results showed that the reagents containing hydrazine hydrate or hydrazine sulfate, or both ammonium sulfide and pyridine, produced what Leers called reduced Teichmann's crystals. However, the hydrazine reagents had the regrettable characteristic of producing in the slide numerous minute gas bubbles that obscured the field of view. Leers' crystals did not keep very long: under continuous observation the pigment was leached from the red crystals leaving colorless residue. My second glucose reagent also reduced the crystals and blood pigment hematin in the preparations, but the red crystals that resulted decomposed immediately, and simultaneously numerous hemochromogen crystals appeared. In this experiment two slide preparations particularly drew my attention: the one that was treated with my first glucose reagent containing a small amount of glucose, and the other that was treated with the control reagent containing one part of 10% sodium hydroxide solution, two parts of pyridine, and seven parts of 27
distilled water. Left at room temperature without heat treatment, the former showed gradual reduction and was red all over after one or two hours. It revealed numerous hemochromogen crystals under the microscope. In contrast the latter looked brownish green and showed no hemochromogen crystals; in the center of the lumps of blood pigment that had failed to become hemin crystals, however, a red color and a few hemochromogen crystals sometimes developed. The contrast between these two slides was quite pronounced, and it proves what I stated earlier, namely, that glucose in my reagents plays a considerable role in the formation of hemochromogen crystals. Also noteworthy is the fact that, in cases in which blood pigment has changed into hematin, my first glucose reagent produces hemochromogen crystals without heating in spite of its small glucose content, though it takes somewhat long.

I should mention here Florence's work in which he used honey to remove indigo that obsured the spectrum of blood pigment. He reported that, if blood stains in an indigo-dyed fabric were treated with a small amount of honey and then soaked in 33% potassium hydroxide solution and blood was reduced by honey to become hemochromogen, which could be detected by the microscope or the spectroscope. Although hemochromogen was produced by this method, its crystallization did not take place. Florence used honey, and I used glucose, to reduce blood pigment; the purposes were different but the ideas were quite similar. This work by Florence was found in Leers' lecture called the present trend in medico-legal blood testing given at the meeting of Prussian Medical Doctors Association on April 27, last year. Since it gave no reference we cannot read the original. As I mentioned earlier, my reason for using glucose was quite unrelated to Florence's work. Although I report on these glucose reagents for the first time today, I already applied this method to identify blood pigment in January of last year when I was ordered by the Kurume District Court to test blood stains related to a murder case. I described the method in the written expert opinion that I submitted on March 6. Thus I learned about Florence's use of honey for hemochromogen production long after I had used glucose for hemochromogen crystallization. This is beside the point but 28
I mention it.

In the method of identifying blood pigment by producing hemochromogen crystals, how do my glucose reagents compare with other published reagents? Other reagents have some advantages and disadvantages, as I found out in the comparative study that I made using the reagents published by Donogany, de Dominicis, and Mita. In one part of the study I used the chemicals exactly as the authors described; in the other part I mixed the chemicals to make solutions, and when they failed to mix sufficiently I added some distilled water. The reagents used are given in the table below. Except for ammonium sulfide and 10% ammonia water, all the chemicals I used for this experiment were made by Merck, Darmstadt. I should also mention that, in this comparative study, I heated the slides with great care after adding reagents because careful heating always gives better results.

1. Mita described in detail the disadvantages of the rea- 29
gent that consists of one drop of pyridine and one drop of ammonium sulfide, which were used by Donogany and others: (1) the yellow color of ammonium sulfide makes the detection of hemochromogen crystals difficult by obscuring their cherry red color; (2) sulfur crystallizes out around the cover glass; (3) an unpleasant odor is given off; (4) if blood stains are on metals, especially iron, the detection of the test crystals becomes difficult or impossible because of numerous, strongly colored crystals of sulfide that are produced. Hummel stated that sulfur crystallized during the heating process to obscure the field of view and that the crystallization of sulfur might obstruct that of hemochromogen. Methling also wrote that sulfur from ammonium sulfide formed crystals and made the slide yellow and difficult to examine.

2. In de Dominics' and Mita's reagents, hydrazine hydrate has a very strong action in reducing blood pigment into hemochromogen. When treated according to Mita's procedure or with the mixed solution, dried blood turns instantaneously red and the blood pigment becomes hemochromogen. But it does not produce hemochromogen crystals as well as hydrazine sulfate does. Therefore, among the mixed reagents listed above, No. 7 and No. 11 are the best and No. 9, No. 6, and those containing ammonium sulfide are the next best. The disadvantage of hydrazine hydrate and hydrazine sulfate is that they cause minute gas bubbles to appear on the slide, especially in and around the test objects. This tendency is somewhat stronger with hydrazine hydrate. Hummel reported that using hydrazine hydrate he succeeded only once in producing hemochromogen crystals from dried powdered blood, but from liquid blood they were easily produced. He speculated that this might be due to gas bubbles.

3. The most serious disadvantage common to ammonium 30
sulfide and hydrazine reagents is that they either fail to

Table

	10% NaOH solution	10% ammonia water	NaOH 5; distilled water 100 hydrazine sulfate 5;	pyridine	ammonium salfide	10% hydrazine hydrate	saturated hydrazine sulfate	absolute alcohol	distilled water
1 (Donogany)	-	-	-	5.0	5.0				
2 (Lochte)	-	-	-	5.0	5.0			5.0	
3	2.0	-	-	2.0	5.0				
4	2.0	-	-	5.0	5.0				5.0
5 (Mita)	-	5.0	-	5.0		5.0			
6 (Mita)	-	5.0	-	5.0			5.0		
7 (de Dominicis)	-	-	10.0	5.0					10.0
8	2.0	-	-	5.0		5.0			10.0
9	2.0	-	-	5.0			5.0		5.0
10	3.0	-	-	3.0		10.0			
11	3.0	-	-	3.0			10.0		
12	-	-	-	5.0		*			10.0
13	-	-	-	5.0			5.0		
14 (Heine)	-	-	-	4.0			6.0		

*5.0 of 50% solution

crystallize hemochromogen or crystallize them with difficulty when blood has permeated the tissue to paper or cloth and cannot be separated. Mita stated that when blood had not dried on the surface but had penetrated such materials as linen and filter paper, his hydrazine reagents sometimes failed to produce crystals. In such cases, he recommended, blood should be extracted with glacial acetic acid, and the extract be dried at room temperature to obtain a residue, with which hemochromogen might be crystallized. Lochte also reported that repeated testing with one drop each of absolute alcohol, pyridine, and ammonium sulfide failed to produce the crystals in blood adhered to wool, cotton, or linen fabrics, when intact or teased tissue was treated. He said that blood should be extracted on such occasions with water, ammonia water, 10% soda water, or 10% sodium hydroxide solution, and the residue be used for the crystallization of hemochromogen. Hummel used pyridine and ammonium sulfide and confirmed Lochte's results, but extraction with water as Lochte suggested frequently gave him negative results.

4. My glucose reagents, both the first and the second, are as effective as the best of other reagents in producing the crystals from dried powdered blood. With the glucose reagents the detection of the crystals is easy because the field of view is clear—unlike hydrazine or ammonium sulfide reagents, they do not produce gas bubbles or sulfur crystals.

Although my glucose reagents cannot be proved far superior to other reagents as far as powdered blood is concerned, it becomes clear that they surpass all the others when tests are made on blood that has permeated fabrics or paper and cannot be detached. As Mita, Lochte, and Hummel admitted themselves, their reagents frequently fail to produce the crystals with such test objects, while my glucose reagents easily crystallize hemochromogen. Although the reagents of the following compositions produce hemochromogen crystals fairly well from blood that has permeated fabrics when used in the same way as my glucose reagents, the results are far inferior:

1. 10% sodium hydroxide solution 3.0 + pyridine 3.0 + saturated aqueous solution of hydrazine sulfate 10.0.
2. (sodium hydroxide 5.0 + distilled water 100.0 + hydrazine sulfate 5.0) 10.0 + pyridine 5.0 + distilled water 10.0.

It is medico-legally very important that blood can be easily 31
identified from a minute amount of sample without loss of material, and the superiority of my glucose reagents over the others resides in this point. Actual slides are exhibited in the other room; I hope you will look at the proof firsthand.

I must point out, however, that there are some cases in which even my glucose reagents fail to produce hemochromogen crystals. In some cases the spectrum is demonstrated even though crystallization does not take place; in some others, both give negative results. In such cases not only other reagents for hemochromogen crystallization but also reagents for hemin crystallization fail. For example, in 1907 I could demonstrate hemochromogen crystals in the tissues of blue Chinese silk crepe, red cotton cloth, thin red silk, newspaper, and thick Japanese paper that had blood from a human corpse adhering to them; I could also produce the crystals in the part of indigo-dyed cotton cloth that had comparatively abundant amounts of blood, but not at all in parts with a little blood. On July 7, 1907, I demonstrated the spectrum in slides prepared from the plaster and the floor wood of a lecture hall that had been stained with blood of a domesticated rabbit, although I could not produce the crystals of hemochromogen. On the other hand in May, 1908, I failed to produce either the crystals or the spectrum in slides containing blood and powdered iron (1:4) or blood and ash (2:10). Also to be noted is the fact that, when treated with the glucose, hydrazine, or ammonium sulfide reagents, red fabrics sometimes produce red, needle-shaped crystals that resemble hemochromogen crystals; one red dye gives a thick absorption band that corresponds to the first of the two absorption bands of hemochromogen. When test objects are colored fabrics, therefore, control tests using the parts without blood stain must not be neglected. I expect to report on

this subject on another occasion.

Thus far I have talked about dry blood. The method of identifying blood in liquids depends on the nature of the liquids: residue may be tested after evaporation; blood precipitate may be collected according to a certain method; blood pigment may be changed into hemochromogen by adding sodium hydroxide solution to make the liquid strongly alka-
32 line, then dissolving a suitable amount of glucose and pyridine in it, and heating the mixture. In this last method the spectrum is examined, and if precipitate is formed, it is collected using a Spitz glass or a centrifuge at a slow speed and is examined under the microscope, since precipitate sometimes contains hemochromogen crystals.

Lastly, a few words will be added for the clinicians' interest. As early as 1897, Donogany reported that hemochromogen and its crystals could be used for identifying blood in urine, stool, and sputum. Neuberg also mentioned in his recently published *Der Harn* (The Urine) (Vol. 1, p. 936) the applicability of the hemochromogen crystallization method to the test of blood in urine. I speculate that blood in urine may be precipitated with tannin, zinc acetate, or glacial ethylacetate as O. Schümm has done, collected on a filter paper, and tested, together with the paper if the quantity of blood is small. In case of stool, blood may be extracted with glacial ethylacetate as Über has done, and the residue may be used for hemochromogen crystallization. Blood may then be easily and certainly identified by the presence of the crystals, or the spectrum if the crystals fail to form. How about trying these tests sometime? Our laboratory will supply the reagents.

—Lecture given at a meeting at Kyushu Medical College on May 16, 1912.

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On the Behavior of the Coloring Substance of Blood in the Spectrum of Sunlight*

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446 As a result of the experiments of D. Brewster, Herschel, and Muller,† the behavior of different dye stuffs with respect to different segments of the spectrum has been determined. In these experiments, it was shown among other things that light of specific refrangibility is so completely absorbed by a large percentage of dyes that, if one lets the rays of the spectrum pass through very diluted solutions of these dyes, dark, rather sharply outlined bands appear in definite places. One can observe the spectrum after the rays have passed through the solution either directly, or by capturing the spectrum on a white surface. At the same time, the experiments showed that from the color of the solutions one may conclude only that these solutions absorb the colors least which they themselves display in white light. On the other hand, one may not conclude from the colors of the dyes in white light which light is absorbed the most.

The absorption bands which appear in the spectrum when it passes through a solution of dye, are apparently characteristics of the dyes, characteristics which often make possible recognition of the dyes when their solutions are very concentrated. They deserve all the more consideration since there is a lack of exact chemical methods for distinguishing dyes and their variable forms.

Pigment such as blood contains displays the same characteristic as do indigo and chlorophyll, dyes which have been previously examined, the characteristic that it can absorb, in a very pronounced fashion, light of a particular refrangibility, and thus produce dark bands in the spectrum which passes through a solution of the pigment. No other red pigment, including chemically altered hematin, displays such bands. This well known combination of apparatus serves the best to test dyed solutions with the spectrum. A heliostat projects the light through a slit into a darkened space – the darkening can be very slight – and onto an achromatic lens. The slit should be at the focal point of the lens. From there, the light is projected into a prism of glass or of carbon disulfide. One now lets the spectrum, produced in this manner, pass through the solution to be tested; this solution should be placed in a thin container of glass with parallel walls. One may now observe this spectrum either directly with the telescope, or with the unaided eye after intercepting it with a white-paper screen. Hematinometers serve very well as containers for the dye solutions, containers which the optician Schmidt in Berlin has prepared following my direc- 447
tions. In these containers one can test a layer of fluid exactly one centimeter in thickness.

If one now observes a very diluted solution of blood in water in such a container, placed in the spectrum, one will see that, after it has passed through the solution, the spectrum will display two definite dark bands in the yellow and in the green. Both bands are located between the Fraunhofer lines D and E. The band corresponding to the more weakly refracted light is rather close to the double line D; the second band is not so close to E. When the solution is dilute enough, both bands have a width somewhat less than the spectral segment between E and b. If one increases the concentration of the blood solution, or if one lets the spectrum pass through a thicker layer of the solution, the width of both absorption bands increases, but almost exclusively at the cost of the yellow-green light which separates the two lines from one another. When the concentration of the solution is still further increased, the bands finally merge together to form a dark, relatively sharply outlined field. At the same time, more and more of the violet and blue light grows gradually fainter without producing any definite bands. Finally, of the whole spectrum only the segments between E and b, and the red and orange remain. In the case of even stronger concentrations, the green also grows faint and disappears so that only the red with its beautiful Fraunhofer lines remains. I have not pursued the question of the refrangibility of the least red light. While, according to these observations, the blood pigment absorbs with unusual strength the light in the areas indicated between D and E, it leaves intact the sections between A and D as well as between E and b with the same clarity as it absorbs the other sections. The sharpness of the contours of the absorption bands described here results from the fact that the most strongly absorbed sections are closely outlined by the sections most weakly absorbed.

Undissolved blood cells also absorb the parts of the spectrum described here. In order to observe this, it is sufficient to project a spectrum, shining from a prism, by means of the concave mirror of a microscope. (The concave mirror must

* Translation of: "Ueber das Verhalten des Blutfarbstoffes im Spektrum des Sonnenlichtes."
in *Archiv für pathologische Anatomie und Physiologie und Klinische Medizin* (Virchow's Archiv) 23: 446–449 (1862).

† Poggendorf, *Ann. d. Phys. u. Chem.*, Vol. 72, 76 and Pouillet, *Muller's Lehrb. d. Physik*

be placed so close to the prism that the light which the mirror reflects is parallel or only somewhat convergent). The spectrum must be projected upwards through the opening of the microscope stage onto a thin layer of blood. This layer has been fixed here between the slide and cover slip. If now one removes the tube of the microscope, and looks down perpendicularly at the blood layer, one will recognize most clearly the two absorption bands.

Watery solutions of the blood from the silver-scaled fish, from the *Testudo mauretanica*, as well as from dove, dog, ox, sheep, and pig, all behave in exactly the same manner regarding the absorption bands in the spectrum; these bands are thus to be generally regarded as characteristic of blood from vertebrates.

Arterial blood as well as venous blood shows both of these bands. Lengthy treatment of the blood solutions with carbonic acid does not alter anything. I have also observed that the solution is equally unaffected when the blood is treated 448
with carbon monoxide, hydrogen, hydrogen sulfide, arsenic hydride, nitrous oxide, ether, carbon disulfide, chloroform, caustic ammonia, or arsenic acid. Blood, dissolved in caustic ammonia, still showed the two absorption bands on the following day with undiminished strength. After treatment with hydrogen sulfide, a third band (in the red) appeared in addition to these two bands (a green solution of iron sulfide in a liquid containing ammonium sulfide, such as one would obtain by adding a very diluted iron sulfate solution to ammonium sulfide, does not produce this line in the red).

Drying the blood at normal temperature does not alter the state of the spectrum.

On the other hand, the absorption lines disappear very rapidly if one adds either acetic acid, tartaric acid, or strong alkalis to the blood solution. The acids work faster than the alkalis in producing this effect. The hematin solution of von Wittich no longer produces the two bands; in sufficient concentration it displays other absorption lines, the stronger of which is found between C and D, close to the last of these two bands. The von Wittich solution corresponds to the blood with regard to the rays of the spectrum which are absorbed the least.

Blood, treated with an excess of cold alcohol, produces a precipitate which, when dissolved in ammonia, no longer displays those absorption bands in the spectrum. Turpentine oil also causes them to disappear. Likewise, the hematin solution, which is obtained by extracting the dried blood with cooking alcohol and sulphuric acid, does not show these bands in the spectrum.

Blood, precipitated with powdery, carbonated potassium hydroxide, retains for days a beautifully arterial coloration, if no heating takes place; if one pours alcohol over the substance, the red coloration soon changes into a dirty brown, and only then does a solution of hematin appear. This solution no longer has the absorption bands. If, on the other hand, one dissolves the moist precipitate in water instead of in alcohol, one obtains a solution which displays both absorption bands just like fresh blood. Likewise, a blood solution with sodium carbonate will not be changed weeks later with respect to the condition of the spectrum.

In none of the fluids which failed to display the absorption bands could these be reconstituted by treatment with alkalies, etc.

If one precipitates the blood solution with an excess of lead acetate, filters it, and then, by using sodium carbonate, precipitates the lead from the filtrate, one obtains a solution which produces most sharply the absorption bands in the spectrum.

If one induces hematuria by injecting gallic acid salts into the veins of dogs, the urine does not display the absorption bands in the spectrum, although one is able to produce hematin from such urine; this urine also does not turn red on contact with oxygen.†

From the behavior of unaltered blood, as well as from that of blood treated with various reagents, it turns out that the contents of the blood cells (the serum displays no noticeable absorption in the yellow and green, when the layer of the serum is not thicker than one decimeter) very strongly absorb the indicated parts of the spectrum, as long as the 449
albumin substances of this liquid have not coagulated or been transformed into alkali or acid albumin. Now, since a substance which shows such definite absorption cannot appear colorless as do the well known albumin substances, one would thus have to assume that this very substance, which gives to the contents of the blood cells its red color, also produces this absorption. Further, since this absorption capacity appears to be independent of the most varied color alterations which the blood undergoes when treated with oxygen, carbonic acid, carbon monoxide, arsenic hydride, etc., and, on the other hand, since this absorption capacity is destroyed by relatively weak processes which, however, affect coagulation or the altering of all albumin substances, the following assumption seems to be justified. All those changes, which produce the gases described in blood pigment, do not destroy the pigment. Moreover, one may now hope to find a means by which altered blood can be transformed again into normal blood.

As a result of the reactions mentioned above, it also appears certain that there is a compound in the blood cells which produces the pigment of blood, and causes these absorption bands. It is not precipitated by lead acetate; it dissolves much more easily than albumin; and, when acted upon by acids, caustic alkalis, etc., it breaks down into an albumin substance and hematin, which is contained in the von Wittich solution. Without a doubt, this is the very body which forms the Funk crystals. If this representation is correct, then it follows that the attempt to obtain uncolored blood crystals is in vain, although it might be possible that, when the corpuscle breaks down, substances can form which are equally capable of crystallization. I am now carrying out the purification and chemical testing of this "blood red".

For the forensic identification of blood in stains on cloth-

† One can conclude from this that, in the kidneys, the transuded blood pigment is probably decomposed by a secreted acid.

ing, etc., one already possesses a rather large number of test methods which are in part exact. One can naturally also make use of the testing method presented above. Bloodstains, which are not extracted, and are on white linen or transparent paper, must be somewhat moistened and then reveal, in a sunlight spectrum, the bands described, when they are placed so as to intercept the rays of the spectrum between the prism and the eye.

Tincture of Guaiacum (Guaiacum Officinale L.) and an Oxone Vehicle as Reagent for Very Small Amounts of Blood, Specifically in Cases in Forensic Medicine*

J. van Deen

228 The smallest blood volume of any age, even with a substantial admixture of other substances, stains blue when tincture of guaiacum and an ozone vehicle (for example: oil of turpentine) are added.

The following experiments are intended to confirm the above:

1. A minimum amount of aged, fetid blood, which had been stored for approximately 8 to 9 months, was diluted with distilled water until the fluid became nearly achromatic. When a few drops of this mixture were added to oil of turpentine, which has a high ozone content, intensive blue staining soon became evident.

The blue staining was also definitely determinable when the blood was initially left standing for 24 hours with oil of turpentine and tincture of guaiacum was subsequently added. When the blood, mixed with oil of turpentine, is filtered, the tincture of guaiacum had no effect on the filtrate, 229 probably because the turpentine did not pass through the filter, and its ozone content was not absorbed by the blood, prior to the admixture of the tincture of guaiacum.

2. Blood was left standing for two years with glacial acetic acid and was diluted in minimal parts with water until the fluid appeared nearly achromatic; then a few drops of oil of turpentine and tincture of guaiacum were added. The blue staining developed immediately.

3. When more glacial acetic acid was added to the blood mentioned under 2, followed by filtration, a minimum of the filtrate still stained blue with oil of turpentine and tincture of guaiacum. However, in some cases the staining soon vanished. Boiling did not inhibit the blood reaction.

4. Minimal quantities of blood which had been stored in alcohol for two years and which contained numerous coagulated particles were treated with oil of turpentine and tincture of guaiacum; blue staining developed immediately. The blue staining could not originate from the alcohol; it could, however, originate from the solid particles in the same, despite their microscopically small size.

5. A three-year old desiccated calf blood clot was finely pulverized; 0.1 g of the same was mixed with 400 g water and repeatedly agitated. A few drops of the above mixture soon stained blue with oil of turpentine and tincture of guaiacum.

6. A drop of the mixture mentioned under 5, mixed with five drops of water still reacted, even when 1/6th of the mixture was subjected to the procedure.

7. When the last-mentioned dilution was doubled, so that 1 drop contained not more than 1:40,000, the reaction still took place.

When very small considerably diluted blood volumes are used, the start of the reaction will take place after a few moments' delay only.

Since Schoenbein demonstrated that the iron in the blood 230 probably transfers the ozone from the ozone vehicle to the tincture of guaiacum, control tests with iron preparations had to be performed.

Ferric oxide, ferric hydroxide, caput mortuum and ferrous carbonate were tested with negative results.

Moreover, it was found that iron filings, ferrous oxide, hydroferrocyanic acid, calcium ferrocyanide and ferric phosphate do not act as ozone transmitters. However, the following act as such:

a) to a moderate extent: ferrous sulfate, ferric lactate, ferrous iodide and ferrous sulfide;

b) to a significant extent: ferrous acetate, ferrous citrate and ferric chloride, in particular the last-mentioned salt, which is no less effective in this respect than old, fetid blood.

$CuSO_4$ and cuprous acetate were recognized as ozone transmitters as well, but not to a very great extent.

Various preparations, in particular the red lead and antimony preparations, yielded negative results. The same applies to red dyes such as logwood, brazil wood, sandalwood and carmine. None of the iron preparations which act as ozone vehicles has a color similar to the color of blood; mistakes therefore do not occur readily. Ferric acetate is red, but its color is of a much brighter shade than the color of blood. Furthermore, it is easy to determine with ammonia water whether the investigated substance is an iron preparation or blood. When tested with the above-mentioned substance, iron preparations show significant clouding which soon deposits in the form of a red precipitate. Blood, on the other hand, will show a greenish-yellow discoloration. A minimum of iron content results in a yellow solution, but the substance remains clear, while fluids containing blood are never transparent. Not too much ammonia water should be used for the test.

* Translation of: "Tinctura Guajaci und ein Ozonträger, als Reagens auf sehr geringe Blutmengen, namentlich in medico-forensischen Fällen", in *Archiv für die holländischen Beiträge zur Natur- und Heilkunde* 3: 228–231 (1862).

The difference, as compared with the blood reaction,
/231 specifically the fresh blood reaction, was less evident only in case of ferrous sulfate. But the difference nevertheless became determinable a few hours later, and especially after a few days. Potassium ferrocyanide prevents mistakes, i.e., mistaking a blood reaction for a copper reaction.

Aged blood which has become fetid, especially when stored in fluid condition, has a stronger capacity as ozone vehicle than fresh blood.

The Reaction of Certain Organic Compounds with Blood, With Particular Reference to Blood Identification*

Oscar Adler and Rudolf Adler

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I.

59 Among methods used for blood identification, the preparation of hemin crystals is to be considered primary; under certain circumstances, albumin and iron determination can be significant as well for blood verification. Clinically, the guaiacum test is widely used for blood determination in urine, in gastric juice and in feces. Recenlty Vitali[1] spoke warmly in favor of the test. As known, the guaiacum test is based on the fact that hemoglobin can transfer oxygen originating from turpentine oil or from hydrogen peroxide into the active ingredient of guaiac resin, named guaiaconic acid by Hadelich; a neutral substance, or a substance which stains blue in acid solution will result, and is named a guaiaconic acid ozonide.

We used *guaiacin* successfully for blood identification instead of guaiacum tincture. The substance, prepared by Schmitt[2] from guaiacum wood, proved to be more sensitive. We mixed the fluid to be analyzed with a small volume of hydrogen peroxide and covered the mixture with a layer of an alcoholic guaiacin solution.

More recently Rossel[3] recommended Barbados-aloin for blood determination in urine. According to Utz[4], the test is less sensitive than the guaiacum test.

60 The purpose of present study is the systematic investigation of numerous chemical compounds which show chromatic reaction as a result of oxidation, in the presence of blood (when hydrogen peroxide is added). Some of these substances were formerly used for the verification of ozone[5] and oxidizing ferments (oxidases)[6]. We considered the sensitivity of the reactions, as well as the behavior of control reactions; our results could, therefore, perhaps be of interest for the study of oxidizing ferments as well.

II.

We report the result of our systematic investigations below. A large number of substances is to be considered in this context; we limited ourselves to the aromatic amido-substances, phenols, aromatic acids and to the diphenyl- and naphthalene groups[7]. We studied the behavior of the reduction products of certain tar dyes (leucobases) in conjunction with the above.

The blood used for the experiments was collected from the carotid of the animal (rabbit), immediately defibrinated, and a specific concentration was obtained for investigation purposes by diluting the blood with distilled water. The reaction took place as follows: at the start, the desired blood concentration of 0.001% (i.e. a 100,000-fold dilution of the blood) was obtained; then hydrogen peroxide was added and the test continued until a definite chromatic reaction occurred, or until a chromatic difference as compared with the control became evident. As the following table shows, we define this point of the test as the sensitivity limit of the reaction.

The following is added for the clarification of the Table: all reactions were performed with the aid of controls (identical testing conditions, but without the presence of blood). When the control showed no chromatic modification, the result was defined as negative. But whenever the control reagent showed a chromatic change due to atmospheric oxygen, only the color difference could, of course, be taken into consideration.

III.

As the Table above indicates, the reactions of the individ- 63
ual representatives of the listed groups (amido-substances, phenols, acids) vary during the described procedure, i.e., the higher members of the series generally proved to be more sensitive.

In conjunction with these experiments, we investigated some readily oxidizable leuco-bases of the triphenylmethane series. We found that the malachite green group (malachite green, brilliant green, acid-green) and the pink aniline derivatives (dahlia, methyl violet, crystal violet) are preferable for the above-mentioned purpose. The rest of the triphenylmethane dyes (alkali blue, ketone blue, patent blue, cyanin, Turkey blue) and the eosins and rhodamines do not yield satisfactory results.

We wish to convey thanks here to the Dye Works (formerly Meister, Lucius and Brünning) for making many of their products available.

In view of the fact that all above mentioned reactions occur because the blood pigment transfers oxygen originat-

* Translation of: "Über das Verhalten gewisser organischer Verbindungen gegenüber Blut mit besonderer Berücksichtigung des Nachweises von Blut".

in *Hoppe-Seyler's Zeitschrift für Physiologische Chemie* 41: 59–67 (1904).

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Substance	Reaction of the Solution	Color Reaction	Sensitivity Limit (%) Blood Solution	Control
A. Amido-substances				
a) monamines				
aniline	acid (HCl, H_2SO_4)	black-green	0.1	negative
monomethylaniline	acid (HCl)	dirty violet	0.1	"
dimethylaniline	acid (HCl)	light yellow	0.1	"
diphenylaniline	acid (acetic)	green		"
p-toluidine	acid (HCl)	red	0.01	"
xylidine	acid (HCl)	brown-red	little sensitivity	"
b) diamines				
o-phenylenediamine	neutral	brown	0.007	discoloration after standing for some time.
m-phenylenediamine	neutral	violet	0.007	
p-phenylenediamine	neutral	brown	0.007	
dimethyl-p-phenyldiamine	neutral	red	0.009	early color balance
tetramethyl-p-phenylene-diamine	neutral	violet	0.009	
B. Phenols				
a) monohydric phenols				
phenol*	neutral or alkaline (NaOH)	red brown	little sensitivity	negative
p-amidophenol	alkaline (NaOH or Na_2CO_3)	violet	0.008	early color balance
o-cresol*	neutral or alkaline (NaOH)	red brown	little sensitivity	negative
m-cresol*	neutral or alkaline (NaOH)	red brown	" "	"
p-cresol*	neutral or alkaline (NaOH)	brown red	" "	"
thymol	alklaline (NaOH or Na_2CO_3)	brown red	" "	"
b) dihydric phenols				
pyrocatechin	alkaline (NaOH)	green	0.005	color balance develops after some standing
guaiacol†	"	yellow brown	0.05	negative
resorcinol	"	greenish	0.01	color balance develops after some standing
hydroquinone	"	brown yellow	0.005	
orcin (methyl resorcinol)	"	red	0.002	color balance develops after prolonged standing
c) trihydric phenols				
pyrogallol	"	brown	0.005	early color balance
phloroglucinol	"	violet	0.005	"
C. Aromatic acids				
benzoic acid	alkaline (NaOH)			
salicylic acid	"	brown	very little sensitivity	negative
pyrocatechuic acid	"	pink (vanishes gradually	0.001	negative (yellowish)
gallic acid	"	brown	0.005	color balance
Diphenyl Group				
benzidine†	acid (acetic)	green‡	0.001	negative
tolidine†	"	red	0.05	"
		green	0.25	"
Napthalene Group				
α-naphthol	alkaline (NaOH)	brown	not tested	negative
β-naphthol	"	brown yellow		"
α-naphthylamine†	acid (HCl)	dirty blue	little sensitivity	"

† alcoholic solution
* behavior of creosote from Buchholz tar is, of course, similar
‡ stains blue after prolonged period of time

ing from hydrogen peroxide to the respective oxidizable substances, the logical conclusion is reached that other substances can trigger analogous reactions as well.

Among the substances to be considered we mention the following here: iron-oxide salts (Vitali[8]), the thiocyanate salts (Tarugi[9]), certain oxidizing ferments (indirect oxidases[10]); all these substances are able to act as indirect oxidizing agents in the presence of hydrogen peroxide. In animal fluids containing leukocytes (urine, saliva, pus) oxidizing ferments which are destroyed by boiling are pre-
764 sumably present. As a matter of course, pus containing blood, as usually found in medical practice, reacts after boiling as well. No conclusive experimental studies are available as of now concerning the behavior of pus without any even minimal trace of blood.

In contrast to the substances mentioned earlier, other reducing substances can have a disruptive effect. Uric acid, for example, can inhibit the sensitivity of the leuco-malachite test (see below); but such an effect can be eliminated by proceeding according to Weber's guaiacum test (see below). Finally, it should be pointed out that substances which trigger a secondary reaction (iron salts: yellow staining of malachite green; nitric acid: formation of diazo-substances) should be taken into due consideration.

Summarizing test results obtained as of now, we feel justified in saying that even minimal blood traces (dilution: 100,000-fold) are determinable with some of the above-mentioned reactions. Therefore, whenever test results are negative (see below), it is reasonable to assume that no blood is present.

A few suggestions for the practical utilization of the blood tests described are listed below.

IV.

Blood Identification

The chemical identification of blood is of foremost importance, chemically as well as forensically. Accordingly, efforts were made for a long period of time to devise methods for blood verification even when the amount of blood is minimal. However, besides the importance of positive blood identification, definite proof that no blood, not even minimal quantities of it, is present, can under certain circumstances--be of great significance as well.

It is known that the negative outcome of one of our finest methods: the preparation of hemin crystals, does, under certain circumstances, not represent definite proof of the ab-
765 sence of blood. Cases are known when the presence of blood was verified despite the negative outcome of the Teichmann test.

We list a few tests below, intended for the identification of blood stains, the identification of blood in water, urine and in feces.

Identification of blood stains. We used leucomalachite green (leuco-base of malachite green) for this purpose. The test was performed as follows: the stain to be investigated is thoroughly soaked with the reagent (see below); then a 3% solution of hydrogen peroxide is poured over the stain. If it is a blood stain, the stain will immediately turn green. The reaction is also well-defined when minimal, hardly perceptible, blood traces are present. The reaction will also occur with boiled blood stains.

We preferred to prepare the reagent as follows: a concentrated solution of completely achromatic, chemically pure, leuco-base of malachite green (tetramethyldiamidotriphenyl-methane)[11] is prepared in glacial acetic acid. A minimal green color will develop in most cases even when a completely achromatic preparation is used; the green color is eliminated by adding an equal volume of chloroform. Water is then added drop by drop while the mixture is carefully agitated until the chloroform precipitates entirely. The green chloroform is then separated from the supernatant reagent. The eventual clouding of the reagent, which can be caused by the precipitation of the leuco-base, is eliminated by adding glacial acetic acid. If traces of green color are still evident in the reagent, these are removed by shaking the reagent with a small quantity of chloroform. The reagent, prepared as indicated, should be entirely achromatic.

We pointed out earlier that substances other than blood can cause a positive result of the test. The possible presence of such substances should therefore be taken into consideration. I refer in this context to statements made in Part III, page 63 and following pages.

As for the negative outcome of the test: we found that the presence of iron salts in ample quantities can prevent green staining, even when blood is present; yellow staining will 66
develop instead. Irons salts should therefore be excluded (iron tests).

When all precautions are taken into consideration, the negative outcome of our test leads to the conclusion that not even minimal traces of blood are present.

Identification of blood in water

Due to their high sensitivity and due to the completely negative outcome of the control tests, the following substances are suggested for blood identification in water: leuco-malachite green (see reagent preparation), crystal violet leucobase and benzidine.

For the benzidine test we used alcoholic benzidine solution, concentrated while heated and filtered after cooling.

For the implementation of the test, we mixed the water to be investigated with a small volume of hydrogen peroxide and a few drops of acetic acid; then a few cubic centimeters of the benzidine solution were added. A spendid green staining develops when blood is present.

The test succeeds also when performed after boiling the water containing blood.

Regarding the precautions, we refer to Part III, page 63 and following pages.

The high sensitivity of the leuco-malachite green test and of the benzidine best is significant, as compared with the spectroscopic method and the Teichmann test. The Teich-

mann crystals can be obtained when the dilution is at least 20,000-fold.[12] Moreover, blue staining develops in the guiaiacum test in a 500-fold dilution.[13] On the other hand, the leuco-malachite green test and the benzidine test show definite color reactions even in a 100,000-fold blood dilution.

Blood identification in urine and feces

67 *Urine:* the aforementioned tests with leuco-malachite green and benzidine are suitable for blood identification in urine as well, when proceeding according to Weber's modification of the guaiacum test.

10 to 15 cc urine is mixed with half that volume of glacial acetic acid, whereby hemoglobin is converted into hematin. Agitation with ether follows, and the hematin mixes with the ether. If the ether in the above mixture forms an emulsion, the liquid can be separated by adding a few drops of alcohol. The ether is removed; then the leuco-malachite green and a small volume of hydrogen peroxide is added. Should some of the leucobase precipitate during this process, the precipitate can be dissolved by adding a small amount of glacial acetic acid.

Instead of the leuco-malachite green reagent, the following can be added to the ether: an alcoholic benzidine solution, some hydrogen peroxide and a few drops of acetic acid.

When the urine contains blood, the color reactions mentioned earlier will develop.

Feces: A small quantity of the feces to be investigated is slightly diluted with water. Then 3 cc of the diluted and unfiltered feces is mixed with 2 cc of the aforementioned benzidine solution and with 2 cc hydrogen peroxide (3%); a few drops of acetic acid are added. Intensive green staining develops when blood is present.

Notes

1. Vitali: *Giorn. di Farmac. di Trieste*, 1902, vol. 7, p. 193
2. Schmitt: Le Bois de Gajac, Thesis at Nancy, 1875, and *Mercks Bericht*, 1902, p. 75.
3. Rossel: *Schweizer Wochenschr. Chem. Parm.*, 1901, No. 39, p. 557ff.
4. Utz, *Oesterreich. Chem. Ztg.*, 1902, vol. 5, p. 558.
5. See C. Arnold & C. Mentzel: *Ber. Dtsch. Chem. Ges.*, 1902, **35:** 1324 and 2902; Chlopin: *Ztschr. f. Untersuch. v. Nahrungs- und Genussm.*, 1902, **5:** 504
6. See Neumann-Wender: *Chem. Ztg.*, 1902, **26:** 1217 and 1221
7. Thallium hydroxide also indicates the presence of blood under similar conditions. We have not considered the other inorganic compounds.
8. Vitali: *Giorn. di Farmac. di Trieste*, 1902, vol. 7, p. 193.
9. Tarugi: *Gazz. Chim. Ital.*, 1902, 32, 1 vol. 505.
10. See Bourquelot: *Congr. Pharm.*, Paris 1899, Répert. Pharm. and Neumann-Wender: *Chem. Ztg.*, 1902, **26:** 1217 and 1221ff
11. A leucobase especially prepared for this purpose can be obtained from the Chemistry Laboratory of the wholesale pharmacy Wilhelm Adler at Karlsbad, where the prepared reagent is also available.
12. Hager: *Handbuch der Pharm. Praxis*, vol. II, p. 879.
13. Hager: as cited above, p. 881

The Chemiluminescence of Hemin: An Aid for Finding and Recognizing Blood Stains Important for Forensic Purposes*[1]

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225 In the unraveling of capital crimes, the discovery and proper evaluation of a blood stain, the identification of blood as such, as well as the determination of the blood type often play an important role, indeed frequently a deciding one.

226 Blood stains are very different, depending on the type of injury involved, and on the kind of material to which the stains adhere. For the expert who thinks and works comprehensively, in accordance with the principles of criminalistics, the proper evaluation of the blood stains present will always be of the greatest importance. From the general type and the direction of the blood spatters, and from the place where the stains were discovered, it is often possible to reconstruct essential details of the crime.

The presence of even the smallest blood spatters at the scene of the crime, on the clothing of the assailant, or on the criminal himself can be of decisive importance. It is, however, not always easy to identify blood traces at the scene of the crime, or on the weapon, especially in those cases which involve an old weapon or old blood stains.

Temperature, substratum, sun rays, moisture, as well as artificial washing, and chemical changes of the hemoglobin can fundamentally alter the external state and color of blood stains. Frequently, blood stains are covered over with dirt smudges which makes it difficult to recognize them. On the other hand, sometimes the dried remains of red fruit juices, for example, or of tobacco saliva, fungus, and mildew, are not unlike blood stains.

While *fresh blood traces* can naturally be identified with ease, usually by the microscopic identification of blood corpuscles, to identify *older blood stains* always requires special aids.

The hematin, which has been formed in old, dried blood demonstrates the well-known dichroism which simplifies the recognition of blood stains. Especially in the sunlight, an older blood stain glimmers with a green hue in reflected light, but with a reddish color in direct and indirect light. Blood stains, however, do not always lie exposed to the light of day. There are, accordingly, limits set in recognizing a blood spot by means of the optical appearance of dichroism. For example, the blood could have been removed from the substratum by cleaning, while it might still be present in the smallest portions in the dust in floorboard cracks, in the seams of shoes or in the seams or hems of a suit, or washed through to the suit lining; it could also be lodged in the smallest openings or notches of a tool or some similar instrument. Not infrequently, one must carry out a test for blood traces in wash water or the remains of liquids caught in goose-neck pipes in the relevant drains or wash basins.

Blood traces outside can become unrecognizable to the naked eye within a short span of time as a result of meteorological or mechanical conditions, or, among other causes, as a result of frequent downpours. One must resort to preliminary chemical tests to discover such hidden blood traces. 227
These tests are all based on the catalytic effect of blood, the effect of transferring oxygen. One thinks of the hydrogen peroxide, tincture of guaiacum, and benzidine tests. A positive result of these tests is, however, not conclusive proof of the presence of blood. Moreover, carrying out these tests has the disadvantage that some of the material is lost.

The hydrogen peroxide reaction must be characterized as very dangerous, especially since steel rust, which unpracticed observers can confuse with blood traces, produces a catalytic decomposition of hydrogen peroxide.

On the other hand, the positive results of microchemical reactions, by means of which the characteristic crystals of hemoglobin and its derivatives (*Teichmann* hemin and hemochromogen crystals) are produced, are proof of the presence of blood. The *Teichmann* crystal test, however, is bound up with a number of difficulties, in so far as the crystalization does not take place when insignificant mistakes have occurred in carrying out the reaction, or when different chemicals are present. Moreover, this test gives a negative result when the solubility of the hemoglobin is reduced.

The identification of blood can only be considered as cer-

* Translation of: "Die Chemilumineszenz des Hämins, ein Hilfsmittel zur Auffindung und Erkennung forensisch wichtiger Blutspuren."

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tain if one of the characteristic blood absorption spectra is obtained from a suspected stain. For a long time it was only possible to link the hemochromogen test directly to the spectroscopic test. After carrying out the other preliminary chemical tests, the use of spectroscopy is no longer possible. According to K. Gleu and K. Pfannstiel[2], when 3-aminophthalhydrazide in alkaline soda solution and hydrogen peroxide or diluted sodium peroxide solution is added to hemin, an intensive chemiluminescence takes place. In pursuing a suggestion made by these authors, we tested to what extent and with what results this light reaction was useful in discovering and characterizing blood traces of forensic significance.

Proceeding from the model test with crystalized hemin (Figure 1)[3] [figures not reproduced in the translation], we thoroughly tested fresh and old blood traces for their behavior with the test solution. As part of the wide ranging experiment, such stains were included which could have simulated
228 the presence of a blood trace. Two reaction solutions were used to carry out the experiments. The composition of the solutions is as follows.

1. About 1/10 g luminous substance, 5 g calcium carbonate, 15 cc 30% hydrogen peroxide in 100 cc of distilled water.

2. 0.1 g luminous substance in 100 cc 0.5% aqueous sodium peroxide solution.

Both solutions are usable for the experiments and are, in themselves, free of any luminescence in accordance with the requirements—the distilled water must be free of oxidizing agents such as hypochlorite.

In the course of the experiments, we found that it was expedient to alter solution No. 1—should this one glow lightly contrary to our expectations—with a trace of "indazolon-4-carbonsäure" in order to obtain a completely problem free, non-glowing reaction solution.

Both test solutions have shown no differences in their reactive capability, so that in practice one can work as well with one as with the other.

Fresh blood at times induces only a weak glow. *Dried blood traces,* however, call forth a bright, blue, long-lasting chemiluminescence when they come into contact with the test solution. The older the blood trace was, the more clearly the light effect showed up. This was caused by the hematin in the blood which had separated from the globin in the course of aging.

229 The objects to be tested for the presence of blood were sprayed with the solution, which at first did not glow. It is an advantage if one uses for this a glass sprayer. The many tests[4] which have been carried out demonstrated that even the smallest blood traces produce a strong luminosity. The identification of blood traces by means of chemiluminescence can be characterized as specific, since sperm, saliva, urine, excrement, pus, and other body fluids do not react in this fashion. Milk and coffee stains as well as starch, red-dyed jellies, inorganic and organic dyes (such as eosin, Sudan red, scarlet red, and others), carpets, tissues, leather, skin, fungus cultures of the most varied types, moldy bread, oils (oil paints, varnish, mineral oil), and colored waxes do not induce a glowing reaction when blood is not present. Samples of earth (humus, clay, sand), stone, wood, and metal samples (copper, steel, brass, lead, zinc, and others), grass, and foliage, fail by themselves to demonstrate luminosity. In particular, rust and other metal oxides, which occur frequently in practice, and which are not infrequently encountered together with bloodstains, and have a strong dissociating effect on hydrogen peroxide, could not stimulate the luminous substance to give a blue luminosity.

In accordance with these findings, it was to be expected that blood traces on the greatest variety of substrata would reveal themselves through the luminescence.

The experiments carried out along these lines completely 230
confirmed this supposition. A few photographs show most clearly the findings of the test.

One can see in figures 2 to 5 [not reproduced in the translation] that, at any given time, only the portions of the substratum which were moistened with blood are illuminated. In the comparison pictures, which were taken of the test objects by daylight, the places moistened with blood are from time to time outlined.

Even traces, which have been washed thoroughly by long 231
rain (see Figure 3), and other similar traces on foliage, grass, earth, and stone, which are not visible to the naked eye, glowed with undiminished intensity after being sprayed with the test solution. Other, even longer-lasting effects were observed. After a successful reaction, the glowing phenomenon could be induced again by repeated spraying with the test solution.

Likewise, it was possible using chemiluminescence to identify small blood traces in large quantities of water, in soapy water, and in other waste water.

Thus, the presence of blood in liquids can certainly be 232
identified, namely in:

(a) 2 drops blood in 1ℓ water pipe water
(b) 4 drops blood in 2ℓ soapy water
(c) 6 drops blood in 6ℓ soapy water
(d) 6 drops blood in 5ℓ dirty water.

In the case of very dirty water, the luminescence appears more clearly only after the suspended particles have settled. One ought to notice that, naturally, the strength of the glowing effect in liquids of very slight blood content is lessened. By adding a trace of caustic lye, however, the glowing capability of the solutions is raised, though the duration of the effect is lessened. A glowing reaction of a minute's duration is the standard for judging the experiment. One must, at this point, also take into consideration that the hypochlorite of tap water or of soap can induce a very weak, but perceivable, luminescence even though it lasts only a second and very rapidly dies away. Figure 6 reproduces the photograph of the test.

Finally, we should mention that blood traces on the hands can likewise be made visible by spraying with the test solution, as Figure 7 demonstrates.

This new blood identification is all the more useful for the

forensic chemist since spectroscopic as well as serological
233 testing and identification of the blood type are still possible after this testing of the materials. After a successful glowing reaction, the extractions prepared from the test objects with physiological saline solution produced clear absorption spectra in every case.

After the duration of action of the test solution on the blood stain, the spectrum of the alkaline or neutral methemoglobin was established. Further, it was possible to obtain the hemochromogen spectrum by adding pyridine and sodium hydrosulfite to the blood solution. Even those blood traces which were no longer perceptible to the naked eye, but were discovered by means of chemiluminescence, were successfully submitted to spectroscopic analysis. Likewise, the *Uhlenhuth* precipitation reaction could be carried out with the traces of blood. From blood mixtures, the individual blood types could be recognized in the usual way. Blood traces whose type was unknown could be positively analysed in the same fashion. Difficulties in the serological differentiation of blood mixtures were not noticed. The precipitations were successful even when the test solution had dried on the blood trace.

Summary. Chemiluminescence of 3-aminophthalhydrazide in soda alkali solution is released with exceptional strength in the presence of small amounts of hydrogen peroxide by means of hemin, but also gives considerable luminosity with dried blood.

The luminosity reaction can thus be applied to forensic practice with success. Even blood traces which are a year old, or even older, excited the test substance to luminosity.

Strong oxidizing agents, such as hypochlorite, ferricyanide manganese dioxide, colloidal platinum, osmium tetroxide, and gold chloride, are also capable of producing a weak luminosity in the 3-aminophthalhydrazide by means of a catalytic dissolution of hydrogen peroxide. The luminous effect, however, is far stronger in the case of the action of dried blood, which as a catalyzer, is able most strongly to activate the peroxide of the test solution. In addition, in forensic practice when dealing with samples used for conviction one need scarcely reckon with the presence of the catalyzers of an inorganic nature, mentioned above. The materials of everyday life, which might simulate a blood trace, demonstrated no luminous reactions under the test conditions given. Far exceeding the significance of the luminous reaction as a preliminary test for the presence of blood is the fact that one can—and here especially lies the worth of the new blood reaction—carry over the blood trace, unaltered by its discovery through luminescence, to the pretreatment processes necessary for characterization, i.e., the spectroscopic and serological examinations.

A further advantage of the experimental method de- 234
scribed here, as opposed to those presently employed, is that, even an extended area where the crime occurred, or a larger piece of evidence, can rapidly be tested thoroughly for the possible presence of blood stains without wasting supplies.

Finally, we should mention that the luminescence of blood traces appears with special clarity in the dark. The intense, uniform, blue light permits fixing the position of blood spots photographically without any further equipment.

For the accompanying photographs of luminescence in the open air, the exposure time amounted to five minutes; for the example experiment (Figure 1) a five to six-hour exposure was sufficient.

We will report at some time in the future to what extent blood traces are still suited for determination of blood groups and factors, after having undergone the luminescence reaction.

Notes

1. Lecture given at the meeting of the Deutsche Gesellschaft für Gerichtliche und Soziale Medizin, September, 1936, in Dresden.
2. I refer to the fundamental works of Gleu and Pfannstiel, "Benzisoxalon-4-carbonsaure und Indazolon-4-carbonsaure" and "Über 3-Aminophthalsäurehydrazid" in *J. prakt. Chem.* NF **146**, 129, 137 (1936). These works give the particulars on pure preparation of 3-aminophthalhydrazide, the so-called white hydrazide, as a luminous substance, the proportions of isomers of the hydrazide, and the procedure for the luminosity reaction.
3. This print was kindly placed at my disposal by Mr. Pfannstiel.
4. I thank my coworkers in the institute, Mr. Koch and Mr. Ahlendorf, for their helpful assistance in carrying out the experiments.

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Section 2. Identification of Body Fluids

Orfila's paper on examination of seminal stains is one of the oldest papers on this subject in the literature. A number of chemical tests were proposed, and microscopical examination for the identification of spermatozoa was rejected because of the poor results. As is made clear in Bayard's paper, Orfila's results were unstatisfactory in part because of the technique used to isolate the cells, and perhaps in part, too, because the microscopes were not that advanced. Bayard's paper, a classic, placed the priority of microscopical examination of seminal stains on a firm footing. Various chemical reactions continued to be used for a long time, though, as is clear from Lassaigne's paper. Lassaigne (1880–1859) was quite well known. Paul Brouardel's paper tends to stress microscopy. Brouardel (1837–1906) held the Chair of Legal Medicine at Paris, and was Dean of the Faculty of Medicine there as well. Cauvet's paper details how a sexual assault case was analyzed at the time in terms of both blood and semen. Brouardel and Boutmy's report was an assessment of nonmorphological dyeing technique for the identification of seminal stains. Charles Robin (introduced in Section 1) and his colleague Ambroise Tardieu (1818–1879) discussed a number of less frequently encountered body fluids in their paper.

Florence's papers discuss in great detail the history of medico-legal examinations in sexual assault cases. He gave a good deal of information about the detection and identification of spermatozoa as well. In the papers, he introduced the now well known Florence crystal test for seminal stains. Florence (1851–1927) spent most of his professional career at Lyon. Barberio introduced another crystal test for seminal stains which enjoyed some popularity as well.

Lundquist's paper in 1945 introduced the acid phosphatase test for medico-legal detection of seminal stains. This test is in very wide use today.

Semen Considered from a Medico-legal Viewpoint*

M. J. B. Orfila

469 I have often been consulted by magistrates, to find out if stains present on linen were formed by semen, fat or matter from discharge of venereal disease, from leukorrhea, etc. Other physicians have been required by courts to give their opinion on similar questions; science, however, possesses no specific ways of facilitating the solution to this problem. This consideration would have been sufficient to involve me in publishing a few experiments I had attempted on this subject, if I had not been provoked into it by reading a report prepared a few months ago by Dr. X . . . in an affair of child molesting. Called upon to confirm the condition of the sexual parts of a young girl of thirteen years and nine months, who was believed to have been violated *nine days* beforehand, this physician concluded that the act of copulation had been consummated, supported, among other facts, by his withdrawal of a *certain quantity of semen from the vagina.* In a consultation asked of me, I was asked: is it possible to allow that semen was found in the vagina of the girl R . . . whose examination only took place nine days after the alleged consummation of the act? It is all the more implausible as, this girl having a mucus discharge, the semen would have been carried out by the matter of the discharge. Besides, how can one be sure that liquid drawn from the vagina was semen rather than mucus? What attempts were made to solve this question? Why not resort to chemical experiments, to exam-
470 ination by microscope? It is necessary to remark in the interest of truth, I added, the author of the assertion concerned did not sufficiently appraise its value before announcing it; he would have seen he could be compromising his reputation in deciding a question of this importance with such levity. The accused was acquitted.

Here now is the procedure I followed in this research: I examined comparatively linen stained by semen coming from several individuals who had had nocturnal emissions, and others who had been hanged, in whom there had been ejaculation. I then studied with several repetitions, on several subjects, the characteristics of stains made on linen by the substance of vaginal discharge in acute and chronic leukorrhea in young girls and adults; and in venereal disease in women incontestably presenting symptoms of syphilis. I also submitted to my examination substance from a discharge of the urethral canal in a case of an internal one-eyed fistula, the sequel of several external fistulae from gonorrhea, five days after cauterization. Finally, to complete this work, I wanted to find out the behavior of linen stained by the matter of whitish lochia, incorrectly called milky, as well as by fat, by saliva and by nasal mucus. It seemed to me that, in establishing the differences between the various liquids and semen, I can consider the problem with which I am occupied as resolved.

Characteristics of semen stains on linen. These stains, which we will suppose are already perfectly dried, are in general thin, faintly yellowish or greyish, little apparent to the point that to see them well, the linen must often be placed between one's eye and the light. Pressed between one's 471
fingers, they are slightly coarse and resist as if starched, whereas parts of the linen not stained conserve their softness; they have no odor, unless moistened, whereupon they quickly emit an odor of semen. If the linen thus stained is brought near a flame, at the end of one or two minutes all the portions sullied by semen *become a tawny yellow,* whereas the other parts do not discolor unless the linen has been placed close enough to the flame to be singed: this characteristic, which did not belong to the substance of any of the morbid discharges I examined, permits the distinction on the fabric of several small whitish stains, impossible to perceive before heating. In this experiment, the semen could only have undergone a great dessication, for in leaving the linen thus yellowed in distilled water for a few hours, it loses its color and the linen acquires all the properties of solution of semen in water.

When immersed for a few hours in cold distilled water, the stained strips moisten completely, which does not happen to the stained parts if soiled by fat. In taking care to press the strips by a glass tube from time to time, they don't delay in discoloring and unstiffening. But they become *viscous* and *emit an odor of semen,* as one can assure oneself in compressing them between one's fingers. The liquid, a milky white, troubled by a multitude of flakes and by fibrils which detach from the linen, delays a lot in clearing: if filtered and 472
evaporated by a very soft heat in a small watch glass, phenomena occur which might be useful in the identification of semen.

1) It is alkaline: it sometimes, however, does not reestablish the color of litmus paper reddened by acid after having been concentrated by heat. 2) If evaporated by a low flame, it presents during evaporation the viscous aspect of a gummy solution; it doesn't coagulate, although it deposits a few glutinous flakes, and its consistency is so particular it is difficult not to accord importance to this characteristic. 3) When evaporated to dryness, it leaves a semi-transparent residue, similar to dried, shining mucus, of a tawny or

* Translation of: "Du Sperme, considéré sous le point de vue medico-légal" in *Journal de Chimie Médicale de Pharmacie et de Toxicologie* 3 (10) 469–480 (1827).

scarcely tawny color, degradable, as all nitrogenous matter at a more elevated temperature. And after being shaken for two or three minutes in cold distilled water, it divides into two parts: one glutinous, of a yellowish grey, adhering to fingers like glue, insoluble in water and soluble in potassium; the other, soluble in water. 4) The aqueous, filtered solution is uncolored, lightly yellowish or yellow, and transparent; it gives a white, flocculent precipitate with chloride, alcohol, acetate, lead subacetate and the corrosive mercuric chloride. *Pure and concentrated nitric acid* brings it a light yellowish tint if it is uncolored, but doesn't render it cloudy, whereas it precipitated or clouded the substance from the various morbid discharges designated above. Alcoholic tincture of gallnut gives rise to an abundant greyish white deposit; the aqueous solution reacted in the same way whenever used not long after being made.

Put in alcohol at 38 degrees for twenty four hours, the linen stained with semen does not unstiffen and the solution does not precipitate with water; however alcohol dissolves a
473 small quantity of matter, for, on evaporating to dryness, a light residue is obtained.

It is easily imagined that no use can be made of observations by microscope in identifying the stains of which we speak: the spermatozoa discovered in human semen by Leewenhoek, frequently observed since by Gleichen, Buffon and Spallanzani, and the presence of which Prevost and Dumas have confirmed in all male animals past puberty, are no longer appreciable when, after dessication of the semen on linen, it is diluted in water for examination by microscope. Indeed, no matter what manipulation was performed in this operation, the spermatozoa are so separated in several points on their body, it is no longer possible to perceive them. It is different in distinguishing semen deposited and dried on a glass slide; the spermatozoa, not having been crumpled or separated, in this case couldn't have been more visible, although without movement; I undeniably identified them in semen dried eighteen years before. But it is especially true immediately or a little time after ejaculation, for example, a half-hour, one hour or even two hours after, that the presence of spermatozoa can be most easily confirmed; for, independent of their form, which resembles that of a tadpole, they execute very marked movements and in the extreme, one can pronounce, solely after the existence of animalculi of this form, that the solution submitted to this examination is semen, for they are not observed with the same characteristics in any other liquid. However, to leave nothing to be desired, the physical and chemical properties, of which I have already made mention, must be sought in this solution. The numerous globules, seen in the humor of the prostates of
474 many animals, manifest no locomotor ability, are always deprived of a tail, and cannot be compared to spermatozoa.

Matter from the discharge of chronic urethritis in several women evidently affected with syphilis. Linen soiled by this matter presents several green, greenish yellow or yellowish stains: among the latter, a few were so little colored, that they could easily be confused with certain seminal stains; that much more, as they emitted no odor and were coarse to the touch. Brought to a burner filled with hot coal, *these stained parts do not become yellow.* Left in cold distilled water for several hours, they discolor; the linen unstiffens and emits an odor particularly *different* from the odor of semen; the liquid was clouded with whitish flakes, and by fibrils detached from the linen. Filtered, this liquid was uncolored, transparent, and reestablished rather energetically the color of litmus paper reddened by acid. Evaporated by low heat in a small watch glass, it furnished a very abundant *albuminous coagulum,* and the solution did not offer the gummy aspect of which we have spoken concerning semen. The product of evaporation to the point of dryness was yellowish white, opaque, clumpy and degradable by fire like all other nitrogenous matter. Treated with cold distilled water and shaken for one or two minutes, it is barely dissolved; the filtered solution gives a white precipitate with chloride, alcohol, lead subacetate and corrosive mercuric chloride, and a yellowish grey one with gall nut, a little like an aqueous solution of semen; but *nitric acid, which does not cloud the latter, precipitates it in white.* The part undissolved by cold distilled water was flaky, non glutinous, and insoluble in 475
potassium at room temperature.

Matter from vaginal discharge in girls and women affected by acute and chronic leukorrhea. All that has just been said concerning the discharge of venereal disease can be applied to stains which this matter forms on linen, except that they are less colored and furnish, when treated with water, a solution in which the reagents already noted provoke much less apparent precipitates.

Matter from a discharge of the urethral canal, in a case of an internal one-eyed fistula, the sequel of several external fistulae. The linen is stained in greenish yellow; the matter was deposited forty days before. It is starched, coarse to the touch, and has no odor in the stained parts; it *does not* yellow like semen when heated. Put in water, it discolors, unstiffens, acquires a particular odor *very different* from the odor of semen. At the end of a few hours, the liquid, slightly troubled, is filtered, to be evaporated at a low heat. Before its reduction to dryness, it reestablishes the color of (litmus) paper reddened by acid; it does not coagulate, and *in no way presents the viscous aspect* of gummy solutions when heated. In treating the very light-yellowish residue coming from evaporation to the point of dryness with cold distilled water, a part is dissolved; the filtered solution gives a white precipitate with chloride, alcohol, lead subacetate, the corrosive mercuric chloride and *nitric acid,* and a yellow one with gall nut.

Matter from a discharge of the urethra in venereal disease, five days after cauterization. The stains this matter forms on linen quite resemble those of semen; the sullied parts were 476
coarse to touch, starchy, without odor; but *they didn't yellow on heating.* Cold distilled water, had discolored and softened the stained portions after a few hours; it had developed an odor different from that of semen. The liquid, clouded by flakes and by fibrils gave a yellowish, alkaline residue, similar to dried egg white, upon being evaporated to dryness. The residue did not appreciably dissolve following two minutes agitation in cold distilled water; in addition, the filtered solution remained transparent when added to chlorine, nitric acid, mercuric chloride, alcohol and gall nut. Now, it is well known that aqueous solutions of semen give precipitates with all these reagents, except for nitric acid.

Whitish lochial material, called "laiteuses." This material forms stains of a dirty yellowish-grey on the linen, having some resemblance to seminal stains; nevertheless, *they do not yellow* upon heating. Treated with cold distilled water for a few hours, they detach and the linen discolors and softens; the scarcely clouded liquid, filtered and evaporated, does not coagulate or deposit flakes, and presents rather the aspect of a gummy solution, a bit like semen treated with water and heated. It is alkaline and recolors litmus paper reddened by acid: however, it becomes colored, and yellows *proportionately to the concentration of the solution* and the dried product is a *deep yellow* similar to "colle à bouche fondue," which doesn't happen with the dissolution of semen. In shaking the dried product for two minutes in cold distilled water, it dissolves in part; the undissolved portion is flocculent, of a deep yellow, and soluble in potassium; the dissolved portion, after filtration, is yellowish and gives an
477 *abundant* precipitate with *nitric acid* and gall nut; chloride, alcohol and lead subacetate give a precipitate and render the solution opaline.[1]

Characteristics of fat stains. They present a fatty aspect, are neither coarse to touch nor starched, and when heated they expand without yellowing. Moreover, they emit their well known odor. Placed in cold water, the linen soiled by the fat *doesn't moisten* in the stained parts; *the fat is in no way dissolved.* If left for a few hours in cold alcohol measuring 38 degrees Baumé, the fat is removed, the alcohol holding it in solution. Water gives a white precipitate and when evaporated to the point of dryness it furnishes a fatty residue. Finally, if the linen concerned is immersed for a while in a solution of potassium, soaplike droplets are seen on the surface of the solution, and the solution furnishes a fatty, white precipitate if a few drops of acetic acid are added.

478 **Linen stained with nasal mucus.** The stains are deep yellow, though the mucus was white when deposited on the linen. Left in cold distilled water for a few hours, it discolors; the fabric is cleaned, the liquid becomes troubled, whitish and flocculent. It is filtered and evaporated by low heat.

[1] In evaporating to the point of dryness the various aqueous solutions furnished by the matter of discharges, of which I have spoken up to now, it is easy to see that the greater part of them furnish an abundant albuminous coagulum, such that the dried product is formed almost entirely by albumin. Then, as this product dissolves in notable quantity in cold distilled water, since chloride, alcohol, gall nut, etc. give a precipitate, I wanted to find out to what point albumin coagulated by heat could dissolve in water. In a watch glass, I evaporated to the point of dryness egg white, diluted in water and filtered; after coagulation, the solution furnished a solid product, which had been dried and shaken for two minutes in cold distilled water. It was once again filtered and the solution gave a precipitate with alcohol, chloride and gall nut; nitric acid also clouded it.

When fairly concentrated, it recolors litmus paper reddened by acid. It presented no trace of *coagulum* during evaporation, and furnished a very small quantity of a whitish, transparent, granulous-like matter. Shaken for one or two minutes in cold water, this matter scarcely dissolves and leaves numerous whitish flakes. The filtered solution is limpid and gives a rather abundant precipitate with chloride, *nitric acid* or alcohol; aqueous solution of gall nut or lead acetate do not cloud it.

Linen stained with saliva. Several linens stained with saliva, coming from six adult individuals, were examined with care: the stains were the result of the reiterated application of saliva on the linen. The characteristics presented not always being the same, we feel it necessary to describe the details observed.

A. Some of the dried stains were starched, coarse to touch and *yellowish,* although the saliva was white coming from the mouth; during dessication, it manifested a particular, disagreeable odor. In exposing the stained parts to heat, those, for example, hardly presenting a yellow tint, acquired a more intense color, and resemble seminal stains treated in the same way. Left in cold distilled water for a few hours, they unstiffened and the linen emitted an odor of semen, especially when pressed between one's fingers. After having 479
been filtered and subjected to a low heat, the very alkaline liquid, troubled by a multitude of flakes, did not coagulate, and furnished a rather abundant yellow residue. This separates into two parts after shaking in cold distilled water for a minute or two: one part insoluble, in the form of thin, yellowish pellicles similar to mucus; the other soluble, which becomes opaline with chloride, nitric acid, or alcohol and with which lead subacetate gives an abundant precipitate, whereas aqueous solution of gall nut doesn't cloud it.

B. Here the linen was white, starched and almost without odor; heated, it *didn't yellow.* Treated with distilled water as was the preceding, it presented a light odor which was *nothing like semen;* the liquid was troubled, flocculent and alkaline. Heated after filtration, it didn't coagulate, and evaporated in the manner of gummy solutions; the product of evaporation was yellowish, semi-transparent and salt-like. Shaken in distilled water for two minutes it separates from the mucus flakes, or rather, the pellicles. The filtered solution *did not become opaline again* with chloride, nitric acid, alcohol or aqueous solution of gall nut.

C. This type resembled the preceding except that heat *yellowed* the linen and the solution became troubled by evaporation, as if it had been albuminous.

It is evident from the preceding: 1) that it is hardly possible to confuse semen stains on linen with those of fat, nasal mucus or the matter of various discharges coming from the vagina or urethral canal; 2) that it is a matter of confirming only the *whole* of the characteristics we have presented in 480
speaking of semen; 3) that it is *sometimes* not so easy to differentiate a seminal stain from a stain formed by saliva; but that it is, however, possible to succeed, the latter liquid not presenting, under any circumstances, *all* the character-

istics of semen. Besides, it is hardly probable that shirts, which one is most often called upon to investigate, have been stained with saliva, especially since deposits of several repetitions are necessary to form an appreciable stain with this liquid, and since it is necessary to wait until the first parts applied have dried, which requires a lot of time.

Microscopical Examination of Dried Semen on Linen or on Material of Varied Nature and Color*

Dr. H. Bayard

Newton was asked how he had made all his discoveries; he replied: *In always searching, and in searching with patience.* I have followed this counsel. . . . *Si parva licet componere magnis.*

Preface

134 When addressing this memoir to the society of the *Annales d'hygiene et de mēdecine légale,* I deposited it before January 1, 1839 in conforming to the conditions of the gathering; but I pursued my research, however, in order to modify my analytical procedures to confirm the presence of spermatozoa without breaking their tails. By means of filtration, I obtained the results I was seeking. Last March, I was called before the society of the *Annales* to repeat a few microscopical experiments, and I verbally communicated the new method of microscopical examination, whose details I present in this memoir.

135 For a long time, the inadequacy of chemical analysis to determine with certainty the nature of semen stains has been recognized. Now, microscopical analysis can furnish certain results which chemistry doesn't offer in legal assessments dealing with crimes of rape, indecent assault and in certain cases of violent death.

Paris, May 15, 1839

Microscopical examination of dried sperm on linen or on material of varied nature and color

Use of the microscope in medico-legal assessment was first recommended by Orfila[1], to determine the nature of sperm in cases of rape and indecent assault; his research did not furnish him with satisfactory results, for he says: *no use can be made of microscopical observation in the identification of seminal stains.*

Since that time, this investigative method appears to have been neglected in its applications to legal medicine, and it is only lately that many forensic physicians have noted the importance and utility of microscopical observations.

Ollivier (of Angers) is the first to have made a conclusive application of the microscope in a medico-legal assessment.

In June, 1837, he was charged with determining *if there didn't exist hair adhering to the iron of an axe seized at the* 136 *home of an individual indicted for murder and, in the case of affirmation, to indicate the color of these hairs.*

He recognized by microscope that the *filaments* submitted for examination were *fur,* differing completely from *hair;* whereas they perfectly resembled the fur of horse, beef or cow comparatively examined; a judicial inquiry confirmed the correctness of his observation.

Ollivier (of Angers) reports in a note added to the article I have just cited that in June, 1838, in a legal assessment, with which he was charged along with Labarraque and Gaultier de Claubry, having as the object of examination a large amount of denatured, adulterated opium, Gaultier de Claubry confirmed by microscopical examination not only the adulteration, but he also discovered by this means the different method of extraction of opium in Smyrna and of opium in Egypt.[2]

In a meeting of the Academy of Medicine on November 20, 1838, A. Divergie read a note on the characteristics of hanging a living man, and added two new characteristics: the first consisting of the presence of spermatozoa in the urethral canal; the second, the state of congestion of the genitalia.

It is this small number of facts to which use of the microscope is limited in legal medicine up to the present.

Now that one is not content with studying in visible texture of organized bodies, but more with discovering unawares, so to speak, their mode of primitive formation, and 137 knowing their intimate composition, the microscope, due to the modern perfections in its construction, will serve to extend the limits of science.

Doctor Donné, in two memoirs appearing in 1837, presented important microscopical research *on the nature of mucus and the substance of discharges from male and female genital organs and on spermatozoa.*

In his last work, Donné was particularly interested in the fluids of "economy", which are appropriate for maintaining the life of spermatozoa for a more or less long time, and from considerations of some of them, he deduced causes of sterility in women. This research is not especially applicable to legal medicine, but I must hasten to point out it is fruitful in application, and will yield priceless information.

Among the ancient and modern authors occupied with the study of human spermatozoa, none, Orfila excepted, observed them with the same objective as myself.[3]

* Translation of: "Emploi du Microscope en Médecine Légale. Examen Microscopique du Sperme Desséché sur le Linge, sur les Tissus de Nature et de Coloration Diverses".
in *Annales d'Hygiène Publique et de Médecine Légale* **22**: 134-170 (1839).

138 Gleichen, Spallanzani, Lewenhoeck, Peltier, Prévost and Dumas, Donné, etc., have observed living spermatozoa, and most of these authors were studying them in a physiological context, seeking to determine their influence on generation.

I considered spermatozoa from an entirely different point of view; I observed them *dead* when they were dessicated as was the liquid in which they were suspended.

The importance of this type of research is now understood, in cases of rape or indecent assault, where stained material or fabric is submitted to examination by experts, to determine the nature of the observed stains.

Up to today, reliance has been placed only on results of chemical analysis; these analytical methods, recommended with wisdom by science, are, however, coarse and little conclusive.

It is true that some microscopical experiments have been attempted, eleven years ago, but with no success, due to the imperfection of instruments and procedures. I devoted myself to new experiments and the CERTAIN results I obtained by microscopical analysis permit me to communicate them. In addition, I am assured that a certain number of obscure questions in legal medicine can be cleared up by this mode of investigation.

It will be very important to determine if spermatozoa exist in all ages in men; I intend to study this question which is of interest at the same time to physiology and to legal medicine.[4]

First Section

139 This memoir is composed of three sections: in the first, after explaining the facts which led me to look into new procedures, I successively study the action exerted in cold and heat on dry semen by:

distilled water,
common water,
saliva,
urine,
blood,
milk,
alcohol,
solutions of soda,
sodium subcarbonate,
sodium subphosphate,
potassium,
potassium subcarbonate,
ammonia.

I end with the enumeration of characteristics presented by dried semen on linen.

Second Section

The second section comprises three series of experiments; but before detailing them, I recommend various procedures I successively employed before resorting to filtration; finally, I set forth this mode of analysis which appears to me the most complete and most certain.

First Series of Experiments

A. Examination of linen stained by simple dried vaginal mucus.

B. Examination of linen stained by semen.

C. Examination of linen stained by vaginal mucus after 140
the act of coitus.

D. Examination of linen stained by vaginal mucus, collected eight hours after coitus.

Second Series of Experiments

E. Examination of linen stained by simple vaginal mucus.

F. Vaginal mucus collected between glass slides.

G. Examination of linen stained by semen.

G¹. Semen collected between glass slides.

H. Examination of linen stained by vaginal mucus after coitus.

H¹. Vaginal mucus, after coitus, collected between glass slides.

I. Examination of linen stained by vaginal mucus nine hours after coitus.

I¹. Vaginal mucus, collected between glass slides, nine hours after coitus.

Third Series of Experiments

J. Examination of linen stained by semen two months before.

K. Examination of linen stained by semen one, two and three years before.

Third Section

The third section is composed of a large number of microscopical experiments on stains of semen, of vaginal mucus with semen, dried on material of

cloth,
cotton,
wool,
silk,

which vary in color.

My research at this moment concerns whether the charac- 141
teristics Doctor Donné assigned to mucus and to the substance of various discharges of male and female genito-urinary organs can be recognized on linen and fabric.

The experiments I have already done on this subject permit me to expect success and confirm in part the important discoveries of this able observer.

Paris, December 25, 1838

First Section

During a legal investigation against Sir Bengnet, indicted for the murder of his mistress, he claimed that the night or morning preceding the murder, the girl, Lecluse, had had sexual intercourse with a stranger, and the despair at being thus wronged by the woman he was to marry in a few days brought him to kill her.

I was charged, with Ollivier (of Angers), to submit any

liquid in the genital parts of this girl to special examination in order to see if there wasn't any trace of semen.

To proceed with this investigation, we had carefully removed the uterus and vagina from the cadaver, in such a way as not to perturb the walls of this canal; it was incised lengthwise with caution, and we wiped the whole inside surface, those parts touching the cervix included, with a very white linen cloth. The linen, moistened by these mucus substances, which were rather abundant, was dried, in order to be submitted to various methods of investigation later.

142 To facilitate this examination, I devoted myself to several series of experiments with the objective of seeing if the presence of spermatozoa can be confirmed by microscopical examination on linen stained by human semen or vaginal liquid mixed with semen and dried. In an article entitled *Sperm considered from the medico-legal viewpoint*[5], Orfila noted chemical and physical characteristics by means of which the presence of seminal stains on linen, or stains from substances of various discharges, can be confirmed.

This author expresses himself thusly, page 473. ". . .It is easy to realize *that no use can be made from microscopical observations in the recognition of seminal stains:* spermatozoa discovered by *Lewenhoek*, frequently observed since by Gleichen, Buffon and Spallanzani and whose presence Prévost and Dumas confirmed in all male animals past the stage of puberty, *are no more appreciable when, after having dried the semen on linen, it is diluted in water for microscopical examination.* Indeed, *no matter what manipulation is used in this operation, the spermatozoa are so separated in many parts of their body, it is no longer possible to perceive them.* It is different in distinguishing semen deposited and dried on a glass slide; the animalculi, being neither crumpled nor separated, in this case couldn't be more visible. Although without movement, I recognized them perfectly in semen dried for eighteen years. But it is especially immediately, or a little time, after ejaculation, for
143 example, a half hour, one hour and even two hours afterwards, that the presence of the animalculi is easy to confirm. For other than their form, resembling a tadpole, they execute very marked movements, and, *in the extreme, the solution submitted to examination can be considered semen from the soil existence of animalculi thus formed, for they are not observed with the same characteristics in any other liquid. . . .*"

The opinion expressed by Orfila in 1827 did not stop my research and, profiting from perfections in microscope construction since that time, I obtained, as can be seen in this memoir, more successful results.

The procedure recommended by Orfila is the same as I have seen still very recently employed, and it is easily understood that it cannot confirm the presence of spermatozoa. If the stained linen *is placed in water*, in *fraying* and *separating* the material, the spermatozoa are broken and the debris is hardly discernible, no matter the magnification of the microscope.

Examination of sperm collected between glass slides right after ejaculation and of sperm gathered in a sizable quantity in a capsule to preserve it as liquid for about six hours, brought me to the use of procedures I will detail below.

I observed that zoosperms between glass slides conserve their life and movements as long as the mucus in which they are swimming stays fluid, and as it became cold and dry, they lost their mobility and exerted only vibratory oscillations, which stopped right after complete agglutination of the mucus, which took place at the end of two or three hours.

I've no need to remark that spermatozoa are always visible 144
between glass slides, for at the moment they are interposed, the mucus is dispersed in an excessively thin layer, the agglutination of which is not harmful to observation. In a capsule where the sperm solution was abundant enough to conserve its fluidity for about ten hours, I could confirm the life and movements of spermatozoa up to the last instant.

Starting with these observations, I devoted myself particularly to recognizing the action of several preservation liquids and a certain number of chemical agents on dried sperm, to distinguish those which disengage the zoosperms most promptly and completely from the muco-glutinous matter without altering them from those which, on the contrary, alter the form of, or destroy, the spermatozoa.

For these attempts, I used semen in which I had identified movements of the animalculi for ten hours; the semen had been exposed to the atmosphere and had dried in the capsule.

In the central part of the capsule the semen is a yellowish color whereas in the other parts, the tint is greyish; it is very dry and detaches in the form of powder.

I was interested in submitting this seminal powder to microscopical examination, using a magnification of about three hundred fifty. A few animalculi, very recognizable by their form, were free and entirely disengaged from the mucus matter; but the greater part were surrounded by a rather substantial thickness, such that the bodies were *semi-opaque*, and the content was distinguished only with great difficulty.

§I. Action of distilled water. A drop of distilled water is 145
placed on this seminal powder; after a few minutes of maceration, the semen swells, disseminates in the liquid, and under the microscope, a large number of free zoosperms in the middle of *irregular*, *transparent bodies* are seen. Lightly heated, these bodies dissolve a bit, and permit perception of the imprisoned zoosperm.

I couldn't better compare the fragments of glutinous mucus than to icicles formed by the cold enveloping all the substances suspended in the water. As previously, they are dissolved by heat and release the foreign bodies imprisoned there.

This dissolution is not, however, complete enough such that there are no remaining fragments of mucus; but they are transparent and it is in their midst that the animalculi are perceived; prostatic monads can also be identified, having a globulous form without a tail. Their volume is infinitely more considerable than that of zoosperm from which they are easily distinguished.

§II. Action of common water. Common water, hot and cold, acts like distilled water. Experiments I've done on river and well water permit me to confirm some rather appreciable differences when the qualities of the water vary; for example, the alkalinity of water activates dissolution of mucus.

A general remark, one which promotes the preference for distilled water, is that common water holds a great number
146 of substances in suspension which deposit between the slides and hinder microscopical examination.

§III. Action of saliva. As soon as dried semen comes in contact with saliva, it swells and disseminates more rapidly than in distilled water. Under the microscope, the mucus is divided into transparent fragments, which partially dissolve if lightly heated; the zoosperm are apparent, but there are very few of them free and they are surrounded by mucus.

I haven't noticed that saliva exerts a singular action on *dead* zoosperm, an action noted by Doctor Donné on *living* animalculi; *their body doesn't become contorted, so that the tail forms a type of knot or eyelet*. In all my experiments, the tail maintained the direction it had at the moment of cont. .t with the saliva.

§IV. Action of urine. Sperm disseminates more rapidly in urine than in saliva, the mucus fragments divide more and are more transparent; prostatic monads are free and visible in great number, heat increases the dissolving action a bit, the spermatozoa are very visible and almost totally disengaged from the mucoglutinous matter. If the glass slides are left to cool, at the end of a few minutes crystallizations of different urinary salts form, which does not hinder the identification of spermatozoa.

I repeated a great number of times these experiments of the action of saliva and urine on semen, because I was aston-
147 ished that urine, which is ordinarily acid, more easily dissolved the mucus, or, to put it more precisely, rendered the spermatozoa visible more rapidly than saliva, which is an alkaline liquid. I always noted the same results, however, even though I used the urine of many people of differing age and sex. The explanation of this difference can be found, it seems to me, in the presence of the abundant mucus existing in saliva and which is added, as it were, to the glutinous mucus of sperm, whereas there is not an appreciable quantity in urine.

§V. Action of blood. It is known that blood, far from exerting a deleterious action on the zoosperm, appears to preserve their life; I have no other purpose in this research than to confirm if the presence of blood hinders microscopical examination. I noted that zoosperm were perfectly distinct in the midst of blood cells; it suffices to add a drop of distilled water and to shake the slides a bit, so that in the movements, whole zoosperm can be identified.

I will return later to the importance of examination by microscope in determining the nature of stains presumed mixed with blood.

§VI. Action of milk. I used mother's milk, and I observed that dried semen put in contact with milk swelled very little and didn't disseminate, which is explained quite well by the multiplicity of milk globules; but as soon as a drop of distilled water is added, the glutinous mucus of semen quite promptly divides, prostatic monads appear, then the zoo- 148
sperms differentiate themselves by their elongated tail.

§VII. Action of alcohol. *Pure* alcohol contracts the glutinous mucus of semen, and no trace of zoosperm is seen; if alcohol is added to a solution of semen in distilled water, this phenomenon does not occur. And as soon as it is gently heated, the mucus fragments divide, becoming transparent, and zoosperm disengage themselves. I made numerous attempts at determining the action of alcohol and I confirmed that *one drop of alcohol to ten drops of water* is the proportion which most activated the division and transparency of muco-glutinous fragments. This dissolving action of alcohol should not be astonishing; it had been noted by Orfila who said in his memoir (page 472) . . . "Placed in alcohol at 38 degrees for twenty-four hours, the semen-stained linen doesn't unstiffen, and the solution doesn't precipitate with water; *however, alcohol dissolves a small amount of matter, for in evaporating to the point of dryness a light residue is obtained*."

Orfila's observations are noted when linen stained with semen is left to itself after being saturated with alcohol. But if lightly heated after the addition of distilled water, the stained linen *loses its stiffness* and recaptures it to a lesser extent after the complete evaporation of distilled water. If the liquid of the solution is submitted to examination by microscope, and particularly that gathered in the bottommost part of the capsule, *spermatic animalculi are found*. It is understood that chemical procedures alone cannot contradict such results.

It is with alcohol that I began a multitude of experiments 149
which, by their successful results, confirmed the certainty of the procedure, and since I've been able to compare the action of several other reagents to this chemical agent, I don't consider it to be of any less genuine value, for the proportions are easily measured and its *useful action* endur for a much longer time. I will have occasion to return to this subject in the third part of this memoir, when I present my research on semen stains of cloth of various nature and color.

§VIII. Action of sodium and a few of its salts. Reflecting that sodium exists as a salt, dissolved in the bodily humors, and that their alkaline state is undoubtedly due to its presence, I repeated many experiments with this substance, either *pure* or as subcarbonate and subphosphate.

In the *pure state*, sodium solution provokes contraction, shriveling of glutinous mucus, and zoosperms are not perceptible; but, remarkably, prostatic monads are free and appear more voluminous than in solutions of distilled water or urine.

If as sodium, sodium subphosphate, or sodium subcarbonate is added to a lightly heated solution of sperm in distilled water, the mucus rapidly dissolves, spermatozoa and prostatic monads appear; but if the appropriate proportion of reagents hasn't been used, the spermatozoa are no longer found at the end of a few hours, while the prostatic

monads are visible.

150 After much trial and error, the proportion which appeared to produce the best effect is 1:20 of the concentrated solution, i.e. one drop of sodium (subcarbonate) solution for twenty drops of distilled water.

Despite the difficulties encountered in use of this reagent, I don't think it should be rejected, for its action is rapid and very advantageous if the proportion is follo.ed closely.

§IX. Action of potassium. I used a solut.n of potassium subcarbonate in the same proportion as sodium and obtained the same effect; I will limit myself to mentioning this, without giving all the details which will only recall what I have previously said.

§X. Action of ammonia. *Pure* ammonia has the same action on semen as *pure* alcohol or pure sodium; but if added to a solution of distilled water and gently heated, the results obtained are conclusive.

On contact with ammonia, the mucus rapidly dissolves; the zoosperm are not altered and are discernible for a rather long time; but at the end of twenty-four to thirty-six hours, on examination of the slides between which the dissolution was performed, zoosperm are no longer found. In evaporating, the ammonia promptly dried the slide, or else this alkali destroyed the animalculi. In any case, they are no longer seen.

The proportion in which this reagent can be used required many attempts. I used one sixteenth of the concentrated
151 solution, *one* drop of ammonia to *sixteen* drops of solution; and, I repeat, even in conserving this proportion, I found no trace of zoosperm after forty-eight hours.

Due to its rapidity, the action of ammonia must be preferred to the reagents already studied when the research to be performed must be done in a few hours. This chemical agent completely dissolved blood. Its use should not be forgotten when a semen solution submitted for examination is to be separated from blood.

In summing up all the preceding observations, it is seen: 1) that distilled water or common water dissolves a part of the seminal substance and that, in gently heating the macerated material, the division of mucus fragments and their transparency is increased and zoosperm are thus rendered more visible; 2) that spermatic animalculi become visible in saliva and in urine, and that these liquids do not alter them, likewise for blood and for milk; 3) that *concentrated* alcohol, sodium, potassium, ammonia, far from dissolving mucus and disengaging zoosperm, cause a very marked contraction and destroy them; that these reagents, employed in *appropriate quantity* and added to the macerated seminal material have a very remarkable dissolving action, by which the animalculi are rendered apparent.

To avoid confusion in the presentation of my research, I previously spoke only of the action of various liquids on *dried semen*; but the objective I set for myself is to confirm that *identification of dried seminal stains on linen can benefit from observation by microscope*.

If linen stained by semen and dried is examined, charac- 152
teristics noted by all researchers can be easily recognized; they are the following:

The stains are thin, of a greyish or yellow-red-brown color, sometimes not very apparent, and, in certain circumstances, of a shiny, gummy appearance. The stains are stiff to touch, the linen rigid as if starched. A remark very important to make is that these characteristics are most usually observed on the surface which had been moistened by the semen and, if the linen is thick, the surface opposite to the stain presents no change in color.

When the strips (also stained) are macerated for a few hours in cold distilled water, they are completely moistened, which does not take place in fat stains; the linen loses its color and unstiffens. The liquid becomes slightly troubled, if the semen is in appreciable quantity; fibrils are detached from the linen and deposit with small flakes at the bottom of the capsule. A spermatic odor is exuded if longer strips are used; if not, it is difficult to appreciate.

During this maceration, *it is necessary to take care not to press the stained linen with a glass tube or any other body nor to dilute it in water*, for what had been noted by Orfila will inevitably happen; *the spermatozoa will be so separated in many points on their bodies, that they will not be identifiable*. If, on the contrary, precaution was taken not to crumple the linen, it suffices to aspirate a few drops of the maceration mixture with a pipette, choosing preferentially the lowermost part of the capsule, and to interpose the liquid between two glass slides for microscopical examination. A few free zoosperm will be identified and a greater number are imprisoned in fragments of glutinous mucus. At this point, using a low heat and one of the reagents, such as alcohol, 153
sodium phosphate, potassium, or ammonia, causes a much more complete dissolution of the mucus to be brought about and a greater number of zoosperm liberated.

These zoosperm can always be recognized by their particular form, just about that of a tadpole. The numerous globules perceived in the liquid of the solution are prostatic monads which are *always* deprived of a tail and are of a much more considerable volume.

Second Section

Before presenting the experiments which are the object of this section, I think it would be useful, to avoid continual repetition, to detail the procedures I found the most advantageous in my research with the microscope. In the first part of this memoir, I presented some considerations which touched on a few of these details; I will, therefore, be as concise as possible.

First procedure for recognizing the presence of spermatic animalculi on linen or fabric stained by semen and dried. It is necessary to place the strips of stained linen or fabric in a glass capsule,[6] taking care, as I've already recommended, not to press or crumple, and still less, to separate the fabric. 154
They must be moistened with water and left to macerate during several hours, then *gently heated* on the flame of a spirit lamp, taking care not to bring the liquid to a boil.

Second procedure. The analytical method previously presented seemed defective to me in many respects, and forced as I was, by requirements of the meeting, to deposit my manuscript before January 1, 1839, I had to limit myself to presenting the first procedure. I did not stop, however, doing new research and I will stop finally at the method of examination that follows, which I presented to the Society of the Annales d'hygiene et de médecine legale, when I was called last March to repeat before it some of the experiments cited in my memoir.

In doing *chemical analysis* of linen stained by semen, I remarked that the maceration liquid became, by filtration limpid and transparent, as cloudy and opaline as it was beforehand and that this change was due, as can be easily imagined, to deposit on the filter of all the animal and foreign matter undissolved in water. I at once applied this observation of microscopical research, and I examined the matter thus deposited on the filter. I distinguished a multitude of spermatic animalculi entire and complete for the most part, but enveloped in mucus or foreign bodies. With the help of heat and some of the reagents already cited, I could disengage the zoosperm which I had thus obtained complete and isolated.

It is known that spermatozoa, due to their specific gravity,
155 gather together at the bottom of vessels containing the liquid holding them in suspension; it is natural then that they deposit on the filter. I ascertained that the spermatozoa were stopped by a simple sheet of filter paper, a fact already recognized, I believe, by Prévost and Dumas.

Mode of analysis — 1) Detach with scissors and carefully remove a portion of the presumed seminal stains; do not crumple the fabric, and place it in a test tube.

2) Bathe the stained fabric in distilled water, and let it macerate for twenty-four hours.

3) At the end of this time, filter this first liquid. Place the stained fabric, already macerated, in a porcelain capsule, moisten with distilled water, and heat by the flame of a spirit lamp until the liquid acquires a temperature of +60 to +70 degrees centigrade. Filter this liquid. Finally, treat the stained fabric with alcoholic water or ammonia in water and filter the diluted solution.

4) When the filtration is finished, cut the filter paper a distance of one thumb from its edge and turn it over on a watch glass, or preferably on a flat glass dish; moisten the filter thus turned over with dilute alcohol or dilute ammonia, which dissolves the mucus and entirely detaches the deposit. If some fatty matter is found mixed in, a couple of drops of dilute ether is used.

Examination by microscope of the capsule or flat glass dish identifies *whole* spermatic animalculi, *without breakage* of the tail, and isolated from the mucus.

I have already performed numerous applications of this method of examination, particularly in *eleven* legal assessments with which I had been charged since February, con-
156 jointly with Drs. Olliviers (of Angers), Moreau and Chevallier. Microscopical examination *gave certain results* each time, which chemical analysis, comparatively performed, did not always give.

The solution obtained is divided into several parts and to each is added 1:10 alcohol, 1:20 sodium or potassium, 1:16 ammonia; after a few minutes a deposit forms on the bottom of each capsule. A few drops must be aspirated with a pipette, and placed between two slides which are placed on the stage of the microscope, and a magnification of 350–600X is used.

Stains of a fatty type are observed between the two slides; these are the stains which must be carefully observed, and here are found zoosperm, which does not hinder, however, the seeing of a multitude of suspended corpuscles in the liquid, and even perhaps some free zoosperm at other points on the slides. A few drops of the liquid thus prepared can be placed on a slide and left to evaporate; if the deposit thus formed is submitted to microscopical examination after complete dessication, the zoosperm are easily identified. In thus working with only one slide, the objects at which one is looking are lighted much more vividly, which is very advantageous when a lit room is being used for sketching.

First Series of Experiments

It was not sufficient for me to confirm the presence of spermatic animalculi in dried seminal stains on linen; I wanted to examine stains dried on linen and mixed with vaginal mucus which flowed during and after the act of coitus.

I succeeded in acquiring such linen collected with care, 157
and devoted myself to the research which is the objective of this second part.

A. Examination of linen stained by simple dried vaginal mucus. These linens were used to wipe the genitals of a healthy woman, who had no discharge, and who had not experienced coitus for over fifteen days.

Rose and light yellowish stains are observed on the linen, more colored on one of the surfaces than on the opposite; the fabric was not starched, but it felt a bit stiff to the touch and seemed swollen. The strips are macerated in distilled water; blue litmus paper is dipped into the maceration mixture, and it reddens a bit, but very weakly; the acidity, however, can be confirmed.

Examined by microscope between two slides, this liquid appears to be composed of a large number of irregular bodies, of which I could not exactly identify the oval form described by Donné (p. 17, Recherches sur la nature du mucus), but I determined without doubt that they looked like small scales. In addition, I observed a good number of rose-colored corpuscles, which did not show a regular form. There was nothing resembling animalculi, of which I made certain in submitting this liquid to the action of the various chemical agents already cited, which dissolved the mucus, altering the form of the scales, but there appeared no bodies analogous to sperm or prostatic monads.

B. Examination of linen stained by semen. These linens 158
wiped the genitals and penis of a man right after coitus.

Greyish, starched, limited stains were noted; these stains,

cut out and placed in a capsule, were treated according to the recommended procedure and submitted to the action of varied reagents. Examination by microscope identified a large number of zoosperm and a multitude of prostatic monads.

C. Examination of linen stained by vaginal mucus after coitus. These linens were saturated by vaginal mucus a little after the act of coitus; in these experiments as in all those reported in this memoir, these linens were *dry* when examined.

The linen presented a light yellowish tint at the stained points; it is firm, starched, presenting the characteristics of semen-stained linen.

The solutions suspend the zoosperm and the prostatic monads; but the papulae and the scales identified in simple vaginal mucus are observed here and are, for the most part, adherent to the spermatic glutinous mucus.

D. Examination of linen stained by vaginal mucus collected eight hours after coitus. It was of interest to me to determine how many hours after coitus spermatic animalculi can still be found in vaginal mucus; I obtained mucus collected from a woman eight hours after coitus without any bathing of the genitals.

159 The linen was stained greenish yellow and was firm, but not rough to touch.

On examination by microscope, I observed a large number of colored corpuscles suspended in vaginal mucus characterized by the scales, and there I found entire zoosperm and prostatic monads more or less ensnared by the plastic matter.

Second Series of Experiments

To verify experiments done in the preceding series, I obtained the same liquids which stained the linen, but collected samples at the same time, between glass slides. It is known that spermatozoa interposed between glass slides can be preserved for a number of years; the examination of what was enclosed between the slides furnished me with points of comparison, and I confirmed the accuracy of my first experiments.

I will not report here the details of these experiments, for that would be a repeat of what I have already presented at length. I successively and comparatively examined:

F. Linen stained by simple vaginal mucus.
F'. Vaginal mucus collected between glass slides.
G. Linen stained by semen.
G'. Semen collected between glass slides.
H. Vaginal mucus collected on linen after the act of coitus.
H'. This mucus between slides.
I. Linen stained by vaginal mucus, nine hours after coitus.
I'. This same mucus between slides.

In all these experiments, I identified spermatic animalculi
160 in the liquid of the solution, and at the same time I saw them preserved between the slides.

I wanted to be sure as to the number of hours spermatic animalculi adhere to vaginal walls, even when washing has been done with simple water. I have identified some in vaginal liquid *sixty-two hours* after coitus; but they were no longer perceptible *four hours* afterward if the woman bathed with aromatic water of eau de cologne. It is probable in the last case that the glutinous matter surrounding the zoosperm and holding them fixed to the vaginal wall at its entrance, was dissolved by the alcohol, and that these animalculi were washed out by the liquid used to carry out the bathing.

Third Series of Experiments

In all the preceding, I worked on linen stained a few days before. I owe to the kindness of A. Chevallier, member of the Academy of Medicine, the opportunity to experiment on linen stained a much longer time before. This chemist procured for me linen stained by semen *two months, one year* and nearly *three years* before.

J. Examination of linen stained by semen two months before. This linen is a fabric of very fine, very white flax; the stains are greyish, starched, the fabric folded, the folds very stiff to touch.

After maceration of a strip of this linen in distilled water and its submission to various methods of analysis, a large number of prostatic monads and zoosperm are perceptible; a few of the animalculi were broken, but a few can be seen 161
which are not entirely dissociated.

K. Examination of linen stained by semen a year and two years before. I did experiments on *five* of these linens, two were of flax, the three others were of cotton, all of them very starched, deeply colored in yellow, one of them rough to the touch and giving the sensation of granulations.

The liquid of maceration has a lightly opaline tint. The whitish flakes, held in suspension for a little while, as well as a type of fine, granulated powder, deposited at the bottom of the capsule.

Under the microscope, the colored corpuscles of irregular form, the glutinous matter of little transparency and prostatic monads are perceptible.

The use of alcohol, of sodium phosphate, . . . accelerate the dissolution, and a rather large number of whole and broken spermatozoa are perceptible, and some whose tails are circularly distorted; the prostatic monads are very apparent.

The glass slide moistened with the solution is dessicated by exposure to the atmosphere, and I am quite surprised to identify under the microscope sodium phosphate and ammonia crystals in pyramids of four faces and truncated peak; I repeated the experiment by leaving a simple maceration liquid of one of these linens open to the atmosphere, and the crystals are reproduced; I am convinced that this salt exists in a state of solution at the time of semen ejaculation.

Third Section

It is not only on linen but on fabric very different by their 162
nature and color where semen stains will have to be investi-

CONTINUED

1 OF 3

gated; it thus appeared important to me to study them when they are dried on material of *cloth, cotton, wool, silk*.

I have previously pointed out the physical characteristics of seminal stains dried on material of cloth and of cotton, either unbleached or white; I will not return to the details already reported, but I believe it useful to present a few of my remarks which I made on material tinted of different colors.

Examination of blue twill duck stained with semen. This material of blue color is shiny, glossy, and supple, although firm almost in its entirety.

There are some parts *dulled* by a dried, whitish coat; at these points the material is starched and does not show the suppleness observed in neighboring points.

Maceration removed the dull color of the stained points on the duck; some fibrils as well as other corpuscles deposit at the bottom of the capsule. The liquid has a bluish tint; treated with alcohol, it doesn't change color and spermatic animalculi can be identified.

If ammonia is used, this reagent alters the strands of threads, without, however, hindering microscopical investigation.

Strands of thread, or their fibrils, are easily differentiated from spermatic animalculi, for the volume of the latter is infinitely less; the strands of thread are straight, transparent,
163 colored like the material, with an external aspect like a tree trunk with its bark.

Examination of colored cloth stained with semen. This fabric, of a rose background, with small points and flowers of all colors, shows no appreciable stains, but in certain parts it is firm, almost starched, whereas there is a lot of suppleness in neighboring parts.

Several strips are cut out of the firmest portions of the fabric. Maceration and moderate rise in temperature of the liquid do not alter the color of the fabric, but it loses its stiffness, becomes less rubbery, so to speak, and an opaline deposit forms at the bottom of the capsule; a drop of alcohol makes the liquid quiver and it recaptures its transparency.

On examination by microscope, complete spermatic animalculi are clearly distinguished; the strands of diversely colored thread are identified by their volume and their particular appearance.

Examination of fabric of cotton stained by semen. One of these fabrics, a twill of blue cotton, shows a more intense hue in the stained parts; the stains are whitish, shiny, rubbery, stiff to the touch.

The addition of alcohol to the maceration mixture is enough to cause the distinct appearance of zoosperm and prostatic monads.

The other reagents have the same action here as in all the experiments we have already reported.

This twilled fabric has the particular characteristic, that
164 it is composed of a few strands of thread for the weaving and of cotton for the rest of the material.

On examination by microscope, the different nature of these substances are clearly distinguished. The thread has the characteristics I have already described: it is straight, stiff, broken almost in splinters at the extremities, with the appearance of a tree trunk. The cotton is wound around on itself, twisted, so to speak, gathered into itself, its extremities clearly broken. Moreover, there is a multitude of small fibrils in the liquid, which are not seen in the maceration mixture of thread material.

Whatever the color of the strands of cotton, this distorted form, undoubtedly from the mode of spinning, is always seen.

I will not report all the experiments I have done on fabrics of cotton of varied colors; these nuances do not hinder the identification of spermatic animalculi.

Examination of materials of wool stained with semen.—Examination of a piece of white flannel stained with semen. There is no perceptible change of color on this fabric, and the stains are not appreciable to touch; instead of it feeling velvety, the fingers feel a sensation of rough dryness. In addition, the flannel is stiff at these points.

These stains, treated according to recommended procedures, furnish zoosperm on examination by microscope, as well as prostatic monads and a multitude of colored corpuscles.

The strands of wool are recognizable by their canalicular form; some of them do not have exactly the same diameter throughout, their surface is sort of wrinkled. In all, the strands of wool have a lot in common with hair, except that their volume is two to three times less considerable.

I also obtained satisfying results in examining sheets of 165 various colors and fabrics of mixed wool and silk.

Examination of dried seminal stains on material of silk. I was able to get silk fabric stained with semen or vaginal mucus after coitus. I am going to report a few of the experiments I have done on this subject.

Examination of a fabric of silk called foulard, of a violet and red color. There are, on one of the faces of this fabric, stains of a greyish appearance, very shiny, of which there is no trace on the opposite surface; the material is stiff and starched in the stained parts.

These stains were macerated in distilled water which had been very gently heated; the solution turns violet. Some strands of silk detached and reached the bottom of the capsule, as well as flakes remaining suspended for a certain time.

Ammonia, sodium phosphate and alcohol equally cause the dissolution of seminal mucus and zoosperm then appear.

Filaments of silk cannot be confused with cotton or thread, for they resemble transparent tubes, having the same diameter throughout, but with no canals, and they have a volume seven to eight times less than hair.

I successively examined *satin* and *velvet*, which had been stained by semen or by vaginal mucus after coitus; I always succeeded in confirming the presence of spermatic animalculi.

I must remark that examination of velvet thus stained 166

requires a very long maceration time and avoidance of its folding on itself, for more difficulty will be encountered in dissolving seminal substances. The use of sodium phosphate, as well as of alcohol always succeeded quite well for me.

Summary of the Principal Facts of This Memoir

1) Spermatozoa conserve life and mobility as long as the mucus in which they swim remains fluid and warm. I have observed them living for ten hours: they die and rest imprisoned as soon as the mucus agglutinates.

2) The dried semen swells, disseminates, and divides in distilled water and in cold common water. It dissolves a little upon gently heating the maceration liquid and spermatic animalculi, characterized by their long tail, are seen with the microscope.

3) Dried semen dissolves in saliva as well as in urine and the animalculi are not altered.

4) Dried semen does not dissolve in blood or milk, unless diluted by a few drops of distilled water.

5) Alcohol or concentrated sodium, potassium, and ammonia solutions do not dissolve seminal mucus; they provoke its contraction and destroy the animalculi. On the other hand, these reagents have a very remarkable dissolving action if diluted with distilled water, in proportions variable for each of them as we have recommended.

6) To identify dried seminal stains on linen, and benefit
167 from observation by microscope, care must be taken not to crumple or separate the macerating strips. In filtering the liquid of maceration, and examining the deposits left on the filter, the presence of complete spermatic animalculi, without tail breakage and isolated from mucus, is confirmed.

7) The presence of zoosperm in vaginal mucus, collected after the act of coitus between two slides, or dried on linen, is easily confirmed.

8) In women not affected with morbid discharge of the sexual parts I have always been able to find spermatic animalculi on linen or slides used to wipe the vaginal walls eight, ten, and even seventy-two hours after coitus.

9) On linen stained with semen and dried two months, one year and nearly three years before, I identified whole, complete zoosperm by their long tails.

10) The nature and color of the material stained by sperm does not hinder microscopical analysis and the confirmation of animalculi; they are identified as well on fabric of thread or cotton as on wool or silk.

11) Microscopical examination permits the distinction of the very different characteristics presented by filaments of flax or hemp, cotton, wool or silk.

References

1. Du sperme, considéré sous le point de vue médico légal. *Journal de chimie médicale*, t. III, p. 469, 1827.
2. *Archives de médecine*, December 1838. Nouvelle application du microscope dans les expertises médico-légales.
3. Without speaking of the work of Devergie, in which this author announced his discovery of spermatozoa in the middle of maceration liquid of old semen stains, a work Bayard could not have known, since it was only published in our issue of January 1839, even though remitted to the committee in September, 1838, a note is found on the same subject, inserted by Ratier in the March, 1837 issue of *Journal de chimie médicale*. In macerating linen stained with semen in a watch glass, and submitting the liquid to microscopical inspection, this physician succeeded in finding spermatozoa; he pointed out in this regard the advantages legal medicine can derive from this mode of investigation. (*Editor's note*).
4. I have begun my research on this curious subject (April 10, 1839).
5. *Journal de chimie médicale et de toxicologie*, t. III, p. 469; October 1827.
6. A watch glass is preferable to every other capsule of a different substance, because the transparency of the glass permits examination by microscope of the deposit which forms after dessication; furthermore, watch glasses heat very rapidly. A glass dish would be still more useful, for the plane surface renders the examination easier.

Observations on Some Reactions Shown by Semen Stains, Albuminous Stains and other Analogous Stains*

J.-L. Lassaigne

405 The similarity of appearance, which certain albuminous stains dried on white cloth, show with seminal stains equally dried, can often confuse, on inspection, the first with the second. It is the same for white material stained by paste,
406 starch, gelatine, gum or dextrin. The parts stained by these various substances present, in regard to certain physical considerations, the appearance of false seminal stains whose analysis and examination by microscope permit establishment of a clear distinction.

Called upon in several instances to determine the nature of various stains deposited on sheets and shirts after indecent assaults, we found that certain chemical reactions, produced on the stained fabric, can orient the examination and add new elements of proof to those invoked in this type of medico-legal assessment.

The tests undertaken by us showed that a drop or two of a potassium plumbate solution applied on an *albuminous stain* provoked a *fallow yellow* color bordering on brown café au lait after a contact of eight to ten minutes at a temperature of +20°. This effect was *not at all* produced either on seminal *stains* nor on *any other stain devoid of albumin,* such as dried stains of *gelatin, paste, starch, gum,* or *dextrin.*

The color displayed on albuminous stains on white linen is due to the formation of lead sulfide at the expense of sulfur, *a constitutive part of albumin,* as has been known for a long time. If a seminal stain, or a stain of another nature, has been deposited on white woolen material, the reagent concerned can develop a color, but only at the expense of sulfur contained in the wool. Thus, this reaction should not be attempted on white material made from this last substance.

In pursuing this work with other chemical reactions applied directly *on the parts of the material stained by the above-cited substances,* we determined different effects between albuminous and seminal stains. These results alone,
407 however, cannot be called upon to establish positively the nature of the stains being examined, but they serve as useful accessories to the scientist when it is feasible to do multiple tests on the stains.

Among the chemical reagents which we used in our research, we will mention: 1) potassium copper subtartrate which, applied on semen and albuminous stains, color the first in *bluish grey* and the second in *pale violet;* 2) ferric sulfate which communicates a pale yellow tint of rust to the semen stain and a reddish yellow tint to the albuminous stain; 3) *gold chloride* reacts more intensely on the albuminous stain and imparts to it a darker *ochre yellow;* 4) silver nitrate blackens the albuminous stain in diffuse light in less than a few minutes while the seminal stain, under the same circumstances, presents a *weak grey hue;* 5) mercurous nitrate behaves as the preceding nitrate, but much less energetically; 6) mercuric nitrate, under the same conditions of light and temperature as mercurous nitrate, exerts no action on seminal stains and causes the albuminous stain to pass to a *pale citrine yellow;* 7) cupric sulfate causes a *pale bluish grey tint* in the semen stain and a *deep sky blue* color in the albuminous stain; 8) finally, nitric acid at 40° causes the fabric stained with seminal fluid to become straw-yellow, whereas the same acid develops on the albuminous stain a *yellow* color bordering on *orange.* All the colors indicated above persist for a long enough time under diffuse light so that the comparative points between them can be established.

The gelatinous stains, those of paste, starch, gum or dextrin are in no way modified by potassium plumbate. As for the other reagents we tested on them, the effects are not appreciable enough that they can be characterized, and 408
the results are not that clear-cut that a conclusion can be reached *a priori.*

We will add to the above-mentioned results another determination we have made in applying heat to the albuminous stains, as Professor Devergie first did on *seminal stains.* The calorie rays of incandescent coal, incapable from a distance of scorching the linen on which is found the stain, causes in these stains a *dark nankeen-yellow* color, whereas albuminous stains do not appreciably exhibit this color or do so very weakly.

This action of heat on seminal stains can be applied to white linen, on which the soluble part of the semen stain has dried. Here we should point out that we have already applied this type of test in an affair of an indecent assault which had been placed in the hands of Lesueur and myself. (The Léandri Affair).

By transferring the substance, soluble in cold water, extracted from an extensive seminal stain on a colored silk petticoat to a piece of white cloth, we could determine the *stiffness of the material and its coloring in pale yellow under the influence of a suitably moderate heat.*

*Translation of: "Observations sur quelques Réactions que Présentent les Taches Spermatiques les Taches Albumineuses et Autres Taches Analogues," in *Annales d'Hygiène Publique et de Médecine Légale* 10 (2nd series): 405–408 (1858).

The facts presented in this report find their application in various circumstances: thus, in publishing them today in *Annales d'hygiène et de médecine légale,* we intended to do a service to all those occupied in analogous work, and desiring to control such work with experiments.

Seminal Stains and Blood Stains*

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117 On June 8, 1874, we were charged with "examining the shirt of a person named Zohra ben Ahmed and the "gandoura" [a long sleeveless shirt] of a person named Brahim
118 bel Belkassem, to investigate by all possible means whether these articles of clothing bore seminal stains, and to say, further, whether the gandoura of Brahim ben Belkassem bore bloodstains as well." We received two packages, labelled as follows:

Shirt of Zohra ben Ahmed (victim)

Gandoura of Brahim ben Belkassem (accused of rape)

1. **Examination of the shirt of Zohra ben Ahmed.** This shirt is made from two pieces of cotton cloth sewn crosswise; it has no longitudinal seam; the two upper pieces are attached to a third near a border, such that there are two unequal openings: a smaller one, for the left arm, a much larger one for the head and the right arm. Made in this way, the piece of clothing was meant to be suspended from the left shoulder, leaving open the left arm on one side, and the head, right shoulder and arm on the other.

The absence of longitudinal seam allows the body to be partly uncovered in certain kinds of movement.

We described the shirt of Zohra assuming that the opening was in front; it is probable, however, that the shirt was worn front-to-back, that is, that the longitudinal opening was in back. This fact might be one of the reasons for the rape. The verbal statement of the doctor stated that Zohra was bent forward, and that she had been seized from the back. If, in this bent-over position, Zohra was only little-clothed, as the facts seem to indicate, if the shirt was simply tied at the waist with a sash, then the greater part of her body was uncovered, especially on the left side; the view of
119 Zohra's form, which the doctor's report said was large and well formed, excited the desires of the one who committed the violation.

The shirt is 1.2 meters long; the material which makes it up measured 2.7 meters along its lower edge. This shirt is extremely filthy, covered by all sorts of stains, mainly stains tinged with blood, generally stiff and starchy, here and there brown with light clots, here and there greyish-red.

The stains occupy the entire width of the material, from its lower edge to the level of the genital organs. On various parts of the shirt, particularly the side which would be worn in front, were noted a large number of green stains, apparently of cow dung.

The widespread stains on the garment are in such quantity that it was impossible to go into a detailed description. In the examination which we conducted, we divided them arbitrarily into groups, according to our best judgment, not every group containing the same number of stains.

Stain No. 1. Located at the uppermost right corner; composed of three masses of brown material, enclosing some fibers of white wool, and apparently formed by a large, dried clot (No. 1).

Stains No. 2. 15 cm from the lower edge and 30 cm from the right edge, a group of stains, pale red, elongate, generally directed from bottom to top and from left to right; one of these, sufficiently dark, to show at its apex a rounded, greyish, starched stain (No. 2).

Stains No. 3. Above group No. 2, reddish-grey stains, some of which are paler than the others.

Stains Nos. 4 and 5. Very large irregular, quadrilateral stain, directed obliquely from left to right and from bottom to top, with a width of 20 cm. length of 28 cm, and diagonals of 35 and 33 cm, extremely pleated, stiff, crinkled, with some 120
clots at the left upper corner, and especially the right lower corner; paler and greyish red on the upper, red brown on the lower, where it appeared formed by blood and vaginal mucus.

This stain is extremely starched toward the middle of the left edge and the middle of the right edge, where it seems to be constituted by a mixture of blood and semen (No. 3).

Three strips of fabric were removed: 1) from the lower part (no. 4); 2) from the left (no. 5); 3) from the right (no. 6).

Stains No. 6. To the left and below stain 4 5 numerous stains, reddish grey or pale red, sometimes a bit tinted in green, of variable size; some comprising simple drops; others, somewhat large, showing that Zohra wiped herself after coitus (no. 7).

Stains no. 7. To the left of stains no. 6, a group of stains of the same form, larger, more numerous, a somewhat deep red, extending from top to bottom up to 60 centimeters from the lower hem (no. 8).

Stains no. 8. Groups of stains: two superior, juxtaposed, on the whole, 34 centimeters long, 18 centimeters wide, especially colored on the lower part, where they presented the

*Translation of: "Taches de Sperme et Taches de Sang." in *Annales d'Hygiène Publique et de Médecine Légale* 44 (2nd series): 117 126 (1875).

appearance of trails of blood, and red brown encircled in grey. Below, various stains, 10 centimeters from the hem and appearing, for the most part, due to spatterings. Between these two groups of stains appeared stains formed especially by drops of blood, the greater part stiff and extremely starched.

Stains no. 9. Group of disseminated stains, somewhat deep red brown.

Stains no. 10. Twenty centimeters from group 9, a not very apparent stain, brownish grey, starched, 25 millimeters long, 1 centimeter wide (no. 9).

Stains no. 11. A large stain situated on the lower hem, 30 centimeters from the left hem, irregularly triangular, 20
121 centimeters wide, 12 to 15 centimeters high, crinkled, deep red brown, with small, disseminated clots, apparently formed by blood and vaginal mucus.

Stains no. 12. Above the stain no. 11, a group of red stains, one of which is central, larger, red at its periphery, from which escaped bloodied trails, greyish yellow, with a sort of grey crust in the middle (no. 10).

This grey matter treated with preserving liquid released carbonic acid. It appears constituted by lime carbonate (chalk), either alone, or mixed with starch.

Stains no. 13. Above stains 12, and about 25 centimeters from the left hem, a group of stains seeming to form but one, 18 centimeters wide, 12 centimeters high, pale red in its central parts, greyish toward the outside. The median covered in places by greyish crusts (no. 11).

Stains no. 14. A large triangular stain with the left helm as its base, 22 centimeters wide, 20 centimeters high, crinkled, stiff, a reddish grey brown (no. 12).

Attentive examination of the stains existing on Zohra's shirt showed that these stains are extremely numerous and widespread over the whole area of the fabric at the level of the genital organs.

They can be arranged in three categories according to form and nature.

With regard to form, they are: 1) very large plaques coming from blood flow effected after coitus; 2) smaller stains resulting from application of the fabric to the vulva; 3) drops of blood.

With regard to their origin, or better, their nature, they appear due 1) to almost pure blood; 2) to blood mixed with vaginal mucus; 3) to blood mixed with semen.

122 It seems then that the aspect and position of these stains should permit the affirmation that Zohra ben Ahmed was the victim of rape, and that this rape was consummated not too long ago for the blood stains still showed small clots in several locations. These stains cannot be attributed to any other cause, the medical report confirming that the victim has not yet menstruated, and that her body presented no trace of violence, with the exception of the external portions of the sexual organs.

The preceding considerations, however, can allow only presumptions about the nature of the stains. Only examination by microscope of those of the stains which seem the best characterized can furnish precise information in this regard.

Examination by Microscope. In stains resulting from rape, the elements to be sought on the shirt of the victim originate from two sources:

1) the perpetrator of the rape; 2) the genital organs of the victim.

If the rape was committed with violence and especially when the sexual organs of the woman are not yet sufficiently developed, a somewhat large amount of blood will be added to material of purely sexual origin.

The elements originating from the perpetrator of the crime are corpuscles normally found in semen which detached them and carried them along during its passage. These are: spermatozoa, spermatic cells, epithelial cells of the urethra, or the epididymis, etc.

The elements originating from the sexual organs of the young girl, other than blood resulting from the tearing of certain parts of the vulva are corpuscles of the vaginal mucus and epithelial cells of the vaginal wall.

To arrive at a determination of these elements of such 123
diverse nature we chose those stains appearing most characteristic to us, as we have noted above.

A strip of material was removed from each of these parts with scissors and placed in a watch glass, then a few drops of preserving liquid of Roussin were added.

At the end of one or two hours, according to the condition of the stain, the strip of material was dissociated and some of the liquid it had soaked in was removed by expression with a glass pipette. Finally, one of the threads was placed on a slide and carefully unravelled, so as to isolate the filaments. Some of the liquid separated with a pipette was then added, and everything brought to the microscope.

After meticulous observation of the preparation thus obtained, a drop of iodine solution was added, and the examination was once again performed.

The first observation had as its purpose the determination of epithelial elements, mucus cells and blood corpuscles, i.e. histological elements, whose color and special form permit an easy determination.

The second observation was to furnish a means of easy recognition of spermatozoa, whose transparency and lack of color render them difficult to distinguish. On contact with a solution of iodine, on the contrary, these organelles take on an evident relief and stand out clearly.

Each preparation, then, was the object of two successive examinations.

Before the presentation of the results obtained, it should be pointed out that, among the microscopic elements observed, we noted only those whose presence offered some 124
interest.

Here are the results:

1) Stain no. 1. Matter of a mucus nature, without well-defined blood corpuscles, with numerous groups of *merismopediae*.

The origin of this matter remains unknown, nothing in the

constitutive elements being able to furnish information in this regard.

2) Stains no. 2.—Blood corpuscles; leukocytes; epithelium (uterine?); epididymal cells (??); no spermatozoa.

3) Stains no. 3.—Several spermatozoa.

4) Stains no. 4.—poorly defined blood corpuscles; no spermatozoa; cuboidal epithelium; cylindric epithelium of the uterus (?) or the epididymis (?);

5) Stains no. 5.—Blood corpuscles; questionable spermatozoa.

6) Stains no. 5.—Blood corpuscles; spermatozoa.

7) Stains no. 6.—Blood corpuscles; no distinct spermatozoa.

8) Stains no. 7.—Blood corpuscles; no spermatozoa.

9) Stains no. 10.—One single spermatozoan seen.

10) Stains no. 11.—Blood corpuscles; a spermatozoan.

11) Stains no. 13.—Spermatozoa; blood corpuscles; vaginal epithelium; urethral (?) epithelium; epididymal (?) epithelium.

12) Stains no. 14.—Blood corpuscles; cylindric epithelium; vaginal epithelium; spermatozoon.

The results just cited permit the presentation of the following conclusions:

125 1) The shirt of Zohra ben Ahmed is stained with blood and vaginal mucus;

2) Stains nos. 3, 5, 10, 11, 13, 14 are formed by blood mixed with semen;

3) Zohra ben Ahmed was probably the victim of a rape.

2) **Examination of the gandoura of Brahim ben Belkassem.** A white woolen shirt, having one single seam, situated toward the middle of the shirt and directed from top to bottom. This shirt is 88 centimeters long, 30 centimeters wide; used, filled with holes and tears; a part of the front left shirttail is missing. It presents only one group of stains located a bit below the waistline, 6 centimeters from the seam and 23 centimeters from the lower hem.

These stains, 5–6 in number, are brown, rather small, a bit stiff. They do not pass through the fabric. The two largest are: one, to the left, 2 centimeters long, 15 millimeters wide; the other, to the right, triangular, 3 centimeters high, 3 centimeters wide at the base.

These stains were submitted to the treatment indicated for those of Zohra's shirt, and the preparations thus obtained were carefully examined by microscope.

This research having brought no results, the presence of blood was sought for by chemical means, but it was impossible to obtain any of the characteristic reactions or to obtain production of hemin crystals.

These negative results permit us the presentation of the following conclusions:

1) The stains observed on the gondoura of Brahim ben 126/
Belkassem are not blood stains and contain no semen.

2) The position of the stains, moreover, causes difficulty in the conclusion that they were produced by blood coming from the sexual organs of the victim.

3) If Brahim ben Belkassem is the perpetrator of the crime ascribed to him, it is not on his gondoura, which is too short, that traces can be found, but on the garment he undoubtedly wore over this gondoura.

On Seminal Stains*

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/332 An important question, and one which is often submitted for evaluation by an expert physician, is that of the existence of seminal stains. We will not be paying particular attention to the description of the procedures or chemical research
/333 formerly employed, their utility and value having disappeared with the discovery of the existence of the spermatozoon, which is the essential, characteristic element of semen. They [the stains] can be of highly divergent dimensions, their tint is a yellowish grey, their form irregular of sinuous contour like a geography map, their edges presenting a deeper shade than the central parts. To examine them well, it is necessary to look at them not only directly, but also by transmitted light. It is necessary to do this as much as possible, not with sunlight, but with diffuse light, filtered by clouds. If it is a matter of experimenting on linen, the impregnated places are much more transparent and permit a better view of the weft of the fabric.

They are most often to be identified on shirts, linen and fabric. In young girls who have been raped, Devergie thought they could be found especially in front. This opinion is too absolute, and it is recommended that they be looked for everywhere; they can even be found on the sleeves near the armpit. If it is a matter of a young boy, it is necessary to note whether they are found on the anterior or posterior part of the shirt. When found on the back part, it is important to see if they are mixed in with fecal matter, indicating the practice of pederasty. Their dissemination has been considered a proof that the young girl had struggled, but it could also be a matter, on the contrary, of repeated venereal acts.

In the Cr. . . . affair, for example, a case of this elderly woman murdered by young people, to whom she frequently surrendered herself, there were three beds, one of which served as the seat of activity. It was the only one which was ordinarily used. On its surface were no less than seventy seminal stains, some of which were not less than 10 to 15 centimeters long. The preliminary examination required determination of whether it was a matter of habitual intercourse, or if the numerous stains could result from repeated acts, recently consummated, during the last 24 to 36 hours. It is easy to understand that a categorical response was impossible.

When there is an assessment to be done on stains and the stained parts of the fabric are cut out, it is good practice to number each strip, such that, if necessary, they can be returned to their place, reconstituting the sheet or linen in its entirety, which permits saying: there is a seminal stain, here, one of mucus, etc. In general, linens submitted to experts are 334
soiled with stains of every type, from which the useless elements must be eliminated. In addition, an important point to know, from the personal point of view for the physician, the shirts and sheets remitted to him frequently contain numerous varieties of parasitic insects, seeking the opportunity to multiply. It is thus prudent not to let these pieces get into his home to avoid this invasion.

It can be necessary to look for seminal stains on substances other than linen, for example, on colored fabric, on various objects and, finally, on the body of the victim. Thus, this very morning, I had to look for seminal stains on the skin of the corpse of a little girl. There were some above the pubis and on the upper part of the thigh. In these cases, a shiny stain is discovered, especially if regarded with certain incidences of light. It is generally rather easily separated from the superficial layer of epidermis for examination. Semen must also be sought in the vagina, the uterine cavity, and the fallopian tubes. Sperm enjoys the property of remaining alive for a rather long time in natural cavities. But when the vaginal mucus is acid, or when it becomes so by alteration, it provokes the destruction of spermatozoa.

On the contrary, in the interior of the uterus, where the secretion is alkaline, sperm is much better preserved. Dumas, the first to describe spermatozoa, found some, living, in the ovaries of dogs seven days after mating. In an autopsy it is then necessary to go further than a superficial examination and to pursue intently the search in the uterus, fallopian tubes and ovaries.

If the assessment is done soon afterwards, the movements specific to spermatozoa can be made to reappear in moistening linen soiled with sperm, and characteristics analogous to those presented by fresh sperm can thus be found. What then are these last-mentioned characteristics? According to Robin and the latest research of micrographers, a spermatozoon is a simple cell furnished with a very long prolongation which is nothing other than a strong vibratile cilium. Whatever it might be, this vibratile cilium communicates very clear movements to the rest of the cell, which seems to direct itself and have an instinct like a true animal. The total length of a spermatozoon is 45 thousandths of a millimeter or μ. Of 335
this figure, the head is only about 5μ in length, 3 in width, and 2 in thickness. In addition, leukocytes, round fatty

*Translation of: "Des Taches Spermatiques." in *Le Practicien* 2 (28): 332 336 (1879).

bodies, other small bodies called *"sympexions,"* and tribasic phosphate crystals are found in semen. It is quite rare that whole spermatozoa can be observed. Most often, linen brought to the expert has undergone changes and crumpling which have most often separated the heads and tails such that only debris are found.

Along with spermatozoa, elements derived from the environment from which the sperm comes can be seen. Thus, in the affair of Mme. Cr . . . , a handkerchief was found on which were numerous stains perfectly analogous to those of semen. Examination by microscope permitted discovery of round, elongated cells, epithelial cells of the mouth, other globular cells of mucus and, finally, cylindro-conical cells of the respiratory tract. It was probable then that at a given moment the semen found itself in the mouth. This hypothesis was strongly confirmed with the discovery of a grain of tobacco mixed in, which left no doubt as to what had happened, the murder victim being the only one who indulged. If there had not been tobacco grains, there could have been a hesitation in distinguishing between cells of the respiratory tract and certain other cells with vibratile cilia found in the epididymis and mixing with semen.

In the frequent cases where there is a mixture of *vaginal mucus,* large, unequal, rolled-up epithelial cells, mixed in with a certain number of pus corpuscles, are found. Leukocytes become predominant only if there is *vaginitis. Fecal matter* is recognizable due to the presence of *twisted fibers* coming from poorly digested muscle tissues, numerous grains of starch and vegetable cells.

Finally, epithelial cells coming from urethral mucosa and epidermal cells with very elongated vibratile cilia are also found mixed in with the sperm. These latter cells are generally abundant in semen of the first few penetrations; thus, for example, they appear only after a continence of about ten days and disappear with repeated coitus on the second or third time.

What is the best procedure to be followed for systematic examination of seminal stains? On dried linen, which had been folded several times besides, and carelessly treated, it is common to see the seminal stain partially peeling off in scales. It is necessary then to collect these separated parts carefully and moisten them slightly with water. But ordinarily, none of the stain can be separated dry, and it is necessary to use one of the following procedures: after strips of contaminated linen have been cut out, they are left to macerate in two or three drops of distilled water, placed in a watch glass. The amount of water must be as small as possible, in order to examine all the parts easily. Another method, attributed to Robin, is to suspend the strips above water in glass test tube such that the strips touch the surface of the liquid, which will imbibe it by capillarity. These procedures for renovation of the seminal stains are equally good, with the condition that one has the time, for 12 to 15 days are sometimes necessary to permit inbibition to go to completion. Once obtained, the surface of the linen is scraped with a scalpel, the threads of material are detached one by one and examined by microscope.

These procedures are applicable when strips or thongs of fabric can be detached with scissors. But this is not always possible, it being either a matter of priceless furniture, or of the stains being located on the skin, for example. These stains are softened by moistening, and scraped however possible. Sometimes the search for spermatozoa is rendered more difficult by the considerable number of epithelia, grains of starch, etc., which clutter the preparation.

It is essential to examine each stain in its entirety. When spermatozoa are not found after examination of the greater part of the stain, one must not give up on their discovery, for they are often found collected in one single point. It is also necessary to develop the habit of always using the same magnification, that of 500 diameters, for example; it is easier 336
then to recognize the presence of spermatozoa. With the same goal in mind, Kasper recommends leaving the liquid presumed to be seminal to dry between the two slides of the preparation, then to remoisten it a little while afterwards. In proceeding thusly, it seems that air fixes to the walls of the spermatozoa and increases the clarity of their contours. Roussin also recommends coloring the preparation with a drop of tincture of iodine.

Even when spermatozoa are not found in a stain having all the macroscopic characteristics of a seminal stain, the conclusion that it is not actually seminal cannot be reached. Indeed, there is a certain number of individuals who are infertile, during a certain time at least, due to an absence of spermatozoa after a double orchitis, for example (as demonstrated by Professor Gosselin). They nevertheless ejaculate a fluid showing every appearance of normal semen. It has also been proposed that spermatozoa disappear in the elderly. This is often not the case, and Dr. Duplay, Jr., has determined their presence in elderly men, 85 years old. Dr. Dieu, a physician of the disabled, has found in autopsies that, after the age of 70, only a quarter of the subjects examined no longer possessed spermatozoa, whereas the remaining three quarters had them. Finally, if coitus has been repeated a number of times, the later penetrations furnish a semen lacking in fecundating properties. Nor will spermatozoa be found if the fabric, on which the investigation is being performed, has been energetically crumpled; it is, therefore, necessary to wrap and fold it carefully.

There are a certain number of cases where distinction must be made between seminal stains and those of gonorrhea. This latter gives greenish stains at its outset, then yellowish and finally uncolored. Their surface area is much smaller than that of seminal stains, their form round and more regular; they are sometimes colored by the coloring matter of blood and contain a large number of leukocytes. The interest in this distinction can be imagined if it is a matter, for example, of a young girl pretending to have been violated, and whose shirt presents stains of gonorrheal mucus.

Thanks to examination by microscope it is almost impossible to confuse spermatozoa with anything else or semen

with any other liquid. However, Hoffmann has pointed out the possible confusion of the spermatozoon with certain bacillary bacteria, also formed by a head and a vibratory cilium, but this latter is ten times smaller than the spermatozoon, and this error will be avoided if the recommendation given above, that of always using the same magnification, is followed.

Report on the Petel and Labiche Procedure for the Detection of Seminal Stains*

Boutmy and P. Brouardel[1]

224 Dr. Brouardel and myself were charged by the Society of legal medicine with examining a work resulting from the collaboration of Petel, an M.D., and Labiche, a pharmacist, a work relating to the use of carmine in the medico-legal examination of seminal stains.

I will communicate to you the conclusions to which the study of this work has led us.

When it is a matter of animal secretions, the experiments it is important to perform to enlighten justice are of two different types, namely:

Chemical experiments;

Anatomic experiments.

225 When both of these types of experiments can be performed, it is possible to arrive at an unequivocal answer to the question; but when, by fortuitous circumstance, one of the two types of experiments cannot be performed, the expert hesitates to assert his opinion absolutely.

This hesitation is understandable, especially when it is the anatomic study which is lacking.

In fact, the chemical reactions which can serve to characterize the liquids of the organism are very restricted in number and limit themselves generally either to coagulations by heating or by a few reagents such as nitric acid, mercuric chloride, phenol and wine alcohol, or to various colorations which certain substances cause to appear in the material being examined.

As a result, the chemical reactions we have just presented, applied to the study of the organism, indicate the class of material rather than the particular identity.

In contrast, in the presence of the anatomic element (which is always unique), confusion is impossible, and the expert can give an opinion with every assurance.

Because of blood cells, for example, blood will not be confused with any other liquid of the organism; and, in addition, every anatomist can distinguish mammalian blood from that of bird or fish.

Further, as with blood, spermatic liquid contains this particular organism, *the spermatozoon*, imprinting it with a very special trademark, and permitting its absolute differentiation from every other liquid of animal origin.

As a result, when one or several spermatozoa can be extracted from any stain whatever, this stain contains semen. A spermatozoon can always be recognized by its very distinct form; and it is precisely this which gives this anatomic element its extreme importance in research in legal medicine.

But, as many authors will note, it can happen that, after 226
crumpling of the material to be examined, the spermatozoa break into fragments, difficult to recognize, and that, as a result, the anatomic examination by microscope will not lead to any precise conclusions.[2]

It is this kind of regrettable circumstance, continue Petel and Labiche, that we would like to avoid by pointing out that carmine can impregnate seminal stains with a special rose color, resistant to washing of the fabric, and to the action of certain reagents.

This method of investigation, proposed by Petel and Labiche, is bound, we believe, to be very useful, and the authors must be congratulated for the care with which they carried out the studies, and for the skill with which they removed causes of error which can arise from the presence in the stained material of elements produced by saliva, nasal mucus, albumin, etc.

But in our eyes, it would be exceeding the limits of discretion to agree with Petel and Labiche that, in absence of spermatozoa, the persistent red coloration, communicated to stains by carmine and remaining on the material, is sufficient to decide on the presence of semen, especially since this fact can engender such serious consequences.

Indeed, in imagining the terrible sentence which a man might receive for the crime of rape, what expert would dare assert that a stain found on material is a seminal stain because this stain colors in rose by the action ammoniacal carmine and then takes 12 hours to discolor in a sodium-carbonate bath, whereas another stain, produced for example by albumin, will discolor in six hours.

Could not a particular circumstance, yet unknown to the authors of this memoir, occur where this stain of albumin disappears only after 11 hours, for example?

Is it absolutely certain that among all the stains which can 227
be formed on fabric, no type will be found which shares with seminal stains the double property of coloring in red with carmine and also resisting alkaline solution for 12 hours?

This 12-hour limit for sperm stains appears to us very arbitrary, and if it diminishes, what will become of the investigative procedure using carmine. In these circumstances, the anatomic element remains the only absolutely certain sign of the presence of semen; and if it is sometimes found that spermatozoa are absent, these cases are so rare that the public conscience cannot be noteceably stirred over it.

It will be agreed that, in a comparable circumstance, it is necessary to be very circumspect, and that it is better to acquit the guilty than to condemn the innocent.

Does this mean that the method of investigation proposed by Petel and Labiche must be rejected? As we have mentioned, we think the contrary, for it brings an additional proof to that already obtained by microscope. This accumulation of proof is always desirable in research as delicate as that with which we are occupied. We thus propose that the society express its gratitude to Petel and Labiche for this presentation of a new reagent, confirming the histological results obtained in the medico-legal investigation of seminal stains.

*Translation of: "Rapport sur le Procédé Petel et Labiche Destiné a Faire Découvrir les Taches de Sperme", in *Annales d'Hygiène Publique et de Médecine Légale* 4 (3rd series): 224–227 (1880).

[1] Meeting of May 12, 1879.

[2] It is also known that the seminal fluid of certain individuals is devoid of spermatozoa.

Memoir on a Few New Applications of Microscopical Examination to the Study of Different Types of Stains *

Ch. Robin and A. Tardieu

Members of the Imperial Academy of Medicine

416 Called upon of late to give our opinion on many cases of legal medicine of great interest, we have had the opportunity to do some new applications very important in the examination of different types of stains. We are eager to include here the principal results which can be useful to other experts and which add to what we already know about the correct procedures for identification of the nature of the substances of which we will be speaking. Additional proof of the superiority of the microscope over ancient methods in the medico-legal evaluation of all types of stains will be found in this study.

Blood stains

Is the stain submitted to an examination by experts from the blood of a man or of a woman? It is not uncommon to see experts called upon to answer a question posed in the same form as that which serves as title of this section. Just such a question was posed in a rogatory commission, in the execution of which we had been committed to examine different articles of clothing, stained with blood from the murder of an elderly man and his domestic, an elderly woman.

417 There have been only a small number of research publications on this question; the treatises of legal medicine lack documentation which might serve to answer it; which is why we thought it might be useful to publish the studies we have done in the circumstances we have just briefly mentioned.

Although by the manner in which the question is posed: "What are the blood stains present on the smock, etc.?", there is no doubt cast on the fact that stains are formed from blood, the experts ought to have assured themselves beforehand as to their exact nature. After having noted that they have the physical characteristics of blood stains, we confirmed with the aid of appropriate microscopical and chemical procedures, that many of the spots actually contain red blood cells, white blood cells and fibrin, the essential characteristics of blood.

Our observations and analyses ought to have been directed principally toward the particular question as to whether the stains present on the smock were formed from the blood of man or the blood of woman.

Knowing that experience has shown that the characteristics which enable a distinction of male blood from female blood must, to be confirmed, even when the quantity of material is sufficient, be investigated as soon as possible after the blood leaves the vessels, and that the objects alleged to be stained with blood and furnished for our examination, were remitted to us twenty-one days after the crime, we immediately conducted a special study of the stains present on the smock in the following manner.

After having removed the portions of material bearing the more important stains, we meticulously cut these to separate the portions of clothing stained and imbibed with blood from those which were not. The bloodied portions were reunited at the bottom of a short, wide test tube; we then moistened 418/ them with a little distilled water; the moistening finished, we added a quantity of sulfuric acid, concentrated, pure and uncolored, equal to about half the substance to be tested; having mixed and compressed everything together in order to render the action of the acid on the stained material even and complete, we tried recognizing the odor emitted from this substance. We noted a light odor of human sweat. Despite repeated attempts under the preceding conditions with gradual increases of the amount of acid, it was impossible to obtain an odor pronounced enough for the comparison of the odor presented by the blood of an elderly man with the blood of an elderly woman, treated in the same way, to give us conclusive results in either direction.

It is known, moreover, that, if a sufficient amount of blood or stains big enough and recent enough, treated as previously, give a particular odor for each animal species which experiment permits a distinction, this characteristic is not sufficiently pronounced for permitting verification that the blood comes from one animal rather than another. Only in the case where the examination has been conducted within an appropriate period of time, and this characteristic is lacking, is it necessary to suppose that the blood does not come from the presumed animal.

It is also known 1) that, if blood from man and blood from woman in sufficient amount or forming stains big enough and recent enough, treated as previously, give an odor comparable to human sweat; 2) that, if this odor is less strong or a bit more bitter in bloodied material coming from a woman than from a man, these characteristics become more and more analogous, then similar, with time. They are not even pronounced enough at any time, that, provided with such a small amount of material as had been submitted for our

*Translation of: "Mémoire sur Quelques Applications Nouvelles de L'Examen Microscopique de Diverses Espèces de Taches", in *Annales d'Hygiène Publique et de Médecine Légale* **13** (2nd series): 416 442 (1860).

/419 examination, it would be possible to affirm with certainty that a human blood stain comes from one sex rather than the other.

In summary: the age of the stains, dating twenty-one days, submitted for our examination; the small amount, relative to the question to be resolved, of the bloodied matter which formed the stains; the natural and constant similarity between blood of man and of woman, which differ only temporarily and by weak degrees of a given odor, comparable to that of sweat, make it impossible for us to decide by the light odor of sweat emitted by these stains if it is the blood of man or the blood of woman which forms them; but nothing authorizes a denial that the blood comes from a person of feminine sex.

A note on the distinctive characteristics, from a medico-legal viewpoint, of blood stains and stains from fly droppings. In a medico-legal investigation, a smock bearing stains, allegedly blood, was submitted for our examination, for the purpose of determining if they actually contain elements characteristic of blood, to which they presented a superficial resemblance.

Near the lower border of this smock were three circular stains, of width from 1-2 mm, forming a thin glaze on the material which they did not saturate for the full thickness; they were of a reddish brown, fairly shiny on their surface and a bit stiff, almost starched. Studied according to standard procedure in search of elements of blood, we found no instance of them. They indisputably presented, on the contrary, the microscopic characteristics and the parts constituting fly droppings in all their aspects. Like these droppings and like the substance of stains which they form on furniture /420 and material, they are composed of a material homogeneous, amorphous, transparent, uncolored, swollen, dissociated by, or dissolved in, water, holding together the coloring granules of these droppings. These granules formed, as always, the greatest mass of the material of the stain, in which they were almost contiguous. They were of a yellow brown, some with a greenish reflection, the others with a reddish reflection, faintly pronounced. They all strongly refracted light, clear at the center, dark on the periphery, as fatty bodies; also like fat granules, they were insoluble in water and in acetic acid and almost all dissolved in hot alcohol and in ether. Some small crystals in the form of short needles of undetermined chemical composition accompanied them.

These characteristics can be found, as one can easily be assured, on almost every fly dropping examined. This permits us to conclude that it is a matter, not of blood stains, but of stains formed by fly droppings.

A medico-legal note on the stains of varnish which show all the physical or superficial characteristics of blood stains. We were entrusted, on 16 December, 1859, to proceed with the analysis of stains present on the smock confiscated from the home of Mr. B. . . ., accused of homicide, and with determining: 1) if the stains are of blood, of smoke or other substance, 2) if they are of human blood or cow blood, 3) if they are recent, with regard to their existence, of fifteen months, as the accused asserts, and that since then, the smock must have undergone two or three complete washings. And, before proceeding with the analysis, to examine the material of the stains, and determine: 1) if they had not been superficially washed, with the goal of making them disappear; 2) if they do not conserve a gummy nature inside the 421 smock, indicating that they have not undergone any washing on the inside.

This smock was on the whole of blue cloth, a bit whitened from decay and use; especially on its exterior surface, the back as well as the front, on the sides of its slit. It was patched near the neck and on the sleeves.

Stains offering the superficial aspect of blood stains. On the right side of the smock, in front, on the chest, shoulder, the upper part of the sleeve on this side, and a little underneath the seam of the armpit, could be seen very small stains too numerous to be counted. There were also some, exactly the same, on the sleeve on the left side. They were of a width of 1-18 mm; the greater part were separated from each other; some were joined together at their ends. Almost all were round or oval; the others were polygonal, with rounded angles. All ended in a distinct border as dark as the rest of the stain. All traversed the entire thickness of the fabric and were as discernible on the side of the smock turned toward the body as on the outside. Most of the ones on the side of the smock turned toward the body presented an aspect slightly brilliant, gummy, that were found only on a small number on the outside surface. All gave to the fabric a stiffness, comparable to that which starch gives to shirts, and to that which blood stains and other sorts of mucus and albuminous liquids of the human body give to different sorts of linen. None formed a crust on any face of the smock whatever. All these stains presented a reddish-brown taint, slightly shiny or gummy on one side, as was just pointed out; an aspect similar to that which blood stains present. This reddish-brown color lost its reddish tint in all the stains located in the portions of fabric colored in dark blue; but the gummy aspect and the stiffness peculiar to starched cloth 422 was still clearly evident.

These stains were evidently duller on the outside surface than on the side turned toward the body; the appearance which they presented in this context could be compared on the outside surface only to that which an incomplete washing, or even better, a scraping after dessication, gives to blood stains. On the side turned toward the body, these stains showed all the superficial characteristics shown by stains formed by blood, and, on the outside surface, the superficial characteristics of the same stains when they have been incompletely washed or rubbed and scraped without having been spread out.

One single physical particularity was lacking in them, it being that, in the obscurity of night, the light of a lamp and of a candle did not render the stains appreciably more shiny, nor more visible, while this occurs, on the contrary, for blood stains.

Nonetheless, their similarity to stains actually formed by

blood was such that we had to proceed with their analysis as one does in the case of stains strongly considered to be formed by blood.

Microscopical and chemical analysis of stains presenting superficial characteristics of blood stains. We cut out the stains and, according to the procedures known to science, plunged them alternatively either in a sodium phosphate solution, or in a sodium sulfate solution. These liquids, which should slowly swell and soften the substance of the blood stains, with the purpose of then isolating the constitutive elements under the microscope, had absolutely no effect on the stains. Water itself in no way modified them.

We then submitted the stains to prolonged immersion, then to repeated washings in cold water and in hot water,
423 both pure and soapy. These washings changed nothing in the taint nor the starched state of the stains.

These facts sufficed at this point to show us the stains we were analyzing were in no way formed by blood.

The immersion and then the washing of many of the stains, done separately in liquid ammonia, in carbon disulfide, in alcohol and in ether removed the gummy aspect from the stains on the inside of the fabric; they caused the starched state to disappear, but not completely, and the mark of faded stains distinctly persisted on the two surfaces of the fabric. The evaporation of the alcohol, the ether and the ammonia in which many of the cut-out stains were immersed for eighteen hours, left a residue of only small amount, which presented no crystals. This residue dissolved in sulfuric acid, but not acetic acid. The small amount prohibited us from submitting it to the action of other chemical agents.

This resistance to the action of pure water, of soapy water, of ammonia, of alcohol, of ether, and of carbon disulfide, tends to make one admit that the stains could not be recent, could have an existence of fifteen months, and that they could have resisted two or three complete washings that the smock would have undergone since the time of their formation, but without it being possible to assign a date to their formation.

The absence of the gummy aspect of the stains on the outside surface of the smock with conservation of this aspect on the inside surface could be due to repeated rubbing of the stains and the wearing out of the smock, which had already modified the general color; but the resistance of the two sides of the stains to the action of water and of the chemical solutions we employed is in opposition to the theory that it might be due to a washing done beforehand.

424 The amount of substance coloring the fabric and giving it the starched condition was so small, that it was impossible for us to retrieve by chemical means an amount sufficient for precise determination of the type of resin, or of varnish, forming the stains. From this point, our only recourse was the use of the microscope to see what type of material was present.

We proceeded to the microscopical examination of the material of the smock, and of the substance forming the stains, examining it between the filaments of fabric, because it did not form a crust on the surface. We discovered the presence of a transparent, homogeneous, reddish substance, such as is shown by particles of dried-out, bloody crusts seen by microscope. This substance was in no way crystalline, it filled the interstices of the threads of the smock and formed a varnish around the microscopic filaments of hemp composing the threads of the fabric, a fact which explains the very evident starched condition of the fabric in the area of the stains. This substance was a bit sticky and the thin, angular fragments conserved on their surface the impression of the microscopic filaments from which they were separated.

But in contrast to that which happens: 1) for residues of brown water of dried-out dung; 2) for fragments of bloody crust taken from stains actually formed by blood, the substance of the stains submitted for our examination did not soften at all in water nor in solutions of sodium sulfate or sodium phosphate. Hot and cold acetic and hydrochloric acid equally left them completely intact. Ammonia and solution of potassium blanched and softened the fragments of the substance of the stains, but without dissolving them. It was the same for ether and carbon disulfide, the action of which, however, was less pronounced. Hot concentrated sulfuric acid dissolved all the fragments of the substance of the stains quite rapidly, as it does for most varnishes and resins, at the 425
same time swelling and softening the microscopic filaments of hemp without dissolving them.

In summary: this resistance to the action of water, acetic acid, hydrochloric acid, with complete solubility in hot sulfuric acid, and incomplete or insolubility in alcohol, ammonia, potassium and ether, proves that the substance of which the stains are composed is not blood, even though it formed on the fabric of the smock stains possessing all the physical and superficial characteristics of blood stains. These characteristics, the only ones which the small amount of substance permitted us to confirm, were, on the contrary, those which belong to material of resins, of dried-out lacquer and other analogous substances, of an origin different from substances of the human body.

The stains were not, then, blood stains. Nor had they any of the characteristics of solubility and composition of residues of water from dung.

They were formed by a substance analogous to that of resins and of lacquer which had congealed and would have dried out after having saturated the fabric of the smock.

By reason of their chemical nature and their resistance to external and chemical agents, they could not have been recent and could have resisted two or three complete washings of the smock.

In another case submitted for our forensic examination, the stains present on an iron axe, an alleged instrument of crime, had been considered in the investigation as probably formed by blood. They were numerous, reddish, without an ocre taint, and of width of about 1–6 mm. Some were circular, with a very finely jagged periphery; most were irregular. Also were found some of the same taint, of a poorly determined periphery. All were very thin, not projecting above the surface of the iron. The jagged border of those presenting
426 this characteristic were the only parts projecting slightly. All were not very shiny or of a dull tone, except the very fine tracing slightly projecting at the periphery of some, which was shiny, and of a crystalline appearance.

This dull appearance of the stains became even more evident when they were examined at night by the light of a lamp. Here, instead of reflecting the light, showing a taint of shiny, brown-red, they stayed a duller tone than that of the polished iron bearing them. Their surface by magnifying glass and by the naked eye was delicately rough. Subjected to the action of water, they did not change their appearance; hydrochloric acid dissolved them, returning to the iron its brilliance. The powder obtained in scraping them, and submitted to microscopical examination after the appropriate procedures, showed no trace of red or white blood cells, nor of fibrin. But it allowed us to see small, irregular, angular fragments, similar to those described by M. Lesueur[1] and by one of us, which showed the reactions specific for iron rust.

Note on blood stains mixed with epidermis and lanugo of a new born. The microscope shows in the blood stains the very anatomic elements which themselves constitute blood, and thus permits determination of their nature with more precision than methods based on the simple phenomena of coagulation and coloration; but it permits in addition recognition of the nature of foreign bodies, other than blood, which can be mixed in with the material of the stains and can sometimes furnish previous medico-legal indications, in the circumstance where the nature of these bodies oppose the findings uncovered by chemical reagents.

427 The following case, where we had been called upon with M. Lesueur to determine whether a newborn had been enveloped in a skirt, is a striking example, supporting the preceding remarks, which apply to cases whose number is more than likely to increase greatly.

On the skirt sent to us, we noted that, of the stains it bore, some are reddish, more or less pale, like stains formed by mucus or bloodied serous liquid; these are of the greater number. Most of them starched the material a bit. The other stains, smaller, are of a deep brownish red, like stains a bit old and formed from pure blood. Many of them are superimposed on the preceding stains and stand out by their deeper color, besides, some of them strongly starched the cloth and some of them even form a crust. It is not difficult to recognize, particularly for people accustomed to practicing childbirth, that many of these stains lie on larger stains, very pale, of a diffuse periphery, slightly yellowish, like stains, formed from urine or water from the amnion. They emit, besides, an odor of urine, a certain flat odor, like a mixture of urine and the waters of childbirth, a very pronounced odor, which is accentuated on leaving the skirt rolled up for twenty-four to forty-eight hours in a slightly humid place. Some of the bloodied stains, superimposed on the odoriferous urine-like stains, had their edges blended into these stains as if the blood had touched the cloth while it was wet and had immediately mixed with the moisture of the latter.

The back part of the skirt bears bloody and urinary stains of the same appearance as the preceding; but they are larger and their edges are more blended together. These stains are situated toward the middle of the upper part of the skirt, on either side of the vertical seam which runs down it, and 428
reminding one by their situation and general disposition of those which the buttocks, wetted by a bloodied liquid, would produce if a person were seated or lying down. Still on this side, but lower, nearer the hem of the skirt are four large irregular stains, of a width of 4-12 cm; one to the left of the seam, three remaining to the right. They are reddish, like stains formed by blood running on a wet cloth, and their edges appear as if washed, blended into the large, slightly yellow stains with a strongly urinary odor, by which almost all of this part of the skirt is impregnated. They also strikingly starch the cloth. All these stains traverse the fabric, but are more marked on the side of the skirt turned toward the body than on the opposing side.

The outside of the skirt is soiled by tracks and stains of grey mud, evidently coming from rubbing against mud or earth or dust while still wet.

After having cut out appropriate strips, taken from the principal stains, we dipped them into as many watch glasses containing sodium sulfate with the addition of a few drops of glycerin. The substance of the stains, once softened and gradually swollen by saturation without being dissolved, were removed with care by scraping, and submitted to examination by microscope.

The material of the deepest, thickest stains forming a crust showed *red blood cells,* some intact, biconcave, circular, others a bit swollen, becoming almost spherical and a bit jagged, as they become in blood exposed to air; but they were still immediately recognizable.

These stains showed in addition a rather considerable amount of fibrin, which the action of pure water permitted 429
us to isolate and discolor by washing out the red blood cells in such a way as to render their fibrillary aspect clearly evident. In the fibrin were some white blood cells, small in number, clearly recognizable. Now, it is known that fibrin does not form fibrillary clots in menstrual blood, which flows normally and mixes with mucus of the matrix, and that fibrin is not found in stains produced by menstrual blood on cloth. Besides, we did not find in the material of any of the pale or dark stains which we examined, white corpuscles called mucus corpuscles which accompany menstrual blood in great number, and which are easily found in the stains in a proportionately greater amount as the stains are paler, have more mucus and are less bloody.

The reddish stains, paler than the preceding, not forming a crust on the cloth, which however they starched a bit, showed red blood cells like the preceding stains; but they enclosed neither fibrin nor white blood cells.

The material of the stains additionally demonstrated ele-

ments which, even though coming from the surface of the human body and its membranes, are foreign to blood. These are *epithelial cells,* polygonal, finely granulated, with an oval nucleus, isolated for the most part, some, however, united in sheaths as a result of their imbrication. These cells are similar to those of the vagina and external genitals of women. They could have been carried out during childbirth, and deposited on the skirt either by the blood which flows in such a case, or during the passage of the infant. They are, it is true, similar to those one finds in menstrual blood; but they were not accompanied, on the stains of the skirt, by the
430 mucus corpuscles which always accompany menstrual blood in great numbers.

In the material of these stains, were found other epithelial cells, cuboidal, thin, transparent, of pale edges, non-granulated, some folded, others marked by fine, irregular lines. The greater part were united in epidermal strips or laminae of a width of 0.1-0.5 mm. These strips or laminae were larger and more numerous than those which naturally detach from the human body and adhere to the parts of clothing immediately contiguous to the body. In the thickest of these strips, formed by many rows of superimposed cells, the most pronounced were smaller than the others and provided with a nucleus. These are characteristics belonging to the still thin *epidermis of the foetus* when removed by fairly rough scrubbing or scraping, and which one does not find on epidermal sheaths naturally detached from the surface of the human body; these latter, indeed, never show nucleated cells.

Along with these small, epidermal sheaths, whose structure leads one to consider as coming from the surface of the body of a foetus, were found a few free *lanugo hairs,* detached from their follicles, small in number, but easily recognizable. These hairs had the characteristics of those found on the surface of the foetal body during childbirth. They were pale, uncolored, faintly striated lengthwise, without coloring material in their thickness, a width of only three-hundredths of a millimeter, without a medullary canal, of pointed end, a bit irregular, with a small tapered root. These characteristics, as one knows, are in no way those of pappus of the adult human body, the diameter of which varies from 6 to 8 hundredths of a millimeter, the free end of which is a bit flat,
431 the substance provided with coloring matter, and the center hollowed out by a medullary canal, interrupted or continuous, and filled with a granulous marrow more or less opaque.

In addition, we have found in the material of these stains, as in all the others of which we'll be speaking, a few *grains of starch* and some irregular grains, of variable volume, which the appropriate reactants have shown us to be of a mineral nature. Of these diverse corpuscles, found in almost all dust originating outside the human body, we limit ourselves to pointing out their existence, without pursuing the matter further; for no conclusion whatever can be based on their presence, any more than on their absence.

We had to study an oval, irregular stain, 12 mm wide, found on the large hem on the bottom of the skirt. It was greenish brown, becoming greener when scraped. These external characteristics being those found on stains formed by meconium, we studied it according to tried procedures for the examination of the latter. We found there polyhedric cells filled with a greenish-brown, granular material. These cells had all the characteristics of those of plant parenchyma; some isolated, others still united in variable number; bundles of punctured vessels and trachea of herbaceous plants, such as one finds in sorrel, spinach, or the parenchyma of various crushed and ground or cooked leaves. This stain enclosed no elements of meconium at all, and its color derived from grains of chlorophyll or green coloring matter of plants, noted in the cells of which we just spoke, and having, by dessication, lost in part the vivacity of their green color.

In summary: from the preceding examination it results that: 1) the stains of this skirt contain, besides blood, elements which could have been mixed in only by immediate contact with the sexual parts of woman; 2) the disposition of these stains in back, in the area of the buttocks, the presence 432
of large stains also in the back part, much lower than the genital organs, demonstrate that all, or almost all, these stains come from blood which flowed from the genital parts of a person giving birth when they were formed; 3) the presence in large blots of these pale stains, of a very pronounced flat odor, similar to that of a mixture of urine and amniotic liquid, together with the presence in the material of these stains of sheaths of epidermis similar to that of a foetus, and especially of lanugo hairs of a foetus, show that these stains come from the blood of childbirth, and that the foetus must have been in contact for a more or less long time with, or even enveloped in, the skirt which bears them.

Mucus Stains

Examination of a stain allegedly of the nature of meconium and formed by the material of bronchial and pharyngeal expectorations. In the assessment mentioned in the preceding section, with which we had been charged, along with M. Lesueur, a bed sheet, on which the childbirth allegedly took place, showed a stain which its appearance and various circumstances mentioned in the investigation led us to consider as being formed by meconium. After having observed the external characteristics of this stain, we conducted our examination of it following the procedure we outlined in a preceding work.[2]

Twelve centimeters from the edge of the sheet was an irregularly circular stain 2 centimeters wide, a pale greenish yellow. The portion of fabric bearing it was cut out, and one 433
end of the cloth, upon being plunged in water, notably softened and swelled when the liquid reached the stain by inbibition. Examination by microscope showed it was composed: 1) of a transparent, homogeneous mucus, striated like that of viscous expectoration, produced in the case of chronic laryngitis and in expectorations called *"hem";* it held in suspension only a small number of molecular granulations;

2) in addition this mucus held in suspension cuboidal epithelial cells, similar to those of the pharynx and mouth, but small in number; 3) other spherical epithelial cells, 2-3 hundredths of a millimeter wide, of which some were very granulated and showed their central nucleus only after the action of acetic acid which blanched or dissolved the granulations. These cells are always found more or less abundantly in expectoration coming from the bronchi, larynx and the back of the throat; 4) this mucus contained especially a large number of leukocytes called mucus corpuscles, irregularly spherical, one hundredth of a millemeter wide. They were accumulated either in irregular masses, or in lines more or less long, parallel to the mucus striations. Their characteristic nuclei, at first invisible, showed up most evidently on contact with acetic acid. This acid rendered the mucus more clearly striated, and gave it a fibroid appearance more pronounced than that which it had beforehand. This reagent has the property of modifying mucus, permitting a distinction between semi-solid and solid mucus and coagulated fibrin; for it swells fibrin, making it lose its striated aspect and its fibrillary disposition.

These characteristics being those one finds in products of pharyngo-bronchial expectoration, and not in meconium and other mucus materials, we concluded that this stain was
434 produced by expectoration which had accidentally fallen on the edge of the sheet.

Seminal Stains

Note on the distinctive characteristics of seminal stains and stains of fecal matter. Stains of a seminal nature were found on the shirt of a young girl, less than eleven years old; which were accompanied by stains of another appearance, which, at first look, had been considered as *natural and physiological exudations of female genital organs.* Starting with this idea, the first expert concluded that these stains were all located on the tail of the front of the shirt.

We were judicially committed, by request of the first expert, to verify the nature of these stains. On one of the tails of the shirt, near the edge, we found two greyish, pale, irregular spots, slightly starched, penetrating the fabric by absorption, but more marked on the side turned toward the body than on the outside. One was 5 millimeters wide, the other narrow, with jagged edges, of the same width and 32 millimeters long. On the opposite tail of the shirt was a large, irregularly semicircular stain, with the edge of the shirt as base, 13 centimeters wide, and 11 centimeters long. All around it for a distance of about 10 centimeters, but mainly above, were many small stains varying in width from a few millimeters to 2 centimeters. Their form was not very regular and their periphery sinuous, or jagged, in places. Those nearest to the large stain merged with it in places, rendering the periphery irregular. These stains were pale, greyish, slightly darker at the edge than toward the center without a yellowish taint. They were more easily perceived at night by
435 lamplight than by day, because they compared more strongly with the tone of the fabric, without shining, however. This characteristic is found in diverse stains produced by mucus liquids of the human system. They traversed the fabric, being, however, a bit more marked on the side turned toward the body than on the outside of the shirt, even though these were whiter and less dirty than the other. The disposition of the hem, in addition to the manner in which the shirt was stained, enhanced the distinction between the inside and outside of the shirt.

All these stains slightly starched the fabric, the smaller more so than the larger; even though the starching was evident, the folds of the cloth, once formed, were conserved more in the area of the stains than in non-stained areas. The large stain had in addition to its greyish tint a sullied shade, which the small ones did not have, and which rendered the larger one darker.

On the large stain, a bit to the side, on the side of the tail of the shirt turned toward the body, were irregular stains or mackles, brown or greenish-gray, dull, like mackles of excrement; they were thin, without forming, or almost without forming, a crust. There were, principally, three of them, 8, 15 and 23 millimeters long, of a lesser width, and variable in their different points of extension; they were united by irregular streaks of the same appearance, seemingly formed by rubbing the substance of the principal stains while they were still fresh. Their edges were jagged, like those of impressions left on folded cloth by a colored substance.

After having noted the various external dispositions of the stains, we cut out a certain number which we then separated into two halves, with the purpose of submitting one to microscopic examination, and the other to the action of chemical reagents.

Examination by microscope of the substance of the pale stains, presumed to be of seminal nature. We cut out in thin 436
thongs the portions of cloth bearing each of the stains whose nature we wanted to determine by microscope. We then dipped them by one of their ends into as many watch glasses containing a little distilled water. At the end of an hour or two, the water having slowly wet the cloth by absorption and capillarity, the stains were swollen and projected a bit from the surface of the fabric, a fact which required much attention to be seen.

At the same time, they became shinier than they had been, and the pale, gray stains, suspected to be of seminal nature, took on a bit of a mucousy or gelatinous appearance.

We then scraped each stain with a clean bistoury to remove the substance covering the fabric, and that which penetrated between the threads by absorption. We then submitted the matter thus removed, separately for each stain, to microscopical examination. The microscope showed us in the substance obtained, as it was just pointed out, a certain number of filaments presenting all the characteristics of those which compose threads of hemp. Between them we easily perceived a great quantity of homogeneous, scarcely grey, transparent substance, such as one finds in semen and

other mucous substances of animal origin. They showed up either in flakes of sinuous contour, such as we have represented in the design attached at the end of this report, or in more extended layers interposed between intertwining filaments. We saw at the same time a large number of very small, threadlike, pale, greyish bodies, 5 hundredths of a millimeter long, one thousandth of a millimeter wide, ending in a swelling or head of darker contour, ovoid in the form of a flattened pear, 5 thousandths of a millimeter long. These characteristics are those of spermatozoa, elements characteristic of semen, and one finds them in no other substance coming from the human body.

They were as numerous and as concentrated as in seminal fluid, such as it is when just ejected by ejaculation. They were whole, flexible, and there was only a very small number broken by the maneuvers of preparation. Having added to this a drop of dilute acetic acid, we saw the mucusy substances in which were suspended the elements characteristic of semen, dissolve and these spermatozoa stayed intact and were more clearly perceptible than before.

We found, in addition, in the material of these stains placed under the microscope, some rare, prismatic, elongated crystals terminating in a point, reminiscent of magnesium phosphate crystals. As is known, these crystals are often deposited in semen during its cooling after having been ejected by ejaculation. We also noticed some mucus corpuscles and some polygonal, flat cells, provided with nuclei, as are epithelial cells of the urethral canal, elements often carried out in small number during ejaculation.

All these elements being found in semen, and accompanying here microscopic filaments called *spermatozoa* which essentially characterize sperm; these existing in as great a number as on the fertilizing liquid, and furnished exclusively by the genital system of a man having reached the age of puberty, we concluded that:

The gray, pale stains submitted for our examination as possibly being of a seminal nature, are indeed composed of elements characteristic of semen, such as one finds in semen cooled or dried out after ejaculation.

438 We also found mixed with these elements: 1) some microscopic grains, irregular, dark, such as one finds in most dusts originating outside of the human body; 2) some rare starch granules, such as one finds on the surface of many whitened fabrics and in many dusts; 3) polygonal, thin, folded, transparent cells without nuclei, almost without granulation, similar to those which incessantly detach from the epidermal surface of the human body, and most of which remain adherent to clothing applied directly to the skin.

Examination by microscope of irregular brownish stains accompanying the preceding, but which offered the superficial characteristics of stains of fecal matter. Under the microscope the material of these stains showed the elements found in the pale stains described previously as elements of semen. But we encountered a considerably greater amount of the following microscopic particles:

1) Greenish granules, irregular with blunted angles or sinuous contour, such as are contained in bile and fecal matter with which biliary liquid is mixed.

2) Cells and tracheae of vegetable matter, such as most vegetable substances contain which serve as food for man, and which stay in the fecal matter in whose constitution they participate.

3) Yellowish globules and droplets, strongly refracting light, ether-soluble and offering all the characteristics of fat globules, and drops which one finds in a certain amount in fecal matter.

4) Finally, we found a certain number of microscopic bodies, ovoid, about 7 hundredths of a millimeter long, provided with a transparent homogeneous wall, rather thick, the external contour a bit embossed and a regular cavity filled 439
with a granular, greyish substance. These bodies offered all the essential characteristics of eggs of the intestinal worm *ascarides lumbricoides,* which one finds in fecal matter of individuals affected by the presence of these intestinal worms.

By this examination, we were led to conclude that these irregular, grayish stains, considered in the report of the preceding expert as coming from a natural exudate of the genital system of woman, do not contain elements characteristic of these materials, all recognizable by microscope; that they contain the principal elements specific to stains formed by fecal matter; that the stains were actually formed by fecal matter, coming from the anus, cleaned by the shirt after defecation; that the elements of these stains were mixed with those of semen, either because the seminal fluid was ejected onto them, or, on the contrary, that that part of the shirt stained by the semen was stained by the fecal matter.

In addition: 1) the less wear on the tail of the shirt bearing the stains of excrement, compared to the opposite tail, which bore no similar stains; 2) the nature of these stains, actually formed by elements of fecal matter, and not by mucus material coming from the genital system of woman, has led us to conclude that:

The tail of the shirt bearing these stains, as well as the stains of a seminal nature, is not at all the tail of the front of the shirt, but the posterior tail, contrary to indications of the report, following the allegation that these dark gray stains were of a mucus nature.

Study of the chemical reactions presented by the stains submitted to examination by experts. Even though the characteristics previously described left no doubt as to the nature 440
of the stains we had been studying, since we had found the very elements which compose seminal fluid in the human body, we submitted them to the chemical reactions indicated as serving to distinguish different sorts of the suspected stains one from the other; for this we used the portions of stained fabric which we had put aside for this purpose. (See Lassaigne. *observations sur quelques réactions que présentent les taches spermatiques avec les taches albumineuses et autres taches analogues. Annales d'hygiene et de médicine*

légale, Paris, 1858, in - 8°, t. X, p. 405).

The heat of incandescent charcoal acting far enough away so as not to redden the fabric instead of producing a dark nankeen yellow on the stains, as happens when one operates on a white cloth stained with semen, produced a hardly-visible nankeen yellow tint. This might be attributable to the dull tint of the whole tail of the shirt worn for a long time, which showed the stains submitted for our examination.

Cupro-potassium sub-tartrate which, applied to seminal stains on white linen, colors them a greyish-blue, colored what we were studying a violet a bit pale and highly visible, as it colors stains of albumin; this might be attributable to the fact that, these stains being placed near the stains of fecal matter, the fecal liquid portion, which is mucus and albuminous, inevitably infiltrated the fabric; in mixing with the seminal liquid, it modified and masked the chemical reactions without, however, changing in any way the characteristic elements we have described.

Nitric acid at 40° changed the seminal stains, located farthest from the fecal matter, to a pale yellow; this color, at first not very visible because of the pale tint of the fabric, became more pronounced under the influence of heat. On the
441 contrary, this acid changed to a yellow veering toward orange, stains of the same appearance as the preceding, which the microscope demonstrated to us as being of a seminal nature, but which located near the stains of fecal matter, must have imbibed their mucus and albuminous liquid portion.

In summary, the use of chemical reagents furnished us with no new proof concerning the nature of the stains of which the microscope had directly shown us the constitutive anatomic elements.

This is due to the mixture coming from the infiltration into the fabric of the liquid part of neighboring stains, of which the microscope demonstrated the superimposition in places, showing the elements of semen mixed with those of fecal matter in certain places of the stained fabric.

This mixture, which is not rare in cases of an assessment of this type, takes away much of the value of characteristics derived from coloration which the stains show on contact with certain reagents. These characteristics, to which a few authors still attach a certain importance, have, however, no importance in the presence of *spermatozoa.* Their presence is so exclusively characteristic of seminal fluid, that one can claim any stain containing them as being of seminal origin, and one cannot affirm their seminal nature until the existence of spermatozoa has been confirmed.

Moreover, characteristics derived from coloration can only be confirmed if the stains are found on white linen; one cannot produce this coloration when the stains are found on colored fabric; this is what happened recently to one of us (M. Robin) during an assessment in which the only stain to be examined was found on grey wool pants; then, in proceeding as mentioned above, the spermatozoa and other elements of semen were discovered as clearly as in fresh seminal fluid.

Conclusions. The results of our examination have led us to 442
conclude:

That the pale, gray stains, by which the shirt is covered in several places, are of a seminal nature and offer all the characteristics and disposition of stains coming from seminal fluid ejaculation.

That the dark brownish stains, smaller and less numerous than the preceding, and mixed with them, especially with the largest, are constituted by fecal matter, such as would voluntarily or involuntarily escape from the anus of a child.

That the nature of these stains, their situation on the inside of the tail of the shirt which is the dirtier and the less used, show that they are found on the tail of the back of the shirt.

It follows that the seminal stains mixed with them are also on the side turned toward the body of the tail, of the back of the shirt and not on the tail of the front of this shirt.

That these stains, which are of a seminal nature, could have been produced on the tail of the back of the shirt by an ejaculation brought on by the rubbing of the erect penis between the two thighs of a child clothed in this shirt, of which only the tail in front would be lifted.

That the three small seminal stains, which the tail of the front of the shirt showed, could have been produced by semen which remained on the thighs of the child, or by contact with the tail of the back, which was the part principally stained.

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2. Ch. Robin et A. Tardieu. Mém. sur l'examen microscopique des taches formées par le méconium et l'enduit foetal, pour servir à l'histoire médico-légale de l'infanticide (*Ann. d'hyg. et de méd. légale,* Paris, 1857, 2nd series, Vol. 7).

On Semen and Seminal Stains in Legal Medicine*

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417 Determination of the nature of stains has taken on a more considerable importance at the Palace these last years, perfectly justified by the progress realized in this process by chemistry and histology, as well as by the unexpected light it has thrown on famous trials. There is no great risk of being contradicted in affirming that every time a preliminary inquiry must grapple with an intelligent criminal, who appears to have left no trace of his passage, it concentrates its foremost efforts on the investigation of stains and imprint evidence. And this effort is quite often crowned with brilliant success.

By such study, the innocence of the accused can be peremptorily demonstrated; in other cases, the culpability has been rendered evident, circumstances of the crime established or the scene of the murder reconstructed in a more precise and clear fashion than with a witness, often an unknowing child or an unreliable individual. Analysis of stains today is also among the standard procedures, and not the least important, of forensic physicians.

At Lyon, Professor Lacassagne was to annex a perfectly equipped laboratory to his amphitheater, devoted exclusively to this type of research; elsewhere, in lab courses in legal medicine, the students practice special manipulations.

At Paris, Brouardel, Vibert and Pouchet gave great impetus to this research and left us studies of the highest interest on these questions.

418 Blood stains were studied especially, and reasonably so, for if the crude material proof of their nature has today become a sort of game, it is not the same for their *interpretation,* which demands a great prudence and very special wisdom on the part of the expert, so great is the danger in having them say more than they can. Blood abounds, it runs in the streets, a scratch makes it flow and a number of professions cannot be practiced without its blemish. It is not the same for seminal stains, whose presence in frequent cases has an absolutely precise significance and constitutes an irrefutable accusing witness. Their interpretation is extremely easy, for, rather often, the role of the expert consists in saying yes or no as to the presence of spermatozoa. Their identification has been generally considered as very simple, certain exceptions reserved, of course, and I have always reckoned, for my part, that with the precepts decreed on this matter by Roussin, Robin, Lacassagne, Renaut, Brouardel, Pouchet, Gérard and many others (1), in arming oneself, in addition, with a lot of patience and an appropriate microscope, one could always do a creditable job in these affairs. I have in any case considered any addition to these precepts as perfectly superfluous.

I have sometimes had, it is true, considerable difficulties in characterizing certain stains. About ten yesrs ago, Coutagne and myself spent three weeks in isolating a few complete spermatozoa in a case of rape of a four-year-old child. More recently, Lacassagne had a few equally extremely difficult assessments. Notably, one concerned stains dating from a month before, which had been washed by the mother of the little victim herself. It is easy to see that it was impossible to succeed, even though the place occupied by the stain was still visible; the able expert succeeded quite well in isolating some filaments which could have been tails, corpuscles which could have perhaps been heads more or less altered by the washing, but a professional opinion cannot be expressed based on such observations. If, instead of semen, Lacassagne had been working with blood, he would have needed an hour at most to affirm, by the preparation of hemin crystals, that 419 the stain was really made of blood, in spite of the washing, even if it had been more effective. With the reaction of guaiacum, only a few seconds would have been sufficient, if it were negative, to be certain that it contained no trace of blood! After having passed long hours and sometimes weeks in examination of a seminal stain thread by thread, one often arrives at that semi-certainty, as embarassing for the expert as it is useless to the judge.

These reflections caused Lacassagne to wonder if it weren't possible to find a simple and rapid reaction for sperm stains, equivalent to that of guaiacum, and secondly, to determine exactly if, from new notions recently acquired about the anatomy of spermatozoa, sufficient certainty for expressing an opinion could not be attained by examination of a tail or a head as well as of a *whole spermatozoan,* which up to now has everywhere, without exception, been considered indispensable. The results of the research I undertook in regard to this question will now be presented.

The Study of Semen

Semen (σπερμα; seed) is a thick, viscous liquid, a bit flowing, of an odor *sui generis* called "spermatic", and which

* Translation of "Du Sperme et des Taches de Sperme en Médecine Légale." in *Archives d'Anthropologie Criminelle de Criminologie et de Psychologie Normale et Pathologique* 10: 417–434 (1895), continued in the next volume.

has been compared to flowers of a chestnut tree, shredded hoof, sawn ivory, flour dough, gluten, etc. Its consistency is variable: thick, almost a gelatin, in a vigorous man after a long abstinence, it becomes very fluid, scarcely milky, in those who abuse venereal pleasures. In the first case, it is an opaque white, bordering on yellow or grey, almost pearly, clotted, nonhomogeneous, because striations in an uncolored, transparent liquid, appearing dark by a phenomenon of refraction, engulf the masses of gelatin. In the second case, it is rather homogeneous, more or less milky, a little translucent. In a degree of even more considerable impoverishment again, after several repeated acts of coitus in a short period 420 of time, it is transparent and shows only a few small whitish points or striations; at the same time the spermatic odor becomes weaker and weaker and even null. Without knowing the reason, semen can be a pure pearly white, or bordering a bit on yellow which is the most usual hue, or a dull greyish tint, or even, but more rarely, frankly roseate, in the same individual. This last hue is, it is true, sometimes due to blood, but is the exception outside of old age; according to Robin, this coloring substance is formed by vesicles observed by him. Coutagne and myself have had to examine stains of rose-colored semen, and the accused assured me that his semen was always of this color; his interest, however, lay in maintaining the contrary.

Semen is heavier than water: its density can attain 1.037 and oscillates between this number and 1.027 in a healthy man; it is mucilagineous rather than viscous, weakly alkaline. Exposed to air, the thickest semen gradually loses its gelatin-like consistency, becomes fluid and separates into two layers, one upper, transparent and uncolored, the other creamy and white. It is thus that I have seen it in samples I have received from various origins, but certain authors insist that, on the contrary, it becomes thicker. Acetic acid and heat do not coagulate the clear supernatant liquid, but if hot acetic acid is added, the liquid becomes intensely cloudy; likewise, if potassium ferrocyanide is added. I will return to this.

Alcohol immediately gives a white precipitate, milky at first, then curdled. The precipitate, examined by microscope, is very finely granulated, engulfs spermatozoa and sometimes large crystals.

In drying, on glass for example, semen leaves a translucid mass of a fatty, granulated, yellowish appearance; if water is added, the primitive semen is not changed, as has been claimed, due to most of colloidal substances having become insoluble. There remains an abundant, clotted, dull grey, unflowing residue, not more than the supernatant liquid which is scarcely cloudy after being left alone. It is also said that the odor of semen returns under these conditions; I have never confirmed it, even with the help of low heat. Moreover, 421 there is a lot to say about this odor, which I have never found in any of the various animal semens which I could appropriate. It is said that addition of blood to dry semen regenerates the primitive odor, and it is affirmed that it is due to spermine itself, as will be explained further on. Semen of spermatorrhea and of sudden death has no odor.

According to certain authors, its flavor is peculiar, very bitter; according to others, simply a bit salty or flat. It is the last which appear to be true, but it is possible there are variations. Semen, in effect, is a very complex liquid, formed from products of secretions of several glands, each secretion having its very distinct characteristics which the limits assigned to this work do not permit me to present in detail. They are:

1) *Testicular secretion,* formed almost exclusively by spermatozoa, thick in bulk, or pasty, and semi-liquid, sometimes creamy, dull white, opaque, or bordering a little on yellow, non viscous, *without odor.* According to Robin many spermatoblasts are found.

2) Liquid of the *sinus of the vas deferens,* brownish or yellowish grey, with various granulations and numerous epithelial, prismatic cells.

3) The liquid of *seminal vesicles, dense, without odor, alkaline,* clotted or creamy, unflowing, sometimes gelatinous, yellowish grey, semi-transparent, not opaline. In the elderly it is reddish brown, and sometimes contains red blood cells. It appears that this is the first liquid ejaculated in infancy and the last in old age. After repeated coitus it forms almost the totality of semen. It contains polyhedric epithelial cells, leukocytes, *drops of an oily appearance,* coloring strongly with reagents and especially with crocein which permits recognition of the bizarre forms which the drops under the top slide take on, stretching out in every direction. Finally, curious round or cylindrical concretions of a diameter varying from 1/100 of a millimeter to one or even two millimeters, according to Robin who has described them at length, and called "*sympexions*". They are uncolored except in the elderly where they are sometimes roseate, 422 rather often branchy or areolate.

These granulations, noted in all the treatises of legal medicine and which can perhaps be confused with the amyloid granulations of certain German authors, have never been used, that I know of, in the diagnosis of stains, and I will not discuss them further. However, I will recall that the most voluminous often engulf spermatozoa, which can be easily isolated with acetic acid.

4) *Prostatic humor,* or "prostatine of Blainville", is a fluid liquid, neither viscous nor flowing, milky, opaline, cloudy giving a weakly alkaline reaction. According to Robin, it has no odor, but Fürbringer, who recently performed a good study on fifty-one cadavers and twenty-one living adults, constantly found the specific odor of ejaculated semen. It is composed of an uncolored, clear liquid holding very fine granulations in suspension, amyloid according to Fürbringer, fatty droplets, flat and cylindrical epithelial cells, grouped in strips, hyaline balls due perhaps to colloidal degeneration of *epithelium;* finally according to Fürbringer, some constant and characteristic elements, uncolored, refringent, round or oval, rarely angular, to which is due the cloudy appearance of the solution; the largest have the diameter of a red blood cell, whereas there are some exces-

sively small endowed with Brownian movement. According to Robin, it is to the product of the prostate that semen owes its appearance, and though he asserts, in contrast to Fürbringer, that it has no odor, he admits that it is, by its mixture with other humors also without odor, that spermatic odor develops. This is why it is lacking after repeated coitus and the sperm becomes greyish, not very opaline and clear.

5) Humor of the *prostatic utricle* ("*male uterus*" of some others) is insignificant in relation to what interests us and contributes only very little quantity to the formation of semen.

6) That of the glands of Méry (Cowper's gland) is more interesting, for it is often so abundant that, during erection, it wells up in a liquid, limpid-like crystal, viscous, stretched
423 out in threads, salty, alkaline, without odor. This humor often forms starched stain, in drops, but difficult to determine, for up to now, the specific principle has not been found, and besides it contains absolutely no morphologic element. I except from this however a few uncharacteristic epithelial cells.

Histological elements of sperm

1) Spermatozoa (σπερμα and ζωον)

(*Animalculi e semine, vermiculi minutissimi* (Leeuwenhoek, 1677) *Filamenti spermatici—Vers spermatiques* (Spallanzani); *Spermatic Animals* (Procope, 1755; Needham, 1750 and Spallanzani); *Minutae bestiolae* (Halter, 1765); *Spermatozoa* (Duvernoy, 1841); *Spermatic Filaments, Seminal Filaments* (Henle, Koelliker); *Zoosperm* (various authors); *Spermozoaires* (Bory de Saint Vincent); *Zooblastes, nematospermes, nêmospermes* (Bory de Saint Vincent); *spermatozoaires, entozoaire of sperm or spermatobies* (Baër); *Treniadosa pseudopolygastrica* (Ehrenberg); *Macrocerus, of the Cercozoa family* (Hilt); *Cephaloides* (Czermack): *Microscopic cercaria or cercaria of semen* (Cloquet, 1827).

Samenkorperchen (seminal corpuscles) (Schweiger-Seidel); *Samenthierchen* (Koelliker, 1841) *Spermatozoid, Spermatozoon, Spermatozoa, Spermatic particles* (English).

Without wanting to repeat here the well-known history of this question, I will recall however, that it is in a letter dated November, 1677 and entitled *Observations on the small animals of human semen* that Leeuwenhoek first made known the spermatozoa. Ham[2], supposed to have been one of his students, had observed them living in the semen of the nocturnal pollution of a patient with gonorrhea, and hastened to share his discovery with the illustrious professor. He told him that he had already seen them, but dead, after
424 injection of turpentine into the patient. Leeuwenhoek looked for them again and found them in the semen of a great number of vertebrate or invertebrate animals. He compared them to tadpoles of frog, and believed that in man and dog there are two types, perhaps of different sex[3].

He confirmed that they come exclusively from testicles, and an important point, *that they don't come from putrefaction,* as many other small animals, infusoria, for example, a point of great interest then. Nonetheless, for many years they were only considered as foreign animalculi and this is, I suppose, the reason why it is only in recent years that the presence of spermatozoa was considered as the essential characteristic of seminal stains, and acquired a legal value. Procope, in a little book, which moreover, he did not sign (*The Art of Making Little Boys*), pleads the question rather spiritually, and believed that he had proven that spermatozoa are only the accessories, the accidents, so to speak, in semen. He says that Hartsoeker (1678?) a contemporary of Leeuwenhoek, had remarked that semen obtained after several ejaculations contained no more spermatozoa; the semen was, however, not less fertile; as proof, the numerous disappointments incurred by those who speculated on Hartsoeker's discovery. . . . For Buffon, too, spermatozoa and infusoria were of the same origin, or almost, for he often seems to confuse them. Despite the works of Spallanzani, it was in reality Prevost and Dumas (1824) who definitively demonstrated that it is not the odor of semen—the *aura seminalis*—which is the fecundating principle but the spermatozoan. A simple reading of names given to spermatozoa by different authors—names which we reproduced above—is as convincing as a long history as to the ideas they had.

Spermatozoa are filamentous anatomic elements found 425
exclusively in semen; they are uncolored, hyaline, inflated in man and most higher animals in one of their extremities, commonly called the *head,* tapering into a long, extremely tenuous cilium, endowed with its own movements, called *tail* or *flagellum.* They are quite rightly compared in form, and also in movement, to tadpoles of the frog, but their tail is proportionately much longer and finer than that of tadpoles.

The relative and absolute proportions of spermatozoa of man are very fixed, more fixed, in any case, than those of any other anatomic element, blood cells or pus, for example. Also, contrary to what has been professed up to now, the fragment of spermatozoa most easily found in old stains, the head, can by itself, in my opinion, if studied well, and rigorously measured, give absolute certainty as to the presence of sperm.

In relation to the technique of assessment, I will note the influence certain coloring reagents have on these dimensions and I will specify more rigorously the different dimensions obtained. The total length of the spermatozoon of man, more difficult to rigorously measure due to the extreme tenuousness of the end of its tail, seems the only part to vary appreciably; it is between 0.048 mm and 0.058 mm. Dujardin notes that the caudal filament represents quite exactly 9/10 of the total length, say 0.050 mm, whereas that of the head is 0.005 mm. As an average of numerous series of measurements, designed exclusively to find out the ratio of head to tail, I found the head having four divisions of the micrometer, the length of the tail varying between 37 and 45, with the great majority being between 40 and 41. The length of the head is of extraordinary fixity, when it has not been deformed by accident, and I do not believe there are

variations greater than 0.0003 mm, more or less, which is within the limits of precision of our instruments, for the markings of ocular micrometers are too rough. The width of
426 the head, seen flat, is 0.0035 mm; its thickness cannot be determined; face on, it is pyriform, and towards the point about 0.0015 mm, toward the base about half the width.

The tail measures, toward the head, i.e. the middle segment, 0.001 mm thick, then it *regularly* thins to end in a point so tenuous that is difficult to perceive the end with the best instruments if some type of article is not used.

Spermatozoa are always uncolored, even in colored sperm, strongly, but unequally, refringent, hyaline. They *appear* perfectly homogeneous in the whole length of their tail, a bit granulated in the head, which is transparent when flat, and permits seeing the granulations which it can screen. At first sight, it appears formed by a single gelatinous substance, but it will be seen that different parts absorb coloring reagents differently which proves they don't have the same chemical constitution. I have tried, uselessly up to now, to employ polarized light in the study of their structure, for the identification of fragments in the analysis of stains.

The *head* or disc of the spermatozoon presents itself in varied aspects, which it is of consequence to know well. It is represented in treatises and articles of legal medicine, almost without exception, in the form of a pear, the small extremity directed toward the front. This is wrong, for when extracting spermatozoa from a stain at least as many which present their head *face on,* i.e. with the aspect of *regular oval,* are found. I have often seen beginners fail to recognize them thus, such was the classical figure fixed in their minds. Something to note, when very rare spermatozoa are extracted from a stain, when only one or two are found, I don't know why, but they always present themselves precisely face on. I made this observation a long time ago. The head seen face on is quite regularly oval, and often shows towards the union of the posterior one-third with the anterior two-thirds a swelling corresponding to the transverse line of separation about which I will speak.

When semen, even from a stain, is treated with a solution
427 of crocein[4], which of all the numerous reagents which I have tried has given me the most satisfaction, the head of spermatozoa is seen cut just about in the middle, or closer to the base, by a transverse line, separating a little-colored, transparent part from a posterior part strongly impregnated. This line is sometimes distinct, as cut with a knife, but in stains it is sometimes shadowy, blurred, as photographers would say. It is not always straight; I have seen it curved in certain cases concave to the rear, such that the anterior clear part covered the rounded posterior part like a sort of skull cap or crescent. At other times it is oblique or irregular, which must be an accident of alteration or deformation. This separation is variable according to species, and can serve to differentiate sperm of various origins. In man, in seminal stains, the appearance resembles exactly that of an acorn of oak enclosed in its cupula (see Fig. 1) [Note: Figure is not reproduced in this translation]. The contents of the dark part is granulated and with the use of double staining, it is confirmed that the head is formed by an envelope which eosin colors poorly, and a nucleus it colors well, whereas the iodated solution of Roussin and crocein perfectly color this very thin envelope.

The head of the spermatozoon stained with crocein presents a small, brilliant, refringent point, always situated in the clear anterior part (Fig. 1). In moving the tube of the microscope very slowly, one is convinced it is a small *vacuole,* the diameter of which is essentially that of the tail of the spermatozoon; it is sometimes oval, the large axis directed transversely; more rarely there are two, smaller and unequal. This small vacuole was not acknowledged by Ballowitz in the work (*Centralbl. f. physiol.,* 1891) which he recently devoted to the anatomy of the head of the spermatozoon, and I found only one author who said anything about it: Rollin, a fastidious observer.

After having noted that the head can be placed in such a 428
way that the depression in the form of the hollow of a spoon in one of its faces can wrongly be taken for a nucleus, he says, "this so-called nucleus must not be confused with one or several clean, yellowish vacuoles which form more or less early after the cadaveric death of the spermatozoan in the thickness of the disc". This is effectively what I believed, before making the acquaintance of these lines of Rollin, and my friend Vialleton supported me in this idea; but I should have returned to it since I had positively seen this vacuole on living spermatozoa. Little of the rest is of consequence to me; what is of the greatest interest to me is that this vacuole is almost constant in spermatozoa retrieved from stains and stained by crocein.

All these characteristics, combined with the rigorous measurement of the head in length and width, are such in my eyes that I will affirm in all tranquility the presence of semen in a stain by inspection of *one single head*—whereas, I have the conviction that there is the possibility for any number of errors in the idea, accepted as dogma, that a professional opinion can be expressed if one single spermatozoon is complete (that is, a head with a tail), seen as it is recommended to study them, with magnifications that are too weak, where the head is a point and the tail a striation!

There exists no anatomic element which has any resemblance whatever to the head, appropriately examined.

Valentin, Jensen, Furst, Brunn and Ballowitz described a hat covering the head of the spermatozoa. I have only been able to observe it once, and this at a time when I didn't yet know it was a constant organ in the spermatozoon before maturity. I will then say nothing about it. At the point of the head of the spermatozoon of man, there is positively a small, brilliant point and there exists a similar one toward the tail; but they are not very visible, even with crocein. It is otherwise with animal sperm.

What causes the separation of the head into two parts is that the anterior part of one of the faces is hollowed out like a spoon, as can be confirmed when it is presented in a three quarter view. Thus seen, this excavation which can give the illusion of a nucleus by refraction, as already pointed out by

Robin, is readily evident; the other side, on the contrary, is a bit bulging. Seen in profile, the head is pyriform, the large extremity directed toward the tail, the point toward the front, thin and rounded, sometimes inclined to the side in a sort of beak, or even hook. It is the classical figure, which has interest, not only because it is thus the spermatozoon is seen in life, but especially because those of animals do not have the pyriform aspect in this position. They are seen as a small rod, lightly attenuated at the two ends. This is an excellent distinctive characteristic to which I will return.

Insertion of the flagellum is not in the axis of the head, but encroaches on one face, like the handle of a spoon; I have said a word about the brilliant point seen there. The insertion seems *almost articulated;* in reality, there is no cavity, as in the dog, but most often (I am discussing sperm from stains) a slight protuberance ending in a facet, which is not always as pronounced, however, as appears on one of the isolated heads.

The tail or flagellum of the human spermatozoon has been described for a long time as a simple thread. This is effectively how it presents itself in ejaculated semen, if examined with the strongest magnification, and even after the action of coloring agents. But the fact that this flagellum is capable of movement already demonstrates that it cannot be homogeneous, and that its structure is complicated. It is to the works of Dujardin, Henle, Grohe, Koelliker, Ankermann and Schweiger-Seidel for an initial period going up to 1865 that is owed the first notions of the histology of the tail of the spermatozoon. The last of these authors had already at this time distinguished a *head* (and in certain cases a *hat* (Koppfkaffe), a *middle segment* (Mittelstuck) and a *tail* (Schwanz). The middle segment had as its essential characteristic that it was cylindrical, i.e., the same thickness in its whole length, to have a particular luster and a constant length in each animal species. Schweiger-Seidel gives the following table which reports the respective lengths of various animals.

Average length in millimeters

	head	middle segment	tail
mouse	0.008	0.023	0.085
hedgehog	0.006	0.009	0.065
"maulwuye"[1]	0.008	0.020	0.055
guinea pig	0.012	0.010	0.080
rabbit	0.007	0.008	0.041
ram	0.008	0.015	0.055
pig	0.009	0.011	0.040
"inuus"[1]	0.006	0.011	0.043
man	0.005	0.006	0.040

[1] [meanings could not be decided, nor could the original articles from which the table is said to have been taken, be located]

Dujardin (*On the zoosperms of mammals. Ann. nat.* **vol. VIII,** 1837) was the first, I believe, to have studied the accessories of this middle segment; a little later, this study was resumed in Germany, where *nodules* (*Knotchen* of Schweiger-Seidel) and strips (*laeppchen*) were described. Grohe at first, then Schweiger-Seidel, thought they saw two small cavities (Lucken, Vacuolen, Hohlungen of varied authors) serving as a sort of hyphen between the middle segment, the head and the tail. The last author gave a design which clearly represents these two cavities, and on the same plate are spermatozoa whose middle segment presents curious transverse striations, which the author considered as phenomena of alteration. He remarked, in addition, that during the movements of progression of spermatozoa, movements which from that time were the object of much research, the middle segment, as well as the head itself, remained absolutely passive.

The second period was marked by Eisner in 1874 (*Untersuch. über den Bau der Samenfaeden. Verhandl. der phys. med. geo. Zu Wurzburg* **(vol. 6.** 1874). This author discovered what he called the *Centralfaeden,* a name which Brunn replaced with *Axenfaeden,* axial filament. This filament traverses the flagellum of the spermatozoon in its entire length, in a state of extreme tenuousness, Eisner remarked, without being too certain of it. He presumed that the end of the tail (or tail proper) must be formed exclusively by this filament, whereas the middle segment was made from small cubes strung on the filament like little pearls. In 1879, Heneage Gribbes (*Quart. journal of micro. science,* **vol. XIX,** 1879 and **XX,** 1880) discovered a spiral thread which made six or seven turns around the middle segment in mammals, as had been seen long before in the salamander. Jensen confirmed this discovery (*Die Structure der Samenfaeden, Bergen,* 1879) and asserted that this spiral thread is specific not only to the middle segment, but is found also on the third segment, the tail, and pointed out that the filament has a different chemical constitution from that of the spiral thread in the middle segment, but not in the tail, where the two filaments behave the same in the presence of reagents.

Retzius (*Zur Kenntniss der Sperm. Biol. Untersuch.* 1881) finally discovered that the third segment, the tail proper, is itself formed by two distinct parts, one he calls *Hauptstuck, the principal piece,* the other, *Endstuck, terminal piece* or *terminal filament.* He called the middle segment of Schweiger-Seidel, *Verbindungstuck,* joining piece, and believed that what was described as a spiral thread was only a sort of spiral hem around the flagellum. Since then, this question has impassioned anatomists. Brunn (*Archiv. f. mikr. Anat.* **B. XXIII,** 1884) carefully described the fine, regular transverse striations of the middle segment in the mouse that Leydig (*Unters. Zur Anat. und Hist. der Tiere,* 1883) had already acknowledged as a spiral thread, but Brunn could not determine if he was dealing with a spiral thread or simply transverse striations. Kraun (*Der Spiralsaumseder Samenfaeden. Internat. Monatsch. f. Anat. Hist.* **Vol. II,** 1885) was more assertive, and Plattner (*Uber d. Spermatoz. bei den Putmonaten. Archiv f. Mikr. Anat.* **Vol. XXV,** 1885) observed, in his turn, that the middle segment in bulls is spiraled, thus verifying the views of Jensen and Brown (*On spermat. in the rat. Quarterly Journal of Microsc. Science,* **Vol. XXV,** 1885). In treating spermatozoa with gold chloride, he observed a dark, spiral thread which surrounds a little-colored internal substance. Furthermore, Plattner seems to have seen two threads; in any case, one of his designs (Pl. XXIII, Fig. 18) indicates two.

Up to then, no one had seen the unrolled spiral thread. Jensen, at first, Ballovitz, Prenant of Nancy[5] and others after them, proved its existence without doubt. According to Jensen, it suffices to collect spermatozoa from rat testicle, and simply add to them 0.6% saline solution—and even without this addition—to clearly see a regular striation, where each stripe is separated from its neighbors. Toward the end of the median segment, these stripes separate more and more such that they become more and more oblique in relation to the axis of a segment. If the tube of the microscope is lowered, to focus the deeper parts, it is seen that below, the stripes have an inverse obliquity, which proves that it is a spiral thread, and not rings, forming these striations. Putting the spermatozoa in aqueous glycerine (at ⅓) the spiral thread separates. Acetic acid at 1% also isolates it, but this reagent has a destructive action and soon fragments the thread; in certain animals the destruction is slower, and the curious action of this reagent can easily be observed.

Whereas the spiral filament is homogeneous, of fine, clear contour, very slightly refringent, the axial thread (or "axe file") is thicker, very refringent, terminating toward the head in a small button even more refringent again, the spiral filament beginning just after the button. Ballowitz, who has performed lengthy studies on this question, established that the axial filament is formed by two fibrils, each ending in a small button, and he could sometimes observe three or even four, on sperm taken from the epididymis. In the rat, this axial filament shows itself as a packet of twisted wicker, and he could count up to seven well-isolated fibrils in the middle segment. He concludes from his research that this axial filament is formed by two bundles of fibrils joined by a *cement,* traversing the whole spermatozoon and appearing in the free state only at the neck and tail.

In summary of all these works, the spermatozoon is formed by a head and a flagellum; its complete length is traversed by a complex axial filament, which is uncovered at its union with the head where it forms the *neck,* and at the extremity of the tail (Endstuck). This total length is divided first into a *middle segment* (to which is suited the name "body", it seems), cylindrical, formed by a spiral thread rolled in a certain number of turns; then into a *tail* proper, divided into two pieces, the first, principal piece (*Hauptstuck*) which tapers, is also constituted by the rolling of a spiral thread around the axial filament, then this, existing uncovered from the principal piece, forms the *terminal filament* (Endstuck). A curious appendix is inserted in the middle segment; it is the coat, the protoplasmic coat, in the form of a thin, granular, transparent membrane, inconstant in the mature spermatozoa. This membrane is sometimes in an irregular strip, sometimes in the form of a trumpet directed toward the head and encroaching on it.

Up to now, alas, all these interesting notions have not been able to be used in legal medicine, because these delicate structures are only visible in sperm from testicle, the epididymis, or *vas deferens.* As soon as the sperm is ejaculated, the spiral filament is welded so strongly to the axis that all is perfectly homogeneous; this is the opinion of Brunn, and Jensen is not far from agreeing. However this point of view cannot be accepted, for the spermatozoon, having reached maturity, is endowed in its flagellum with rapid movements, which necessarily implies a persistent fibrillary state; a perfectly homogeneous mass could not be endowed with movements in its parts. I do not despair in finding a reagent, not to see all the details of structure which I have just presented, but strong enough to permit at least recognition of segments, for these are not of the same chemical nature. In heating dry semen, kept on a glass slide, to 40° for a few hours in a humid room, and then in treating it with saturated solution of crocein, I have seen the middle segment of separated tails enlarge considerably, curve itself and clearly present the axial filament, having the appearance more of a fine cavity than of a thread. Then the incurvation gradually continues, in proportion to the thickening of the segment, and its two ends finally join and weld together. This strange phenomenon takes more than an hour to finish; the segment then presents the appearance of a disc with a central depression and a very fine circular streak, representing the axial filament, in the middle of the radius.

Moreover, the protoplasmic *coat* is often found, whose resistance to chemical agents is considerable; next, what Schweiger-Seidel described under the name of *nodules,* and which are only forms of alteration of the middle segment, or all the elements of the spiral filament which are visible. Frequently in sperm extracted from stains and treated by crocein, these small swellings of vague contour, four, five and more in number, generally of unequal size, are found on the segment. These small successive swellings, which give a winding appearance to the spermatozoon, have been described by Robin.

to be continued

References and Notes

1. See the general bibliography at the end.
2. I find this name written in various ways.
3. This hypothesis is not absolutely implausible. It was upheld in 1836 with brilliance by Sielold, then by Fraisse, and recently in a masterly article, Brunn demonstrated that Paludina vivipara has two sorts of spermatozoa. (Archiv. f. microsc. Anatomie, 223, p. 413) My friend R. Koehler also described the two forms of spermatozoa of *Murex brandicus* and *trunculus* (*Comptes rendu,* 1888).
4. There exist many different agents which, in the trade of stains called "aniline," bear this name; the crocein I use is furnished to me by the maison Stéphan Girard, of Fontaine-sur-Saone.
5. Note on the *Structure des spermatozoides chez l'homme,* 1888, and Rem. on the *Structure des sperm.* (Revue du Nord de la France), Lille, 1888-89.

Semen and Seminal Stains in Legal Medicine*

(Sequel)

Dr. A. Florence

Second Part

Technique of examination of stains

37 **History.** It seems to me quite difficult to admit that past experts didn't make any use of confirmation of sperm in cases of rape or of impotence, yet I found practically no proof of it, for various reasons: the library at the law faculty in Lyons, recently founded, is relatively poor in old documents of this type; that of the medical faculty, on the contrary, has quite a large number of old treatises on legal medicine, all of which I perused with great care, but without finding one single fact where a seminal stain played any role; most often, the word was not even pronounced. The reason is that up until recent times intervention by respectable matrons was considered much more appropriate in affairs of this type than by physicians, and, besides, much experience was readily attributed to them in this kind of thing. A decree of Innocent III[1]—this same pope who was the first to recommend the examination of the corpse by a physician in murder cases, and introduced this custom into canon law—confided to these matrons the examinations concerning impotence. It was they who sovereignly judged in the proof of sexual union, and it is known that they had to determine if there had been intromission, *et an fuisset emissio, ubi, quid et quale emissum.*[2]

38 Unfortunately they have left us no report on the procedures they followed. It's a shame, for it must certainly have been quite interesting.

During the first period of the Middle Ages, the violated woman presented herself at the court with her torn clothing, and showed the very traces of the awful treatment to which she had been submitted (Pardessus, *Commentaires de la loi salique,* p. 567).

The ancient Fueros of Spain admitted the intervention of matrons: "In regard to this woman who complained of having been forced, if the act took place in the fields, she had to throw down her cloak in the first city encountered, and lie on the ground saying: Such and such a man forced me, if she knew him; if she did not, she gave some information about him. If she was a virgin, she must show proof of rape to the most reliable woman she could find . . . if she has not thusly acted, her complaint is not whole, and the accused can defend himself." She could also prove the crime by intervention of two men, or by one man and two women of honor. (A. du Boys, *Histoire du droit criminel en Espagne,* p. 136). This dramatic procedure, where the violated woman presented herself with her tears, her torn and bloodied clothing, endured for a long time, and it was not until Alphonse X, that she could bring her complaints to the steward of the king (*Idem,* p. 399).

In France, in response to the ridicule thrown at the congress in the famous suit by the marquis of Langeais (in 1659), who, even though declared impotent, had seven children by Diane of Navailles, the Grand Chambre definitively abolished this singular procedure. But the role of the matrons was not stopped by that: on the 25th of June, 1707, a decision of the sovereign council of Alsace enjoined persons declaring themselves to have been *forced or raped* to appear before the matrons.

Much closer to us, Jousse, an authority on these matters for a long time, indicated, in 1771, admissible proof of violence done to a woman or girl.

These proofs, he said, derive from:

1) The testimony of witnesses who saw the violence.

2) Circumstances of the fact, e.g., if the woman's cries were heard, in relation to the violence being done her, or if she was heard to cry for help in a remote place, where the voice made itself heard with difficulty, especially if the person claiming to have been violated is of good reputation (Julius Clarus). 39

3) But the sole declaration of the girl who asserts that one has done violence to her is not sufficient proof (Julius Clarius). All the more reason is she not credible if it is proven that, since the rape, she voluntarily abandoned herself to him or that she leads an ill life.

4) Boérius holds that a young girl is not presumed violated if pregnant(!)[3] (Jousse, vol. III, p. 751; 1771). It is quite necessary to admit that the jurisconsults of that time had profited very little from the words of Voltaire.[4]

It is evident that no one imagined making use of the confirmation of sperm, for even the signs of deflowering did not intervene, even though perfectly known; but it was just as well known that a woman could have been deflowered without appearance of these signs, and also "that she could have her virginity with the supposed marks of deflowering".

* Translation of: "Du Sperme et des Taches de Sperme in Médecine Légale". In *Archives d'Anthropologie Criminelle de Criminologie et de Psychologie Normale et Pathologique* 11: 37 46 and 146 165 and 249 265 (1896). (Contains the second, third and fourth parts of the article, the first part of which appeared in the same journal, vol. 10, pp. 417 434).

medicine had drawn so much mistrust in assessments that it was preferred to forego it. Stains were, likewise, totally neglected for many years, and even though Sue, professor of legal medicine at the Faculty of Paris since the year VII had done an ingenious investigation on blood stains, Orfila did not say a word about it in his celebrated *Traité des poisons,* 2nd edition in 1818.

In his *Lecons de médecine légale,* published in 1823, he treats procedures which must be used to determine if the confirmed deflowering is the result of introduction of the male member or another body; he does not indicate the confirmation of semen in the vagina of the victim; nor does he speak of it when he considers death attributed to rape. He is forced most often to leave the question unresolved as he does in assessments 1, 2, 3 and 4 which he reports, assessments 40 where the most elementary examination by microscope would have peremptorily resolved the question. Finally, in the case of impotence in a man, he did not have recourse to the microscope. Of the rest, Poilroux, in his *Médecine légale* published in 1837, does not behave otherwise, and it is only in his 1848 edition of his *Médecine légale* that Orfila finally indicates the confirmation of spermatozoa in cases of this type.

In 1826, Ollivier d'Angers and Barruel had to examine stains in an affair of rape (*Journal de Chimie Médicale,* 1826, p. 565). These experts heated the stains, which were rose, and determined that they emitted no odor of fat. They moistened it with water, and rendered it milky by shaking; alcohol, added to the maceratum, clouded it even more. The experts then concentrated the liquid, and noted that it turned litmus paper blue; after total evaporation, the liquid left a yellowish residue, and gave a strong animal odor on calcination. The authors concluded that the stains *could have been* the result of the applicaiton of semen to the surface of material due to three characteristics of the spermatic liquid:

1) being partially soluble;

2) leaving a residue which made the material sticky;

3) being alkaline.

At this time, numerous experiments were attempted to try to find spermatozoa in the stains, notably by Orfila, but without success; this was undertaken without much hope, for the spermatozoon was generally considered as ubiquitous, as some type of infusoria. The main experiment of Spallanzani who had demonstrated that filtered frog sperm (and consequently supposed free of spermatozoa), and much older of Hartsoecker, who, a little while after the discovery of the animalculi, found that semen obtained after numerous ejaculations, in which he had not observed spermatozoa, would produce fecundation, were still the authorities in the science, despite the beautiful work of Dumas and Prévost. (1824).

41 Animalcules whose imperfection hardly permitted them to be distinguished from spermatozoa, were also observed; their origin, however, had nothing in common with sperm.

Thus, in the dictionary of Medical Sciences of Panckouke, in the article *Sperme,* appearing in 1821, the animalcules are claimed to have been found in liquids other than human semen, and it is claimed that Spallanzani fecundated frogs with semen without spermatozoa, and this sufficed to destroy the system of Leeuwenhoeck. Likewise, the article *Génération,* appearing in 1817, points out that cercaria found in semen appear extraneous to fecundation, contrary to the opinion of Leeuwenhoeck, of Hartsoecker, of Valesnieri.

The belief in *aura seminalis* endured for a long time, even with forensic physicians. And it is not astonishing that Devergie in 1839 wrote the following observation concerning the examination of the semen of two brothers, both without children, and of which the spermatozoa (?) were of a peculiar form, ovoid corpuscles presenting movement:[5] "If analogous facts in sufficient number were observed, one would perhaps be able to enlighten the question of the cause of fecundation: to determine whether it is accomplished by means of spermatozoa or if, on the contrary, the hypothesis of an *aura seminalis* has some basis."

Orfila presented his research on seminal stains to the Royal Academy of Medicine, at its meeting of Aug. 25, 1827, and concluded it is not possible to find cercaria or animalcules in these stains by microscope. The same year (*Journal de Chimie Médicale,* vol. III, 1827) his important article appeared: *Semen considered from the medico-legal viewpoint.* The illustrious scientist was called to give his opinion on the report of a physician who, on examination of the sexual organs of a young girl of 13 years, 9 months, who had allegedly been raped nine days beforehand, concluded that the rape had been consummated and claimed to have retrieved a certain quantity of semen from the vagina. Orfila, observing that science possessed no adequate means for facilitating the solution to the problem, affirmed that it was unlikely that semen had remained nine days in the vagina of this girl who was afflicted with a mucus discharge, and the 42 accused was acquitted. This assessment convinced Orfila that he should concern himself with the methodical investigation of the diagnosis of seminal stains. Being aware of the physical characteristics, he indicated the following procedures as a technique of examination:

1) Sperm stains brought close to a flame become a tawny yellow and this tint disappears if the stain is immersed in distilled water for several hours. This was, for Orfila, the most important sign.

2) The stains moisten fully, which does not happen to fat stains;

3) The stains macerated and pressed by a stirring-rod become viscous and *emit a spermatic odor* when compressed between one's fingers;

4) The liquid, filtered and evaporated at a very low heat, becomes alkaline; it shows during evaporation the viscous appearance of a solution of rubber, does not coagulate, but deposits a few glutinous flakes, and its consistency becomes so particular that it is difficult not to accord an importance to this characteristic;

When dried, it leaves a semi-transparent residue, shiny, of a tawny color. Put in water again, this residue separates into

two parts; one glutinous, yellowish grey, adhering to fingers like glue, insoluble in water, soluble in potassium; the other, soluble in water.

5) The solution gives a white, flaky precipitate with chlorine, alcohol, lead acetate, lead subacetate, and mercurous chloride; *pure, concentrated nitric acid* gives it a light yellowish tint, if it is uncolored, but does not cloud it, whereas all the other morbid vaginal discharges become cloudy. Alcoholic tincture of gall-nut gives an abundant greyish deposit.

Orfila, speaking of microscopical examination, adds: "It is easily understood that no benefit can be derived from microscopical observations for the recognition of stains. The animalculi are not more appreciable if after drying the semen on material, it is diluted in water for examination by
43 microscope." But he remarks that spermatozoa coming from stains on glass are more visible and he claims to have *"recognized them perfectly in semen dried for eighteen years"*. Orfila does not seem to accord a great confidence to the verification of spermatozoa, for he says that "the existence by itself of animalcules of this form (and executing very marked movements) in the extreme case permits the attestation that the *solution* submitted to examination is semen, since no other liquid is observed *with these characteristics.* However the physical and chemical properties which I have already mentioned *must be looked for in this solution.*"

This page, in which Orfila showed justified prudence, was strongly reproached in 1839 by Devergie in the violent discussions they had on the subject of priority of the use of a microscope in examination of these stains.

In 1834, Chevallier had to examine suspected stains. He did not use the microscope, but operated, in following Orfila's procedures a little, by comparison with seminal stains; he was not pleased with these procedures, especially the action of heat, of which was made great account, having found nothing clear and he was prudently forced not to reach any conclusion.

Devergie did an assessment in 1834 (*Médecine Légale,* 2nd ed., 1840, p. 387) and relied on the yellow color and absence of the smell of burning which the heated stain presented; on the spermatic odor which developed only the following day; on the starched state of the stains after washing with water, dessication, and finally nitric acid. These are the only procedures indicated in the 1837 edition, vol. II, p. 181, of his *Médicine légale,* and they scarcely differ from those of Orfila.

These investigative procedures were a great success, and were practiced for a long time, especially in foreign countries; thus, in the *Praxis* of Fredreich (1855), the author at first presents the procedures of Orfila, then for just as long, those of Devergie, but, something curious, in this very important book, which had several editions, he did not say a word about microscopical research of spermatozoa in the edition (2nd) of 1855, where Friedreich devoted numerous pages to the study of stains.

In 1838, Devergie read a very important memoir entitled:
44 *New signs of death by hanging,* at the Academy of Medicine, in which he used a microscope to confirm the existence of spermatozoa in the urethral canal of those hanged. Before this, he had established, in the affair of the murder of Tessier, in collaboration with Turpin, that no act of pederasty had taken place, for there were no spermatozoa in the urine emitted by the victim before death.† Finally, Devergie announced in this memoir that he had *been able to confirm spermatozoa in semen stains existing for ten months on cloth, a fact that much more important, since the means provided by chemistry for recognizing stains don't have all the certainty one has the right to expect in a medico-legal analysis.* It is, then, Devergie, and not Bayard, as one often writes, who deserves the merit for this discovery. But Devergie had been preceded by almost twelve years: in 1827, Lassaigne, having recounted to Chevallier that he had been the first to retrieve spermatozoa from a stain, Chevallier informed him that Lebaillif, in the affair of the rape of Contrafatto, had determined the nature of seminal stains in this way. Lebaillif did not publish his report, but there was no doubt as to the priority of his discovery: he had a great reputation as a micrographer and all studies of this type were addressed to him. It was thus that Orfila committed himself to find a method for determination of blood stains by microscope (*Journal de chimie medicale,* 1827).

In March, 1837 (*Journal de chimie médicale*), Ratier, in macerating materials stained with semen in watch glasses, and in submitting the liquid to microscopical examination, succeeded in retrieving the spermatozoa and pointed out on this subject the advantages which legal medicine might derive from this mode of investigation.

In 1838, without acquaintance of Ratier's note and even less of the appraisal by Lebaillif, which, not having been published, remains a dead issue, and before the memoir of Devergie had appeared, Bayard deposed at the meeting of the Society of Hygiene and Legal Medicine his beautiful work entitled: *On the Use of the Microscope in Legal Medicine.* (*Annales d'Hygiene,* p. 134, 1839). It is not an at- 45
tempt, a simple affirmation, like that of Ratier, who had never made an appraisal of stains, who undoubtedly succeeded one time in isolating spermatozoa, but who would have perhaps not succeeded a second time, and who certainly did not isolate blood cells from a stain with the impossible procedure which he indicates in the same note. It is a conscientious methodical study, devoted as much to demonstrations done in the presence of members of the Society of Legal Medicine, as to *eleven assessments* done with brilliance. And, there is some merit, if not courage, in disputing the validity of the categorical assertions of Orfila. And the fact that successful attempts were done before his, undoubtedly unknown to him, is no reason to refuse to Bayard the merit of his important discovery. If there were workers of the first hour, it is he, and only he, who was architect of the edifice. It would be, however, an injustice to forget a few scientists

† [victim was a woman].

who, on the whole, inspired all this research and brought it into focus by their works: I speak of Dumas and Prévost, and especially of Donné, whose names must be indissolubly tied to the history of determination of stains.

Following the work of Bayard, and due to the polemic waged between Orfila and Devergie, there appear in Paris, from 1837 to 1839, ten theses on the determination of stains: unfortunately I could not procure them in order to compiete this story.

The Bayard procedure. In the beginning, Bayard simply macerated in distilled water the strips of stains, which he took great pains not to rumple, and whose fibers he did not dissociate. After several hours of immersion he lightly heated without boiling them, then examined the liquid. Not very satisfied with the results, he indicated his *second procedure:*

1) Cut out with scissors and remove with care a portion of the stains presumed seminal; do not rumple the material, and place in a test tube;

2) Bathe the stained material in distilled water, and macerate for twenty four hours;

46 3) At the end of this time, filter the first liquid. Place the stained material, already macerated, in a porcelain capsule, moisten it with distilled water and heat by the flame of an alcohol lamp until the liquid attains a temperature over 60° to 70°. Filter this liquid. Finally treat the stained material with alcohol or by ammonia solutions and filter the diluted solution;

4) When the filtration is finished, cut the filter paper at a distance of one thumb from the edge and turn it over on a watch glass, or, preferably, on a dish of flat glass; moisten the inverted filter with alcohol or ammonia solution, which dissolves mucus and entirely detaches the deposit. If fatty matter is found mixed in, a few drops of ether water is employed. Microscopical examination of the capsule or the dish of flat glass permits recognition of *entire spermatozoa, without breakage* of the tail, isolated from mucus.

Alcohol in water was 1:10, sodium and potassium 1:20, ammonia 1:16, and Bayard made observations at a magnification of 350 to 600 diameters.

What comes out of the beautiful work of Bayard, is that these are things *seen* which he describes, and he has performed a great many times his conscientiously presented experiments. I will point out as proof of the sincerity of his observations only two facts: p. 156, he remarks that if the preparations are dried, the spermatozoa are more visible, an observation later made by Schweiger-Seidel, and, more recently, by Pincus, who made of it a procedure he believed new; then, p. 161, he described the crystals of semen, which he takes to be sodium and ammonium phosphate, crystals known today under the name of *Charcot crystals.* It is then, he who deserves the merit of the discovery of these crystals and not Robin, nor Charcot.

146 The Bayard procedure, that I will call the filtration procedure, seems to have been followed for a long time despite its difficulties; the author reproduced it without changing anything in his *Manuel de medecine legale* of 1844. Yet in the six-year internal numerous works had appeared on this question, particularly in the form of theses at the Faculty of Medicine in Paris, as I have already noted.

In 1848, C. Schmidt indicated his squeezing out procedure, which has as its principal advantage to leave the clothes intact, which can be of importance in discreet examinations. The material is examined with care to assure which side holds the stain. In holding it obliquely, it is easy to see one of the faces more marked than the other and it might even show a projection; then, taking the stain as center, the material is folded in a point or cone, with the stain forming the *outside* peak. This point is then suspended vertically over a watch glass half-filled with water, into which it scarcely dips. It is left in this position for three or four hours, replacing the water as needed; then this is slowly heated by means of a small spirit lamp. A few drops of ammonia solution are added, and the material is picked up, pressed from the top toward the bottom *between the thumb and index* finger, the stain is thus expressed, it disappears, and the water flowing out is cloudy and a bit mucousy. It is examined by microscope. This procedure, on the whole, quite simple, perfectly succeeds when the stain is rich, but in difficult cases, in which it is hardly visible, nothing is obtained, or at best, a few fragments. (C. Schmidt, *Die Diagnostic verdaechliger Flecke in criminalfoellen,* Leipzig, 1848, page 42). 147
This procedure is most often followed by experts of the Russian Ministry (*Anleitung zur Untersuchung verdaechtliger Flecke,* etc. Saint Petersburg, 1871).

Koblank[6] in 1853 (Casper's *Vierteljahrschrift,* III, p. 140) gives another squeezing out procedure which enjoys a certain favor undoubtedly due to the support of Casper. The stain, cut out, is put in a saucer containing a little cold water, the material is dipped in the liquid with a stirring rod until it is completely saturated. After a quarter hour, a drop of this water is observed under the microscope; the presence of spermatozoa is easily determined. The material must be pressed with the stirring rod.

If the stain is complex (blood, fecal matter, etc.) and the liquid too cloudy, Koblanck recommends adding a few drops of acetic acid which clears the preparations, without affecting the spermatozoa. This procedure, less brutal than the preceding, perhaps better safeguards the integrity of spermatozoa, but it gives absolutely no *result with very meager stains.*

Scraping procedure of Ch. Robin and A. Tardieu (Memoir on a few new applications of microscopic examination to the study of various types of stains. *Annales d'hygiene et de médecine légale,* 1860, p. 434).

The authors cut out the stains, macerated them to saturation in a watch glass, then scrape them with a bistoury. They find spermatozoa "as numerous and concentrated as in spermatic liquid, as it is found when it has just been ejaculated: they are whole and flexible, and there was a very small number which were broken." They add a little acetic acid to dissolve mucus substance. The two experts, though having a

peremptory proof of the seminal nature of these stains, did not think themselves dispensed from submitting them to chemical reactions, such as Lassègne had indicated, i.e., the action of heat, of cupro-potassium subtartrate, and nitric
148 acid. It is true that, little satisfied with the results obtained, they add: "These characteristics, to which a few authors still attach a certain importance, have none, however, beside those derived from the presence of spermatozoa". This scraping procedure is assuredly the simplest, the most rapid, and the easiest of all; we do not hesitate to employ it when the stains are thick. Professor Renaut had formerly supported it with his authority.

But if the stains are meager, it is an absolute disaster, with a high risk of losing it all, while with a less brutal procedure, one would have succeeded perfectly.

Up to this time, the exclusive preoccupation was the *extraction* of spermatozoa, without indicating a procedure to see them better, either by staining, or otherwise. Pincus, having had to do an expert investigation, found nothing in his preparations, but, in looking at them the following day, when they had dried, he saw a great number of them in a perfect state (*Vierteljahrschrift für Gerichtliche Medic.*, 1866, N.F., Vol. V, p. 347). This caused quite a commotion, even though it was nothing new, for Bayard had indicated it in France in 1839, as I have already noted, and besides, Schweiger-Seidel in Germany had also brought attention to the advantages of letting spermatozoa preparations dry before observing them.

This skill was greatly reproached, for it is only a skill and particularly by Ungar. It is, however, certain that if a spermatozoan is not dried out, but if the liquid surrounding the preparation has disappeared, it is in the best possible conditions for being observed, whether stained or not; it is bordered by a little meniscus due to capillarity, a strongly refringent meniscus which gives an extraordinary relief.

Thus when a spermatozoon accidentally finds itself lodged in an air bubble, or if the liquid is aspirated with precaution by blotting paper, the end of the tail is perceived with perfect clarity, something very difficult to delineate exactly without this method, at least without a special utensil. Unfor-
149 tunately, it is of little convenience to wait until the following day to observe the preparations, since all the particles floating in the liquid, spermatozoa as well as the rest, are dragged in a heap during dessication by the meniscus which is pulling back, and everything reaches the border of the thin slide. Here is the danger, for in the accumulation of the amassed material in one place, it is very difficult, if not impossible, to find the rare spermatozoa.

On aspiration with blotting paper, one runs the same risks which can be partially avoided, in strongly compressing with the paper *only the edges* of the upper with the lower slide in such a way that water is always able to pass, and the solid particles disperse according to size in the sinus formed between the two slides. But all this becomes obsolete due to coloring.

Roussin in 1867 was, I believe, the first to have the idea of staining spermatozoa to facilitate their visualization; he was also the first to substitute methods employed up to the present with his *unraveling procedure,* the only one permitting success in every case. I cannot praise too much the remarkable memoir of this able and conscientious expert, who made an immense step forward in this delicate research. We quite often follow his procedure at the laboratory of legal medicine at the faculty of Lyon in the way he described it. I will present it further on. As for his staining solution, it has stood the test of time and has emerged victorious from all attacks. The solution contains:

Iodine 1
Potassium Iodide 4
distilled water.....100

"This reactant", said Roussin, "alters neither the volume, nor the form, nor the external texture of the spermatozoa, which suddenly take on a remarkable relief on contact, and are separated in the field of the microscope with the greatest clarity. The clearly visible portion of the tail increases considerably[7] and the whole preparation takes on a precision difficult to define[8] ". All of which is quite exact. It is only with difficulty that I can account for Hoffman[9] finding no 150
advantage to this coloring, under the pretext that the whole preparation was uniformly colored; Ungar[10] contends that phenomena of coagulation produced by the liquid, in engulfing all the mass, most often hinder finding spermatozoa, and consequently go precisely against the end to be attained.

We often have recourse (M. Lacassagne, M. Contagne, and myself) to the liquid of Roussin, and we have had to make a similar reproach; it is true that we always employ it at the dilution indicated by Roussin, and as we put only a small drop on the preparation, the dilution is thereby reduced to 1/200, while in Germany it is employed, due to misprint no doubt, sometimes one tenth (Maschka, vol. III, p. 126). As to the rest, when the spermatozoon is isolated and perfectly colored by the reagent of Roussin, it is less of a hindrance than one would think that other foreign bodies of the preparation might be equally colored; the eye knows it, is attracted by it, if I might thus express myself, and seizes it in passing when the preparation slides before it.

This reagent admirably fulfills the principal goal, to leave absolutely no doubt in the mind of the expert once the spermatozoon is found, which always comes with patience; it is of little bearing that one looked a bit more or a bit less, the essential thing is that no error can be committed with regard to the nature of the object found and I strongly affirm that in this context, the liquid of Roussin is perfect. It stains the external envelop of the head of the spermatozoon, which is admirably distinct from the background, with vivid and clear-cut contours, and if an objective of higher power is then substituted, there is no possible doubt.

Longuet proposed in 1876 to substitute ammoniacal carmine for the liquid of Roussin which he also reproached for staining everything.

He absolutely rejects the unraveling method used by 151

C. Robin and Tardieu which he accuses, among other misdeeds, of *artificially creating the spermatozoa.*

As strange as it appears, this accusation certainly has some basis; by the brutal unraveling are freed fibrils of material and also granulations which Longuet claims peculiar to hemp[11] which can be perfectly confused with the heads of spermatozoa.

It is common that these so numerous fibrils juxtapose themselves on a granulation to closely simulate a spermatozoon, especially if observations are done with the weak magnifications generally used. All those who have had to examine stains are able to make this observation.

Longuet indicates that this error can be avoided in noting that in false spermatozoa, the tail has a diameter equal everywhere whereas the real tapers toward the extremity. One will see further on other procedures which leave less room for error.

Longuet estimates that maceration must be prolonged forty-eight hours, which, he said, is without inconvenience, due to the extraordinary resistance of spermatozoa, which even ammonia steeping does not destroy. It is only after this time that the spermatozoa have regained all their suppleness and that the unraveling of fibers does not break them. As to the rest, a long maceration is certainly necessary, if one wishes to follow the indications of Longuet, because ammoniated carmine tints spermatozoa rather poorly and slowly, even after forty eight hours; in contrast, says the author, it leaves intact all vegetable elements, fibrils or granulations, "one sees so well at first glance that all which is white is vegetable and all which is become red is of animal nature". The reagent of Longuet is formed from five to six drops of ammoniacal carmine solution, such as is used in laboratories, in five grams of distilled water.

In the following year Petel and Labiche gave a procedure also based on the use of ammoniated carmine, but with the
152 considerable difference that the determination of the nature of the seminal stains passes the microscope by completely, and can then be applied to cases where spermatozoa have completely disappeared as a result of breakage. Suspected stains treated with ammoniacal carmine strongly color in red, and afterwards energetically resist washing with pure water or even more than twelve hours in a solution of sodium carbonate. All the other stains by contrast were discolored in less than six hours according to the authors.

I did not find the formula for the solution of carmine employed by Petel and Labiche anywhere, which is, moreover, immaterial, but what is not at all immaterial is the concentration of sodium solution, which plays here a major role. The Society of Legal Medicine charged two of its most distinguished members, M.M. Brouardel and Boutmy, to report on this procedure (Meeting of May 12, 1879). One reads with great interest in the Annals (1880 p. 225) the remarkable account rendered by these two able practitioners who, though acknowledging the benefits able to be drawn in certain cases by this coloring of stains, quite wisely pointed out how imprudent it would be to base the condemnation of a man solely on such a frivolous sign, a sign still uncertain; where, in short, the basis is but a matter of more or less about six hours to twelve hours.

It is then absolutely wrong when Vogel says that MM. Brouardel et Boutmy confirmed the assertions of Petel and Labiche (*Viertel. f. ger. Med.* 1882, p. 160, Boutmy and Brouardel, bestaetigen dieses Verhalten). The contrary would be more exact, it seems to me. Vogel criticized the procedure, and said he could establish that varied stains, of vaginal mucus, or white flowers, discolored like those of semen, as much in a concentrated solution of sodium carbonate as in a dilute solution. He ends by saying that the procedure can at best serve as negative proof and demonstrate that a stain which refuses to color itself with carmine is not formed by semen. But, he adds, a long time ago Hager (*Untersuchungen,* 1871, p. 461) indicated picric acid for this same end; on the other hand Hager very prudently remarked that many other stains behave like 153
semen, for example those of vaginal mucus, eggs, flour, nasal mucus, an important fact, he said "because it is a very common habit of the women of the population to blow their noses in their shirts(!)"

Destruction Procedure. Vogel (*loco citato*) found fault with the procedure of Petel and Labiche and also that of Longuet, which he reproaches for staining especially the other elements of semen, precisely for leaving intact and uncolored, even after fifty-four hours of maceration, the spermatozoa themselves. These scarcely have a little bluish tint due to a phenomenon of refraction. In the face of the insufficiency of these procedures, Vogel indicates one which is certainly unexpected. Discontent with all stains: picric acid, aniline blue, Methyl violet, picroaniline, fuchsin, eosin, Bismarck brown, etc., he simply destroyed the support of the stain, or its debris, in respect to the spermatozoa. The stains, he said, are moistened with water, then scraped by knife, taking care to remove only the least possible material, but a few threads are no problem, for they will be destroyed. On the bottom slide, he adds concentrated sulfuric acid to the product of the scraping, then after two minutes, one or two drops of tincture of official iodine. He stirs softly with a stirring rod and puts on the top slide. All is destroyed except the spermatozoa, which are vividly colored in brown by the tincture of iodine. Unfortunately, the preparations, as one might suppose, keeps hardly two days at best, even after washing. It is assuredly abusing the resistance of spermatozoa to attack them with the most violent of our corrosives, uniquely to destroy a few fibrils which can hinder the research, but to which with a little practice, one pays almost no attention. Moreover, the considerable heating produced by the mixture of the water of the preparation with concentrated acid can compromise everything in certain cases.

Staining with Eosin. Ammoniacal carmine was generally unsatisfactory, staining inconsistently, sometimes rather well, other times very badly, and eosin was accepted with 154
great favor in all the laboratories, as its manipulation is particularly convenient. It always instantaneously stains

spermatozoa in a splendid, vivid rose; the head especially is remarkably discernible, and takes on a lively refringence under the influence of the reagent. It is generally accepted that Schnitter was the first to propose the use of eosin in legal medicine, in a memoir written in Polish in 1883. But a long time before that this reagent had been indicated by Professor Renaut in our laboratories at Lyon and in 1819 M. Clément made it the subject of his conferences in legal medicine (conferences published in 1880. J. B. Baillière and son, 1880, page 192).

I textually reproduce the procedure of M. Renaut, who distinguishes himself from others as well by the use of 1:3 alcohol instead of water.

The stain will be cut in fragments of one square centimeter, each fragment placed in a watch glass and moistened with 1:3 alcohol which has no action on the spermatozoa, whereas water swells them, blanches them, and even dissolves them.

The fragments are left under a bell-jar until well saturated; one hour suffices to attain this goal.

The two faces of material are scraped with a scalpel and the scrapings placed on a glass slide, then the scraped material is dissociated on another glass slide, and the granular liquid thus liberated is mixed with that of the scraping. It is good to do the two operations just described separately and successively for all the fragments; then to individually examine the series of numbered preparations obtained.

The liquid coming from the scraping or the dissociation is finally mixed with glycerine saturated with eosin at 1 part in 200. The top slide is placed, sealed with paraffin and the preparation examined.

It is rare under these conditions to find completely isolated, non-fractured spermatozoa. But numerous fragments
155 of dried sperm are encountered in the preparation; the action of the 1:3 alcohol has not sufficiently softened them to render them diffusable. These fragments are colored an intense rose by the reagent; they present breaks of conchoid appearance almost characteristic; it is these in the end which most often contain the most characteristic spermatic filaments in the state of the most complete integrity. The spermatozoa are entirely or partially engulfed in the coagulum and can easily be seen with a wide-angle objective. The head is always characteristic. An oval point of magnificent carmine red, to which is attached a filament tinted in rose, like the whole of the dried sperm, but differentiating itself by its refringence. I insist on the point that it is indispensible in preparations made as just described, to find at least one whose head is not separated from the caudal filament. Observation of isolated heads might cause confusion, and remove from the medico-legal verification all its precision.

The use of eosin is quite simply and incontestably a great progress in legal medicine as well as in general histology. Its intervention renders an enormous service in every stain, for it gives an incredible character of clarity to thousands of doubtful particles; to convince oneself, it suffices to color any type of stain with it, any mucus stain whatever, and one will be struck by the relief taken up by epithelial cells for example.

M. Renaut, in a fear a bit exaggerated, it seems to me, of destroying spermatozoa, counsels alcohol at 1:3, if this liquid is taken at a degree higher than indicated by the wise professor of Lyon, there is great risk of totally losing the stains; thus even those on glass do not give spermatozoa with strong alcohol which reduces them into fine granulations.

It is not hard to imagine that the techniques used in the laboratories of histology and especially bacteriology have been tried just about everywhere in legal medicine and at about the same time; quite particularly the double staining, 156
so fertile in the research on microorganisms, has been tried with varied success.

I will cite especially in this context the idea of the work of a team of Ungar, in collaboration with Steilberg. These authors macerated stains for about five hours in water acidified by hydrochloric acid, one drop per cc, liquid which, according to them, conserves the spermatozoa, renders them more resistant, not without shriveling them a little. The stain is then taken with tweezers, and softly rubbed on the upper slides, which are then exposed to air until complete dessication, and finally heated three times. Ungar stains the preparations by letting them bathe in solutions covered by a bell-jar to avoid evaporation.

The stains used were: 1) eosin in saturated solution and hematoxylin (formulae of Friedlander or, better, of Boehmer). But it is necessary to leave the preparations in hematoxylin at least six days, a redhibitory defect; 2) eosin and carmine steeped in alum water (formula of Grenacher); 3) vesuvin and eosin. The solution of vesuvin contains two grams of this coloring substance, sixty-six grams of water and thirty-four grams of alcohol.

I do not insist on the inconveniences of double colorings, impractical when one finds a spermatozoon only with great difficulty, and useless when, on the contrary, there are many of them. Besides, Ungar, moreover, seems to have understood, for he proposes to simply stain the preparations with methyl green to which is added hydrochloric acid (methyl green, 0.15 to 0.30; hydrochloric acid, five to six drops; water, 100 cc.). The preparations are very beautiful if left to dry according to the procedure of Pineus.

Methods of the Laboratory of Legal Medicine of Lyon.

If I believed it necessary to report all the procedures which I have just presented, it stems from the conviction that in one case or another knowledge of them can render great service to an expert. Not every material lends itself equally well to 157
the procedure of choice; *unravelling* of velvet, for example. Besides, it is not always on material that one might have to look for spermatozoa: stains on leather, on felt, on a solid body are not treated the same as those of material. The expert, then, needs to know all the procedures, and knowing which to choose as the most convenient is to his great merit. Orfila and even Donné, who in the first half of this century had the well-deserved reputation as the most able histologist

of his time, and who made himself known precisely through his studies with spermatozoa, contended that finding them in a stain was impossible. If these authors could have been acquainted with this group of procedures, which I have just briefly presented, they would certainly have changed their opinions, for they would have succeeded with one or the other of them.

When a stain is thick, all the procedures are successful: in this case we followed for a long time, and we still sometimes follow the simplest of all, that of Professor Renaut, such as we described it on page 154, but with slight modifications.

The stain, largely cut, is placed in a watch glass with some drops of water to which is added a little *aqueous* eosin solution. It colors intensely in carmine rose and after a little while small glaring masses, swollen by the liquid, can be perceived, more colored than the rest of the stain. These are delicately removed with a scalpel, or a cataract needle, and examined under the microscope. If necessary, the mucus is dissolved in a drop of 1/20 ammonia solution, and the action of the reagent is favored by light movements of the upper slide. The spermatozoa are very visible, and it becomes easy to preserve the preparation as material evidence. For this, the top slide is raised and the water evaporates in great part, then a very small drop of glycerinated gelatin, dissolved in a waterbath, is added, the top slide replaced and lightly pressed for more exact application against the bottom slide.[12]

158 But if spermatozoa are only rarely found, it is better to seal the preparation immediately with bitumen, without the risk of losing them in the manipulation which can happen in the use of gelatin.

The stain itself is then submitted to scraping with a scalpel.

But when, to the contrary, the stains are meager, imperceptible, or even difficult to find, we still use eosin to find them the most quickly, as Professor Lacassagne presented in his article *Stains* in the dictionary of Dechambre; but certain that the scraping will not give us good results, we proceed to unraveling.

Miscalculation on one hand and, on the other, the interminable hours which we are often obliged to devote to suspected stains, which we sometimes leave without positively having acquired the certitude that they could not have been formed by semen, prompted me to look for a chemical procedure, capable of eliminating suspected stains. The well-known reactions of spermine gave me no result: the most important — the spermatic odor it gives with gold chloride and magnesium — is too vague to be used in legal medicine, and besides, I once obtained it with egg white; flour paste gives it better than semen itself. One knows, moreover, that spermine has been found almost everywhere, and that this makes it lose all significance in the case in point. But it seems to me that a liquid whose physiological goal is so exalted, and which in so little volume must fulfill the highest of all functions, must be infinitely more complex than the poor analyses which have been made indicate.

It was impossible for me to believe that semen did not contain one or many bodies, alkaloid or other, perfectly specific. Even more, it seemed natural to me to allow different principles in the semen for each species of animal, 159
exactly as in the liver, the stomach and the humors of animals in general; chemical principles very similar, but however distinct for each species.[13] This admitted, my path was traced: examine under the microscope the action of all the known reagents on semen, varying the experiments in every way, such as concentration of solutions, etc. I had the satisfaction of thus finding a certain number of reactions which I will later present, only being able to present one here, which appeared to me particularly adapted to achieving the purpose I was pursuing, due to its extreme simplicity and clarity.

I had tried the reagent of Bouchardat (iodated potassium iodide) with no result, when I had the idea to try potassium triiodide, the reactions of which on the alkaloids I had formerly studied. I obtained absolutely nothing on my first attempts; I don't know why. On the recommendation of M. Bouvault, with whom I was discussing it, I prepared the reagent in a sealed tube, with the idea of assuring a more intimate union of iodine and potassium. I was not any happier, undoubtedly because, too impatient, I used the liquid while still hot.

I rationally proceeded in the opposite direction, i.e., in leaving the reagent in icewater during twenty-four hours and I obtained this time, with the semen, crystals which on first sight appeared so identical to those of hemin, that I first asked myself if, by distraction, I had not used slides used for blood research and poorly washed them!

Preparation of the reagent. It is most simple and I can only explain my first failures because I must have tried it by error on non-seminal stains.

Ordinary iodated potassium iodide does not give crystals with semen; quite on the contrary, it instantaneously dissolves those produced by my reagent; but as soon as the richness in iodide increases, so that the liquid contains potassium triiodide, the reaction is produced. This is not the place 160
to discuss the state of the bodies in the solution thus obtained; I will be content to say that that which appeared the most convenient is formed simply of:

pure potassium iodide	1.65 g
iodine (washed beforehand)	2.54 g
distilled water	30 g

This richness in iodine, which corresponds to KI_3, is not indispensible, for perfect crystals can be obtained with a solution containing 1.65 g potassium iodide, only 1.27 g iodine, a formula corresponding to KI_2. The reagent is prepared cold, very rapidly, is stable for a very long time, and is absolutely exempt from caprice. It is necessary to put it in small emery bottles whose stoppers terminate in a stem serving to obtain the drop necessary to each operation.

Use of the reagent. A very small fragment of stain — one thread suffices in a strict sense — is put in contact with a drop of pure water on the bottom slide; after an instant it is removed, then with the stem of the stopper or a stirring rod,

a drop of reagent is left *beside* the droplet left on the bottom slide.

In placing the cover slip, the two liquids are mixed, in which cloudy, ochreous striations form. The crystals appear almost instantaneously; if the stain is very meager, they take a bit longer to be produced, and one can assist their appearance. When it is very hot, it is necessary to chill the reagent in ice water.

Appearance of the crystals. Because of the extreme sensitivity of the reaction, there is always an infinity of sizes and varied forms, but clearly characterized by a brown tint more or less dark, according to the thickness of the crystal. Addressing myself to experts, if I simply say that these crystals appear at first sight so like hemin that one can be mistaken,
161 I would be describing them better than by the longest, most minute, scholarly description. Curious thing, the form and color of hemin crystals were regarded as absolutely specific up to now; no one knew of a histochemical reaction able to cause confusion with them. And here, semen shares with blood the privilege of giving crystals so similar that description of one can serve to describe the other, without any error being able to be produced, due to the very different methods followed to attain them.

I believe then, that the fundamental type of semen crystal is identical to those of hemin: a brown on yellowish brown lamina, five or six times longer than wide, terminating at its two extremities by a hook, forming an angle variable with the direction of the crystal, a figure recalling a plank of oak flooring, called *fern leaf.*

It is the classic figure of hematin crystals, known by all: these are known to often have at their extremity a second facet, of different obliquity, making a sharp angle with the first: these I called *notched crystals*,[14] *cristales con escotaduras* of Carlos Demarias.[15] This form is also found in semen crystals, exactly as in those of hemin.

Again as hemin crystals, they fuse rather often in crosses or stars, as can be seen in photographs, and the resemblance is perfect.

But the *typical* form is not here the most common. Very often the crystals are acicular at least on one side, whereas the other terminates in two points, which gives them the aspect of a lance-head, instead of a very elongated parallelogram of primitive type.

Sometimes the crystals are united in parallel, in groups of a few, recalling *bifurcated* crystals of hemin. When the preparations are very rich, or when the crystals are nourished,
162 they become great enough to be visible to the naked eye; or, more often, thin, yellow plaques stick against them, representing parallel macles.

It was a preparation of this type that Professor Offret wished to examine; he, in our University, makes with a rare devotion his exceptional competence available to everyone, thus giving priceless services to our laboratories. Here is the note which my knowledgeable colleague wrote me:

"The substance to be examined presents itself by microscope in *natural light* in the form of fibers of rather variable size, of a yellow brown color. The extremities form an angle of about 60° with the length of the fiber, giving an aspect of a very elongated parallelogram. The internal contour of the fibers completely disappears in this fusion and the extremities have a jagged aspect.

Examination in parallel polarized light. These fibers and the laminae coming from them polarize intensely in their thin parts, giving a yellow color rather like the color of the crystal in natural light. This yellow color is from a mixture of the color of the crystal itself and the tint provided by polarized light. In the thick part of the fibers, the absorption of light resulting from the intense color of the substance hinders any examination.

These fibers are rigorously extinguished following the direction of their length during their rotation between the prisms.

Examination of converging polarized light. This examination is very difficult to do because of the opacity of the substance in its thick parts. In using the device of Lasaulx, special for very small crystals (i.e. in removing the ocular of the microscope) the formation of two black branches of an hyperbole is noted, fusing in the center to form a cross equally diffuse, in the position corresponding to extinction in parallel polarized light. During rotation of the plate, the two branches of the 163
hyperbole rapidly leave the field.

The phenomena accessible to observation are then unfortunately insufficient to determine the crystalline system. However, it was good to note the preceding results, which were indeed clear."

M. Offret will later do new research on the crystals to determine their crystalline form which seems to belong to the clinorhombic system, even though not terminated by a facet proper.

Their size varies considerably: the largest attain a quarter of a millimeter in the preparations.

These crystals are soluble in a great quantity of cold water, very soluble in hot water. It suffices to heat the preparations to make them disappear; but they reappear on relowering the temperature. If, without sealing the preparations, one leaves them exposed to air; or, even if sealed, on leaving the reagent present, the crystals disappear bit by bit beginning at the extremities; but if a new drop of reagent is added, they reappear the following day. Ether or alcohol dissolve them instantaneously; it is the same for acids, alkali and potassium iodide. Ammonia in very weak dose leaves them intact.

If the extract of the stain on the bottom slide is completely dried and reagent added to the residue, the crystals form poorly and stay very small.

I will present elsewhere the characteristics of the principle which gives birth to the crystals, which I propose to call "virispermine", as it appears different to me from spermins

described up to the present derived from animal testicles. Its extraction from human semen is most easy; I prepared various salts and I hope to soon be able to combine them sufficiently to establish the formula.

Up to the present, due to lack of raw materials, always starting with stains brought to me, I could only isolate very small quantities. This principle exists in rather great amount in ejaculated semen, for a large stain gave me at least ten
164 centigrams of crystals. In treating them with magnesium, I isolated the principle itself by alcohol, unfortunately tainted by magnesium iodide.[16] It is soluble, acrid, and did not give me the reaction of Poehl's spermin. I will present its properties elsewhere: from the medico-legal viewpoint, it is interesting to know that it is very soluble in stains, and it resists ammoniacal putrefaction. Stains left in water in a humid place, covered in moss and emitting a fetid odor, give crystals just as well as fresh stains.

If one treats the maceration of an entire stain in a test tube with the reagent, a brick red or chocolate precipitate is produced which immediately deposits on the bottom and edges of the tube in the form of small crystals visible to the naked eye and quite remarkable: they are irridescent, russet, glistening, especially if examined in full light or by the sun.

I could not better compare them than with those old Martin polishes laden with spangled gold. In any case, a semen stain can be easily recognized without recourse to a microscope, it is so particular.

Sensitivity of the reaction. It is prodigious: if a small ribbon or simple thread is removed from the stain and impregnated with a small drop of water on the bottom slide, the addition of a drop of reagent gives a quantity of crystals so considerable, it is impossible to study one of them exactly, they are so numerous and entangled. Most of them, often disposed in the form of a cross, cover the field of the microscope, but on lowering the objective, one finds very small crystals in infinite number, like sand on a beach. Photograph no. 5[1] was obtained by treatment of a single fibril extracted from a stain: due to the diffusibility of the substance and eddies produced by application of the top slide, the crystals were dispersed throughout the preparation, but the fibril itself was all shaggy and totally obscured.

It is difficult to fix by number the limit of the sensitivity of
165 a microchemical reaction, for this limit has to correspond exactly to the quantity necessary to obtain a characteristic crystal: thus understood, the limit of the sensitivity would be fantastic here. It is a considerable number of preparations which can be obtained with one single strip taken from a stain: if moistened, it suffices to apply it on the bottom slide and just as soon remove it; the little water left behind gives magnificant crystals, and the same strip can serve another time.

Significance of the reaction. To my thinking, this reaction can serve only to separate suspected stains into seminal and nonseminal: all I ask of it at the moment is to permit me to reject the latter in a few seconds and to then apply all my efforts to retrieving spermatozoa from the former. It will certainly render service in the investigation of stains, and considerably simplify the operations of experts. But it is understandable that I cannot stop myself as of this moment from going a bit further: up to now, *I've found no product of secretion,* no liquid, nasal or vaginal mucus, urine, sweat, saliva, tears, milk, cerebral substance, hydrocele liquid, leucorrheal discharge, pus, etc., etc., which gave me this reaction which is so characteristic. I tried almost all the usual alkaloids, flour dough, foods, a great number of non-seminal stains, and I have never found anything comparable; the white, flowing liquid secreted by urethral glands during erection did not give me anything either. Something more curious, animal semen did not give me any crystals. I was able to try soft roe of various fish, dog semen, horse (ejaculated), rabbit (testicles), guinea pig, hare, and he-goat with no result. I do not, however, hold these results as definitive, not having repeated my attempts.

The spermin of Poehl (extract of bull testicle), that of Jacquet (guinea pig testicle) produced nothing. I believe I am then authorized to expand, until there is proof to the contrary, the significance of my reaction within the limits I will establish further on.

Research on Spermatozoa by Unravelling Technique.

As I have already pointed out, this procedure is incon- 249
testably the *procedure of choice,* to which it is necessary to have recourse in all difficult cases. It is assuredly the most rational and least brutal consequently, the most certain. To have abandoned it in the laboratories (I have no idea why) was a serious mistake, for its failures are ascribable not to the procedure but to the clumsiness of the technician. Scraping was thought to have presented a shorter, but especially less bothersome, method to attain this end. As a matter of fact I am quite convinced that this procedure requires much more time than any other in isolating an intact spermatozoon which has escaped, as if by a miracle, the crushing to which the stain is submitted by repeated action of the scalpel. If it is necessary to intentionally fragment the spermatozoa of a stain, would it not be possible to find a more appropriate means than this violent scraping? I always have to make numerous preparations all things considered, requiring a very long time to find a complete spermatozoon, and this without any other benefit than the meager consolation of discerning numerous fragments which I suspect to have been the heads and tails of spermatozoa.

All this debris encourages beginners a bit, and even others, 250
but it is agreed that they are worth nothing when it comes to conclusions, leaving the expert in annoying perplexity, if definite success does not come to crown so much work. The worst is that this scraping requires a considerable part of the stain, perhaps the entire stain, and all is irreparably lost if it has been unsuccessful! A square millimeter, a simple thread which does not alter the fabric, is largely sufficient in almost all cases, if one proceeds by unravelling.

[1] [Note. Photograph not reproduced in the translation].

Some preliminary considerations would not be superfluous before presenting the procedure itself.

Instruments. It requires the usual instruments of all operations in micrography: very fine dissociation needles, a fine forceps (like used to remove a splinter) to handle the fragments, a scalpel, small pointed scissors, watch glasses of two and a half centimeters in diameter, slides and cover slips, a good microscope, a coloring reagent, etc. I macerate the stains in small watch glasses, very rounded, thoroughly ground, all of the same size in such a way that they fit exactly on top of each other during the macerations. A certain number of these glasses, carefully labelled, are placed under an equally rounded bell-jar during the operations, where they are protected against external accidents and evaporation. Many authors have indicated the magnifications to be chosen for the observations. It is assuredly a very important, even major, point. But magnifications much too weak have almost always been recommended. Every extraction procedure isolates extremely thin fibrils, always terminating in a sort of small head, perfectly simulating spermatozoa even to the eye of a practiced technician, if observed with objectives that are too weak. The photograph† (Plate I) represents a false spermatozoon of this type which would certainly have fooled a beginner. Numerous corpuscles are always swimming in the preparations and often adhere to the extremity of a fibril. Afraid of losing this spermatozoon, sought for so long, the beginner takes great care to impart no movement to the preparation; it is enough, however, to touch the cover
251 slip with a needle to immediately separate the fibril from what is simulating a head, and to convince him of his error. In any case, it is not the magnifications generally recommended which will help him out of this danger and even less the classic figure of spermatozoa found in all the treatises such as I have reproduced (Fig. 2) [not reproduced in the translation]. I have seen in numerous assessments stains which have nothing in common with sperm and so many pseudo spermatozoa of this type that I wonder if they have not often actually caused errors. It is strongly agreed that one can reach a conclusion in the presence of a *whole spermatozoa,* i.e., a head furnished with its tail. All that is possible in this oversimplified recommendation is confusion. This head absolutely necessitates verification of its structure and, if needed, its exact dimensions. This is possible only with high magnification.

First of all, I can think of nothing as dangerous in research of this type as continual changing of objectives and oculars; the operator's eye must absolutely familiarize itself not only with the form of the body to be looked for, but especially with the size under which the optical system shows it to him. If the objectives are often changed, this notion of size is totally ignored, whereas the habit of always keeping the same investigating objective gives the object to be found an idea of real, fixed, unchangeable size which renders confusion almost impossible. In this particular case, I use as an investigating optical system a Verick objective no. 7 and an ocular no. 3; in addition, I always pull the tube of the microscope to a reference line, marked on the length of the tube, such that with this elongation each division of a micrometric ocular corresponds to two μ. I can adequately perceive the details of the structure of the head with this magnification due to the particular penetration of this objective, but I do not neglect in difficult cases to examine the material with an oil immersion objective. However, with most of the strong objectives of our better manufacturers, one can bypass an oil immersion system, and still be rigorous.

Today all confusion is avoided using staining reagents, useful in all procedures, but absolutely indispensible in un- 252
ravelling, which often produces an infinity of fibrils similar to a caudal filament of a spermatozoon. I have already indicated (page 154 of no. 62) [this journal, vol. 11; see in these translations] the most often used. Methyl green to which is added a trace of hydrochloric acid and crocein appeared superior to all the others, but it is up to each of us to make his own choice. As for myself, I find a very great superiority in crocein which gives the details of the structure of the head so clearly without diminishing it as does methyl green in acid solution.

Choice of strips. All those who have done numerous assessments can remark that in the same stain certain small strips do not give spermatozoa, while others, on the contrary, give them in great numbers. Explanation of this very frequent peculiarity is easily given: when the thick and coagulated sperm of a vigorous man falls on a fabric, the fabric acts on the sperm by capillarity, drawing off the aqueous part which extends all around in a zone more or less wide; it is on the edges of this zone that concentrate the soluble principles forming the translucid border in form of a *geography map.* The spermatozoa, despite their mobility, don't appear to follow the liquid in great number; they remain fixed to the middle of the stain. It is necessary, consequently, to always cut small strips from the center of the stain, and as only a very small quantity is necessary in proper procedure — a simple thread, for example — the stains can be left with all their physiognomy intact, which is not without importance. If not, the border, saturated in the diffusible principles of sperm, is perfectly suitable for obtaining crystals by potassium triiodide.

Procedure. I feel obliged to give *in extenso* the procedure as first described by Roussin (*loc. cit.*, page 158), when it actually became possible, due to his iodated iodine solution.

"With very fine, very clean scissors, a small square, of a
253 half-centimeter on a side, is cut from the center or the edge of each stain, taking the precaution not to impart any tugging to the fabric nor to cause appreciable crumpling. Two drops of distilled water are deposited on the bottom of a watch glass and taking the small suspicious fragment with the tweezers, it is gently placed on the surface of the liquid, which impregnates it bit by bit by capillarity, and completely moistens it. Experience has taught us that maceration must be prolonged for about two hours. During this time, the watch glass is covered by only a small glass plate, to inhibit evaporation and prevent contamination by foreign bodies. Care must be taken not to engender any movement in the fabric; after an hour, it is turned over and completely immersed in the water droplets. The moistening accomplished, a magnifying glass and two fine needles jointed together are used to perform a complete unravelling in the watch glass itself, *very slowly* and meticulously of each of the threads forming the warp and web of the material. A very clean glass slide (bottom) is then chosen, on which is deposited a little of the liquid of the preceding preparation: more simply, we take all the unravelled threads with the point of the tweezers and softly touch the surface of the glass with the small humid packet. The droplet thus deposited is swiftly covered with a thin glass slide (cover slip), avoiding the capture of air bubbles as much as possible, and the finished preparation is brought to the stage of the microscope, set at an appropriate magnification.

The observation must be slow and, especially, patient: the movements engendered to the preparation to bring all of its points successively into the field of the microscope, must be methodical and extremely slow; each visible corpuscle should be studied for a long time, alternatively placed at the center and on the edge of the field; the incidence of light is frequently changed by the greater or lesser obliquity of the mirror, and the focus of each object corrected and varied by almost continual movements of lowering and raising the tube of the instrument, which is moved by a very finely threaded screw.

254 If a certain number of cylindroconical corpuscles are discovered, and *a fortiori,* a few small isolated piriform bodies, it is almost certain, admitting that the examined stain is actually produced by semen, that an attentive and prolonged observation will bring about the discovery of a few intact zoosperms.

It happens, however, that observation of the liquid coming from the maceration gives only doubtful results: in this case, it is necessary to turn to observation of a few of the unravelled threads, and here is the best method to follow: one of the unravelled threads accompanied by a drop of liquid is deposited directly on the bottom slide, then, with a magnifying glass and two needles jointed together, unravelling is done very gently, by a movement of slow traction, to completely separate and spread out over a surface of about one square centimeter all the fibrils of hemp or cotton composing it; the preparation is covered and examined by microscope. Direct observation most often results in discovering zoosperm, if there are any; the greatest number of them are always broken; only a few can be observed intact or almost intact. The manipulation and observation is begun again on a second and a third thread, if the first is negative or insufficient. It is especially in the case of these doubtful results that the iodine solution whose formula is given above is useful. It suffices to deposit a very fine droplet on the bottom slide at the time of covering the preparation and to observe it immediately afterwards."

Here are the few modifications to which we subject the Roussin procedure at the laboratory of legal medicine:

In the middle of a stain, moistened beforehand with a drop of water and placed on the bottom slide and which gave crystals by use of a potassium triiodide solution, a small strip is cut out which must not be more than three millimeters on a side, only two if a very fine cloth (cambric) or, not wanting to alter fabric, a simple thread of three millimeters long is extracted, a rather simple operation, if after having sectioned the thread with the point of a scalpel, a thin forcep (like that for removing a splinter) is used. The strip is intro- 255
duced into a very small water droplet to be moistened, and left there for about two hours. But, in general, after a much shorter time, the first attempts can begin, in delicately detaching a thread with tweezers or a needle. It is placed in a droplet of aqueous, concentrated crocein solution, where it is left for a few minutes. This solution is simply placed on a bottom slide and can be examined itself, if need be. The thread is then dissociated on a slide in a *drop of pure water* with two very fine needles. On a thread of this length, in presence of a sufficient quantity of water, the thread unravels by itself; in two or three small pulls, the filament should be resolved into elementary, well-separated fibrils, uniformly dispersed in the droplet, I should say disappeared, for one can scarcely see them. If the strip was too big, unravelling is accomplished only at the expense of considerable pulling: the fibrils mix, intertwine, cover one another, unite in groups impossible to examine, the least inconvenience of which is too big a separation between the slide and cover slip. The top does not lie flat against the bottom, and observation with an immersion objective is just about impossible. It is quite otherwise when the thread is short: the fibrils disperse themselves, separating so well they can be examined one after the other in following their widths, even under the strongest magnification. Under these conditions, it is not possible that a single spermatozoon can escape being observed, and it is astonishing to find so many in such a small fragment, when a strip of one centimeter does not give them by scraping.

In following these conditions to the letter, a thread of only one millimeter will suffice to obtain first crystals, then spermatozoa, without altering the stain itself in any appreciable manner.

The examination. The preparations must be carefully, methodically studied; the liquid imprisoned between the
256 fibrils is examined first; in general, it contains very few spermatozoa, if one was too hasty in proceeding with the unravelling, but once the maceration is sufficient, they detach more and more and float into the liquid. If the procedure was performed delicately, they are ordinarily entire and intact, or if fragmented, the rupture has taken place in the length of the caudal filament almost as often as at the insertion into the head. But it is especially the fibrils which must be followed; as they are not colored, or only insignificantly. The head is very easily seen, even when adherent to their surface, and the tail appears as easily as if the spermatozoon were free. Once the maceration is sufficient, most of them, still

† [Not reproduced in the translation.]

held to the fibril by most of the caudal filament, present the head as entirely disengaged, but suspended by a fraction of the tail like a fruit by its stem. A whole packet of filaments emerging in bouquet from the same point is often thus found, where they are retained by a mucus mass colored in crocein. Even when the staining has not been very intense, and the spermatozoa are very weakly colored, they are easily found to the right and to the left of the fibrils, generally well spaced between each other, and in any case, easy to observe.

It is usually said they envelop the thread in great numbers like a sort of sleeve; this is an exception which must be quite rare on vegetable fibrils, because, for my part, I have never observed them, but this good fortune often happens with wool. I have seen some covering the fibril with an extraordinary regularity without causing any difficulty with the intimate observation of their structure, despite their number.

If a great number of spermatozoa are found, it is not any less necessary to verify them by an immersion objective, and all the more reason if only a few or only one are found. For this, the preparation fixed, the objective is changed and, in appropriate light, all the details of structure I previously presented are sought. If the head is disposed in profile, it is piriform, and the vesicle is not seen; but its aspect in this position appeared so charactersitic that almost all authors have thus exclusively described and represented it, and rightly so.

257 It is then necessary to delicately touch the bottom slide with a needle, a slight maneuver, which most often places the head full face. If this was not fixed to the fibril by a part of the tail, and if there was fear of losing it, it would be prudent to attempt this only at a low magnification, which, giving a wider field, permits keeping the spermatozoon in sight during its flight. But a few heads conveniently positioned are almost always found, if sought with patience; the head is then of an oval configuration, the anterior part round and thin, very pale, transparent, furnished with a little vesicle—sometimes with two, small and unequal—then the transverse line, generally very clear, sometimes *obscure,* separating the posterior segment from the more colored, thicker head, less transparent, and containing, near to the insertion of the tail, a luminous, refringent point; this point is often not so visible in spermatozoa of old stains on cotton or plant material in general; finally, the small appendix, which, like an apophysis, bears the articulation with the tail. This appendix is sometimes very short in which case the facet is not less visible, or the appendix can even be nonexistent. When it extends rather far, it flares a bit to receive the articulation with the tail, whose origin is clearly indicated by the almost complete absence of color. Sometimes the head is connected to the tail, not by the juxtapositon of two facets, but by a very thin thread, the axial filament. The tail, when adherent to the head, assuredly presents the best of characteristics, and at the very most, for more security, the dimensions can be taken (plate II). But when isolated, detached, can use be made of it in legal medicine? Yes, but simply as an indication of probability, if its dimensions correspond to those of human spermatozoa, if it tapers in a regular fashion. Up to the present, means have not been found to discern, in the case of stains, the spirals of tails, or even their division into segments. By crocein in concentrated solution, by iodide, by glacial acetic acid, the first segment can sometimes be seen, but in a fashion too inconstant for the hope of making any use of it in a difficult case.

It is necessary, for greater precision, to take particularly 258 the diameter of the head; this operation is neither long nor difficult and gives an additional security that it would be a serious mistake to neglect; it would seem to me as necessary to indicate these measures as those of blood cells in an assessment of blood stains. If the preparations are left to dry without any other care, they become quite splendid and conserve indefinitely if sealed; the spermatozoa appear with great clarity; the vesicle does not disappear with dessication, and the transverse line seems more accentuated; the luminous point near the tail is often visible only after dessication. When a very little glycerine, about 5%, is added to the maceration water, even more splendid preparations are obtained.

Observations in a Few Particular Cases.

Stains on white satin. They are fatty, grey, translucid, and bordered by a clearer zone but not by the border of the form of a geography map. They are not very starchy, and the spermatozoa disengage with great rapidity, are splendid and very distinct. Silk fiber is not colored by crocein, so that the spermatozoa are perfectly visible on the fibril; the tails themselves stand out admirably, and are as visible as isolated ones.

Stain on white sicilian. The stain had penetrated through the thick fabric, appeared greyish, more milky than oily, its tint weakly yellowish, but the very fine border is luminous; a stain very starchy, stiff, rather like ribs. Under the microscope, it can be seen that this fabric is woven in cotton, and the search for spermatozoa does not present the least difficulty; a square millimeter of stain suffices.

Stain on blue shot silk; with black tram. The stain was very difficult to perceive; nothing in particular could be seen by transparency; a bit of stiffness marked its place; the spermatozoa stood out well on the blue fibers, but on the black fibers, it was necessary to wait till they were partly detached.

Stain on blue cloth stippled with white thread. (Affair of 259 C. de Charolles). The stain was a bit whitish, and resembled that which one would have obtained by powdered rice. The fabric was very stiff. It suffices in cases of this type to dry-scrape the stain with a scalpel to loosen an abundant white dust, collected directly on a slide. A drop of water, and, after a little while, a drop of crocein, are added. The spermatozoa were quite numerous, set in a sort of viscous, layered matrix, which presented no obstacle to observation, even though colored yellow. The details of the head appear extremely clear. The stain, then treated by unravelling, give numerous spermatozoa adhering to the length of the fibrils, and which gradually detach.

Stains on solid bodies. These are the easiest to examine. I have already described them. Those on iron, however,

merit particular mention, because they can sometimes become very black and fail to be recognized.

In all cases of this type, it is enough to moisten the stains with a little water, and the research can proceed a few minutes later; it is sufficient to remove the shiny matter with the point of a scalpel or with a cataract needle. It is the same for stains on skin or hair agglutinated together by the semen. They are cut and macerated like those of fabric.

Semen in the vagina, uterus, etc. If in an autopsy, it suffices to take a little mucus from the organs, to stain it with crocein and to examine it. If the laboratory is far, the mucus must be collected on slides, or even fragments of porcelain, on which it is left to dry. But care must be taken to collect the mucus as recommended, as done in many assessments, on a cloth used to wipe the cavities! The difficulties are uselessly complicated and besides the risk of failure is high, for, in general, there are only very few spermatozoa in mucus, and it is known how little can be found in cloth stains

260 made with very rich semen. If the operation is on someone living, a small curetage is very convenient, but it suffices, in general to wipe one's fingers on the slides after a vaginal.

Animal semen. The crime of bestiality, quite common in antiquity, which primitive religions even adopted in their temples, undoubtedly to limit its spread by endowing it with a sacred character, was still widespread enough in the Middle Ages to have occasioned numerous trials. These invariably ended in burning at the stake, not only of the accused but also of his victim. This horrible depravation seems to have become very rare with civilized peoples, despite what is said,[17] if the trials which they occasion can be a basis for judgment. They are extremely small in number, and in none of those I have reviewed have the experts used as proof the direct confirmation of the presence of spermatozoa. In the case of Pfaff, an animal hair served as proof; in that of Maschka, it was claw scratches streaking the stomach and thighs of the accused.

Apart from this crime, the expert might encounter animal semen stains made accidentally, among peasants, for example, grooms of a stud farm, etc. Soft roe of fish, which escapes so abundantly and often with force, when the fish is touched at the moment of spawning, should also be noted. Description of these spermatozoa certainly ensues from the framework I set for myself but would carry me too far from the point considering that they are very distinct from each other, even among animals which seem closely related. To cite only one example, there are among the diverse varieties of frogs absolutely dissimilar spermatozoa.

I have been able to examine quite a large number of them, but none of those I have seen can be confused with man's. Dog sperm, however, comes close, for it also has a transverse line, but no vesicle is seen in its anterior part. None of the

261 semen I examined gave me the reaction of triiodide except man's.[18]

In a case of this type, it would be necessary to work by comparison, in taking sperm from the seminal vesicals of the incriminated animal.

Confusion with other morphologic elements. If observations are done at sufficient magnification, after staining, and the results corroborated by measurement, it is not possible to confuse foreign elements with human spermatozoa. Vaginal trichomonas, cercaria, flagellated infusoria, have been described; Frankl and Pfeiffer[19] have also indicated ciliated bacteria (*Trommelsch lagel bacterien*). But once again, there is nothing in these elements which would hinder a diagnosis.

The Conclusions. At this moment I confront a very delicate question. In the present state of science, it is by universal consensus that all authors and experts, without exception, require for the conclusion of the presence of semen that one entire, intact spermatozoon be isolated. I have said that even this minimum standard has always appeared dangerous to me in the hands of a young expert, following classical specifications to the letter, since I, for my part, have found things resembling spermatozoa under these conditions. Observations with magnifications that are too low, without preliminary staining, permit encountering elements perfectly resembling those described, especially that represented in the classical photo which is reproduced everywhere (Fig. 3). But should this reasonable specification, the prudence of which cannot be praised enough, be maintained according to the strict formula I have just written? I would like very much to say "yes," but I do not hesitate to say "no," because if I found this head so characteristic, so specific in its structure, that no possible confusion can take place; if I 262 saw it with the exact size, form, vesicle, transverse cut, articulary facet, in honor and conscience I could not declare that I have not seen the head of a human spermatozoon. There does not exist, there could not exist, a morphologic element resembling this head in all its complexity. Has there ever been any hesitation in drawing a conclusion when a blood cell has been isolated? Has there been an author who has imagined not giving a conclusion in this case? And yet, a blood cell is a simple disc with no characteristics other than its circular form, the simplest figure of all, that of spores and a multitude of other infinitely small bodies. I know well that my affirmations can be dangerous, but at this moment, I am aware only of the concern of scientific truth, and I cannot concern myself with inexperienced experts. In all questions in legal medicine, even those which seem the simplest, pulmonary docimasia, for example, the assessment is only as good as the expert himself, not the procedure. I could not truly be held responsible for the faults committed, if committed by the greatest degree of freedom I dare to give to research; for example, Mittscherlich could be accused if chemists could take the reflections of a poorly covered gas-burner for a phosphorescent glow.

But such a case will never occur. I am sure that in working correctly, whole spermatozoa will always be found, or at least even in the most complicated of cases, a number of heads such that there can be no place for doubt. Moreover, tails will be found, which, it is true, have no major characteristic, but their weak staining, their diameter regularly taper-

ing into an extremely fine point, and especially, their total length, exactly measured, are certainly not to be neglected and will corroborate the convictions of the expert.

And if this extraordinary case occurs, the reaction of triiodide fortunately provides additional means to eradicate all doubt.

I have to examine the case where only the crystals would be produced, for unknown reasons. Since the works of Ratier, Bayard and Devergie, it is unanimous to refuse to give
/263 any value whatever to chemical reactions in the investigation of seminal stains. Tardieu, Casper, Von Hoffman, Taylor, Tourdes, Brouardel, Vibert, G. Pouchet, Lacassagne, Coutagne, Boutmy, Maschka, briefly, all the authoritative masters on the question, are in absolute agreement.[20] At the very most, there was found, formerly an observer who noted the presence of an albumin coagulable by heat—it does not exist in semen—permitting differentiation of this from other mucus; but this has been forgotten a long time. Outside of this, everything has been rejected, and with good reason,
/264 since only vague reactions, in no way justifying the pretentions of their authors, were proposed. But it is no longer a matter of an albumin or a mucus that one must try to distinguish by feeble nuance, but a new characteristic, specific principle, secreted exclusively by the testicle, a principle I encountered nowhere else, not even in animal semen, [which I discovered]; and, until there is proof to the contrary, its significance cannot be refused. No substance is more clearly characterized by its chemical properties, and such a delicate reaction, and none is easier to find. I hope, then, that the excommunication, legitimately levelled against chemical reactions applied to the investigation of semen, is not addressed to mine.

However, I know that a sort of sanction, of consecration, if you will, that only time, and the examination of numerous humors, can give, is necessary to a reaction of this importance. In awaiting it, here is the way I would conclude in difficult cases:

1) If I obtain crystals without any spermatozoan heads, I would say I am *probably* dealing with semen, and I would emphasize in my conclusions: "considering that no known humor other than human semen has up to the present given these crystals."

2) If I obtain crystals and, at the same time, only the heads of spermatozoa *perfectly characterized,* I would clearly conclude in the affirmative.

3) If I obtain only debris of spermatozoa, even with perfectly characterized heads, but without my reaction, I would still remain doubtful, considering that there can be found in animal semen spermatozoa resembling that of man, giving rise to an error, *although I don't believe it* for my part.

If I have not resolved in this work all the questions I proposed, I have no less the conviction of having rendered real service to experts in bringing them, by my triiddide reaction, a method as simple as it is easy for differentiating suspected stains in a few seconds; by the *modus faciendi* which I indicated for unraveling, I gave them the means for looking at large numbers of preparations, and finally, with
/265 crocein, I gave them the possibility of confidently concluding the presence of semen, even when an entire spermatozoa has not been isolated.

I am certain they can finish in less than two hours assessments which, before would have required perhaps many weeks, and that they can attain an absolute certainty in numerous cases where incontestably firm conclusions had been impossible.

Additional note: On April 6, the centenarian P.V. . . . died at the home for the aged at Guillotiere, at Lyon. Born July 20, 1794, he was, consequently, 102 years old. A police record mentioning the curious tatoos of P.V. . . . leaves no doubt as to the exact age of the old fellow, nor to his identity, a rather rare circumstance, considering it is believed that, most often, centenarians simply use the papers of their father. The autopsy was done at the laboratory of legal medicine by Professor Tripier, Doctor Pavot and myself. The seminal vesicles contained a reddish, rather thick liquid, containing spermatozoa, giving with crocein all the characteristics I have stated in this memoir. In addition, we found fatty globules and a large number of rose, blackberry-like, spiny corpuscles of various sizes in this liquid. After dessication, this liquid was revived with a few drops of water to separate the fat, and it then gave crystals with triiodide.

It remains definitive, then, a fact surrounded with all desirable guarantees and duly confirmed, that man's semen contains perfect spermatozoa up to extreme old age.

A. F.

References and Notes

1. Pope from 1198 to 1216
2. Lacassagne: *Précis de médecine judicaire,* p. 104
3. It was believed that conception could not have taken place if the woman had not consented to the act; from the fact that she was pregnant, it was agreed that she was voluntarily taken!
4. "Jurisconsults have judged virginity during fourteen hundred years, as they judged sorcery, and so many other cases, without understanding anything."
5. These globules, sometimes seen immobile and sometimes endowed with motion, are pointed out in almost all the first microscopical investigations of stains.
6. I find this name written Koblauch, just as often as Koblank
7. With our modern microscopes, it can be seen in its entirety
8. *Annales d'hygiene et de medecine legale,* vol. 27, 1867, p. 155
9. *Lehrbuch des Gerichtl. Medicine—Vierteljahrschr. f. gerichtl. Medicine* 1887, p. 318
10. Clément had already made the same reproach (Conférence de médecine légale, 1880)
11. These granulations exist in almost all stains; they are spores
12. Glycerinated gelatin of Kaiser: one part by weight of purest French gelatin in six parts of distilled water is allowed to soften for about two hours. Seven parts of chemically pure glycerin is then added and one gram of concentrated phenol is added to 100 grams of mixture. This is heated for 10-15 minutes with continual shaking, until the flakes formed by the addition of acetic acid have disappeared. This is filtered while hot on a very fine glass wool, still humid from washing by distilled water.
13. The biliary acids of man are different from those of pig, which are not identical to those of goose.
14. Florence. *Des taches de sang en méd. judicaire,* p. 79, 1885

15. *Las Manchas de Sangre,* Montivideo, 1894
16. This is why silver oxide must be employed, giving a pure crystallized product
17. Martineau, Tardieu, Schauenstein
18. Spontaneous crystallization observed in dried semen on glass slides is different, and diverse varieties of crystals can be found even in a single semen: that of horse contains at least two, if not three, which perhaps corresponds to as many spermins.
19. Cited by von Hoffmann
20. Bayard: chemical analysis is insufficient to resolve it . . . but microscopical examination permits better specification of the observed facts (*Méd. leg.,* p. 274).

Roussin (*Loc. cit.* p. 148): There are so many chemical reactions applied to the determination of semen; none of them characterize this secretion. The impotence of chemical means today has been demonstrated so well, and is so universally recognized, that it appears useless for us to insist on it.

Gorup Bezanès (*Traite d'analyse zoochimique,* 1875, trad. de L. Gautier, p. 422). But all these reactions are not sensitive enough to permit drawing a certain conclusion, and stains produced by different mucus give rise to similar reactions. Only confirmation of the presence of spermatozoa by microscopical examination can furnish positive indications.

Bouisin Briand and Chaudé: As for chemical reactions indicated by many authors, they have no value next to the preceding, and experience proves that almost all stains formed by mucus also show them.

Brouardel and Boutmy (*Annales,* 1880, p. 225): In effect, the chemical reactions which can serve to characterize the liquids of the organism are very restricted in number, and are generally limited to either coagulation by heat, or by a few reagents such as nitric acid, mercuric chloride, phenol or wine alcohol, or to various colorations which certain substances cause to appear in the material being examined.

As a result, the chemical reactions we have just presented, applied to the study of the organism, indicate the class of the material rather than its particular identity.

The presence of the anatomic element, which is always unique, by contrast, makes confusion impossible, and the expert is always able to give an opinion with every assurance, etc.

Vogel (*Vierteljahrschrift,* N.F. XXXVI, p. 160, 1882): Under these conditions, it is certain since there are no characteristic reactions that microscopical investigation of the morphological elements of semen stands alone, and that spermatozoa always remains the only certain sign.

Taylor (*Méd. lég.,* trad. de H. Coutagne, 823): There are no chemical reactions on which one can count with certainty for discovering seminal stains

Real Encyclopedie, v. IX, p. 31: Only the finding of spermatozoa by the microscope is a certain sign that one is dealing with a seminal stain.

A New Microchemical Reaction of the Sperma and its Application in Medico-legal Investigations*

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383 The search after a sure method to recognize stains caused by semen has been on account of its medico-legal importance, one of the most interesting and the most debated questions to which during the last years men interested in medico-legal questions have given their attention.

The surest and easiest method, which deals with the recognition of the spermatozoa, very often meets in practice with insurmountable difficulties, without even mentioning those rare, but not impossible cases, in which there is azoospermia (sperma without spermatozoa) and in which this method naturally loses all value.

Among the methods which have been proposed for the purpose of demonstrating in the best way possible the spermatozoa, it will be sufficient to mention those of Bayard,[1] of Schmidt,[2] of Koblank,[3] modified and perfected by Pincus,[4] by Hofmann,[5] by Longuet,[6] and the method of Unger[7] and Steilberger,[8] and these methods are surely the most simple and the most rapid, and are superior to all the other methods by the safety of the results.

384 But the spermatozoon, like other cellular structures, may through different agents, some chemical and others physical, undergo such changes, that it becomes hardly or not at all recognizable, thus making it impossible for the expert to make a decision with the certainty which the law demands. Given this insufficiency of histological methods, the idea naturally occurs to one to find means which are more appropriate and which have a larger field of application. Therefore, we have turned to chemistry and worked on the proposition that the sperma must contain certain special substances which must be capable of giving constant and characteristic reactions.

The first attempt in this direction was made by Orfila,[9] to whom it seemed that dilute nitric acid, although it caused a yellow color in organic liquids which contained albumin, did not change the color of the sperma, which contained none. The reaction of Orfila was not confirmed, and the opinion, that the sperma contained no albuminoid substances was proven to be erroneous by Posner,[10] who has given to the study of the sperma various valuable contributions, and more recently by Slowtzoff.[11]

To this is to be added that nitric acid, as proven by the research of Filomusi-Guelfi,[12] destroys the spermatozoa, thus interfering with histological tests. After the failure of this test, many years passed before this research was taken up again, and it was only in the year 1895 that Florence,[13] in a publication which made a great deal of commotion in the camp of legal medicine, showed a new micro-chemical method for the recognition of seminal stains, strengthening this with numerous comparative tests, none of which showed the same reaction.

The reagent which he mentioned is a concentrated solution of iodine in iodide of potash, in a proportion which makes a highly concentrated iodine salt; the triiodurate of this reagent, on contact with the sperma, causes a formation of strong yellow crystals. The reaction was constant, simple and very sensitive and could be applied with equal ease to dry as to liquid sperma, to fresh sperma as well as to stale sperma, whether in good or in bad state of preservation.

Florence considered the reaction as the product of the 385
action of the trijodure on an alkaloidal substance contained in the sperma, the virispermine, a substance not only specific to the sperma but characteristic of human sperma.

The problem seemed solved in a most brilliant and decisive way, and those interested in legal medicine hurried to repeat the tests made by Florence for the purpose of enlarging and deepening the tests and experiments with sperma.

In a short time the following works saw the light of day: The one of Lecco,[14] of Richter,[15] of Gumprecht,[16] of Struve,[17] of Tamassia,[18] of Mattei,[19] of Tolsky,[20] of Davidoff,[21] of Caneva,[22] of Witalinsky and Horszkiewicz,[23] of Bocarius,[24] of Perrando,[25] of Ponzio,[26] of Dwornitschenko,[27] of Centner and Ramsaizeff,[28] of Mary[29] of Korsunsky,[30] of De Crecchio,[31] of Johnston and Witney,[32] of Cruz,[33] of Grigorieff,[34] of Gutowsky,[35] of Okamoto,[36] of Goldschmidt,[37] of Beumer,[38] and of Kippenberger,[39] a mass of studies sufficiently vast, which in short time has dissected this grave and delicate argument.

The evidence of many of the works just cited points to the result that the claim of Florence is not sufficiently backed up by fact, and that not only the sperma of many animals, but also many different organic liquids, both physiological and pathological, show the same reaction very plainly.

And in consequence of this general opinion, not contradicted by anybody but Johnston and Witney, Strassmann[40] states that the reaction of Florence is not only not specific of the human sperma, but even that it can be absent in the presence of human sperma, when this is mixed with blood.

Other cases in which the reaction gives negative results are those in which the liquid sperma is in a state of advanced putrefaction.

These results have taken from the reaction of Florence the value claimed for it by its author and have limited the same to that of a preliminary test.

386 A negative result, according to Strassmann, does not authorize one to claim that seminal stains are absent, while Goldschmidt and Hager-Mez,[41] claim that the absence of this reaction is a sure proof of the absence of sperma.

In conclusion, the reaction of Florence, which according to the idea of the discoverer, was decisive proof of the indication of seminal stains, has an importance much inferior to that of the histological proof, and cannot alone solve the question.

Having thus shown the present state of the question, I will without further introduction show the result of my studies, which are the fruit of many tests and experiments, made in the course of several years. I have made use of a great deal of human sperma, taken in all cases, except two, from healthy and not from sterile individuals. I have made the tests with sperma not over a few minutes old, as well as with such as had dried on cloth, also with sperma kept in glass vessels which were sealed and with other in a state of putrefaction. The result has always been the same, and causes me to claim that this reaction is safe and belongs particularly to seminal liquid.

The reaction is made in the following way: Put a drop of the sperma on a covered glass. Add to this a small quantity of a watery saturated solution of picric acid, a quantity not more than one-half of the liquid to be examined. After a few seconds the spermatic liquid will become turbid, through the formation of a precipitate, which, limited at first to the point of contact of the two liquids, little by little mixes itself with the two, spreads over the whole drop, which acquires a yellow color and a turbid appearance.

After a few minutes, between two and five generally, the reaction can be said to be complete, and after having placed on top of the liquid a cover glass, one proceeds with the microscopical examination.

If instead of liquid sperma one has dried sperma, all one has to do is to soften it with a little water, the same as one would do, if the sperma were dried on cloth. Then one adds to the water liquid, which should not be diluted with the picric acid solution.

387 If the liquid was very diluted, the formation of the precipitate would be slower; also, if there was an excess of picric acid, or the picric acid solution was too weak, the reaction would not be so clear, and the precipitate might assume a granular form, causing it to lose partly or entirely its demonstrative value.

The picric acid, besides being used in aqueous solution, can also be used in a saturated solution of absolute alcohol; in fact, in certain cases, as for example in cases in which one has to deal with putrefied sperma, the latter gives better results.

But in the case in which one takes the alcoholic solution, one must, to avoid the mixing, which would follow the contact of the two liquids, and which would cause a too rapid evaporation of this hydro-alcoholic mixture, reduce the quantity of liquid to be examined very much, in fact use less than a pin's head, and also reduce very much the quantity of the picric acid to be used.

Thus I would advise to begin the tests with a very small quantity of picric acid, which is to be added to the solution to be tested by means of a platinum needle and then after a few minutes one can add a little more, for the purpose of obtaining a diffused and noticeable turbidity.

Further, it is sufficient to make two or three preparations with liquid sperma or with an aqueous extract of a seminal stain, for the purpose of ascertaining the proper proportions of the two liquids, so as to be sure to obtain perfect preparations.

One thing more in regard to the cover glass. This should be neither too small, so that the liquid does not run over the sides, nor too large. Thus, by placing the cover glass carefully on the drop, which has first been deposited on the slide, the crystals will remain together and are not squeezed out of shape.

The microscopical examination, which is made under an enlargement of from 400 to 600 diameters, shows that the precipitate resulting from the reaction consists of small crystals, yellow and strongly refractive. One look at the attached plate, which reproduces faithfully these forms, designed by means of the camera lucida, will teach as much as the most minute and accurate description.

Without entering for the moment into the question of the 388
shape and of the crystallographic points of these crystals, which would be a rather complex and difficult question, I will only say, that these crystals, which are four or five times longer than they are wide, are very thin, appear like needles with rhombic circumference, and traversed longitudinally by a rifrangent line, which has the appearance of an edge. The obtuse angles seem always to be worn off and rounded, and in not well shaped crystals even the other angles have often the same appearance. In the last case the crystal appears to have the shape of an ovoid body, sometimes more, sometimes less elongated, which in extreme cases assumes the shape of a round disk. From this shape to the perfect rhombical shape we find a whole line of intermediate forms, which represent different stages of development, and which are very numerous and characteristic.

Besides isolated crystals, which prevail in number, one finds also twin crystals, others hanging together in the shape of a cross and others again in clusters. The table shows various forms of collections, as well pure as imperfect formations, in which only polarized light can reveal an entirely

* Translation of: "Nuova Reaziona Microchimica della Sperma e sua Applicazione nelle Ricerche Mediee legali."

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crystalline structure.

I have already mentioned that these crystals are very rifrangent. Now I will state that they are also very much birifrangent. The examination with polarized light gives the proof of this, as also it permits various vivid colors of interference to be observed with an enlargement of 60 diameters.

The exact measurement of the angles was not possible to me, in view of the smallness of the crystals and their imperfect formation, and the values vary from eight to ten degrees. The size of the crystals varies from 5 microns to 20 and more microns, although I do not claim that there cannot be smaller or in good conditions larger ones. The medium size, however, which responds to the majority of forms seen, and which are produced under ordinary conditions, varies from ten to fifteen microns.

The reaction can be noticed as well in acid as in alkaline media, as long as neither acidity nor alkalinity surpass certain limits. So, instead of the picric acid solution the picro
389 citric acid solution of Esbach can be used or a solution of picrate of ammonia.

But I prefer the picric acid solution to all others.

The reaction is very sensitive and sufficient to detect the very smallest quantity of sperma. It can be produced as well in fresh sperma as in dried sperma or in sperma which is in a state of putrefaction. And, in respect to putrefied sperma, the reaction is much more sensitive than that of Florence, so much so in fact, that I have received positive results in cases in which the reaction of Florence has failed. The presence of blood, as long as it is not in excessive quantities, does not disturb the reaction. Neither does the action of heat, within certain limits, make any difference.

At 10 degrees the liquid sperma as well as the dessicated sperma give a positive reaction, even after several hours.

At a high temperature the effect with the two kinds of sperma (liquid or dried) is different. Liquid sperma, which is kept at a temperature of 110 degrees for an hour does no more give the reaction. At 132 degrees ten minutes is sufficient to give a negative result; at 143 to 146 degrees five minutes. Sperma, however, which is dried in the clothing, is capable to stand a heat of 150 degrees for an hour, without interfering with the reaction. But carried to higher temperature the reaction shows less well, and carried to 200 degrees a few minutes suffice to cause the reaction to fail. I have stated that a heating to one hundred degrees does not harm, but I could even say that it helps the reaction, in the sense that the crystals which are formed are more beautiful and better formed.

The best preparations I have obtained from stains on linen, which had been exposed for an hour to a temperature of 130 in a dry oven.

In regards to the age I will submit the following data: Putrefied sperma, kept in well stoppered bottle gives a positive result after eight months. Dried sperma on linen even after three years. This I have verified on sperma kindly furnished to me through the courtesy of the celebrated Prof. Corrado, director of the Medico-Legal Institute of this University, to whom I owe for this, as well as other assistance, the most heartfelt gratitude.

To make a durable preparation the preparation must be 390
dried, then quickly washed in water, dried in blotting paper, again washed with alcohol, bathed in xilol and lastly mounted in Balsam.

The question now arises, whether these crystals represent a definite combination between some organic principle of the sperma and picric acid, or result from a union of the picric acid with the inorganic bases of the sperma, if not only with substances due to the decomposition of the sperma? Which means to say, that either this reaction has a useful value in practice, or owing to a substance which does not belong exclusively to the sperma, has no value whatsoever.

Yet the question must be answered, what is this substance, and whence does it come?

I might multiply these questions, but for the moment I will limit myself to those which form the kernel of the question and which are the most obvious. So let us commence with the most simple hypothesis, that we have to deal simply with picric acid crystals. However, we must observe that the formation of picric acid crystals in consequence of the mixing of a solution of this acid with an aqueous solution which is rich in bases with which it could easily enter into combination is not a fact which can be brought in harmony with the laws of chemistry. And even if the acid had been added in excess, even if only a part remain free, this would remain in solution, and could not become deposited except in consequence of the concentration of the liquid beyond the limit of saturation.

In our case, however, the reaction appears at once and the crystals form before such an evaporation could take place. Besides there exists a noticeable difference between picric acid crystals and those which form through the action of the acid on the sperma.

The acid crystals are formed of rhombic prisms, which show an entirely different formation under the microscope. Besides the different shape of the crystals, the difference in color must be noticed.

Picric acid crystals show under the microscope a very slight yellow color, hardly visible with strong enlargement, while our specific crystals, even when observed with an enlargement of 600 diameters and more, show always a de- 391
cided yellow.

Lastly, I wish to observe that the best solvents of picric acid are Benzel, which at ordinary temperature dissolves from 8 to 10 percent (Fritzsche), and in Xilol, which dissolves even 14 percent.[42] Both Xilol and Benzel, however, are the worst solvents of the specific crystals.

A second objection might be that we have to deal with an alkaline picrate and more especially with picrate of potassium, which among all is the least soluble. One need not think of either the picrate of sodium or of calcium, because they are very soluble in water, the first in from 10 to 14 parts, at 15 degrees, the second in a like measure at a temperature of 20 degrees. One may also exclude the possibility that one

has to deal with picrate of ammonia, as ammonia, as is proven by all the analyses, both of Liebermann,[43] as well as of Slowtzoff, is not a usual part of sperma, and is not formed in the same except as a result of decomposition. And the sperma reacts to the picric acid within a few seconds after ejaculation, and besides, the reagent of Nessler, which is, as everyone knows, very sensitive to ammonium salts, does not show the slightest trace of the same.

The picrate of potassium again is a salt which is nearly insoluble in cold water, one part, according to Post and Mehrtens soluble in 228.17 parts at 15 degrees. My crystals however are easily soluble in water.

It is not easy to determine what the substance may be which causes the sperma to react, as it does, to the picric acid. The fact that the reaction fails, after the sperma has been subjected to 200 degrees, justifies the suspicion that one has to deal with an organic substance. A fact, which must be kept in mind, is that the reaction can be obtained as well in acid as in alkaline or neutral solution, analogous to the one which Popoff[46] has found in the alkaloids.

In regards to the specific character of the reaction I wish to state, that no matter how many substances I have examined, including vaginal mucus, nasal mucus, sputum and so forth, none has given the same result, none have formed the same crystals as the seminal liquid.

392 The only substance, which when treated with picric acid gives similar, however not like results, is Poehl's spermin. This latter, however, heated for half an hour to one hundred degrees loses the power of forming a crystalline precipitate with the picric acid, forming instead a precipitate consisting of granules which appear oily and which possess not the slightest birefringence.

Lastly, I wish to state, that the seminal vesicles after death fail to show the reaction. Thus without stating what causes the reaction, I will only say that it is due to an organic substance, which has nothing to do with the one which produces the reaction of Florence, and which is contained also in the sperma of sterile individuals.

This reaction has, besides the advantage, that it can give durable preparations, also that advantage over the reaction of Florence, that it is absent in the presence of other substances than sperma, and which react under Florence's reagent like sperma.

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On Forensic Identification of Semen and Semen Stains*

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/2131 Among legal medicine's effective investigations, the identification of semen stains occupies a quantitatively dominant role, particularly on clothing. The methods that have been used until now are either chemical or physical reactions or histologic proof of spermatozoa.

The chemical reactions that are most often used, stem from the time around the turn of the century, when knowledge of the characteristic components and complete chemical content of semen was scanty.

The classic Florence test is carried out by having a well-soaked extract of stains brought into contact with a strong iodine-potassium iodide solution on a microscope slide. In this way brown rhombic crystals can appear that presumably consist of iodine. The reaction is caused by the presence of choline, that can be thought to arise as a degradation product of lecithin that is found in semen in considerable amounts. Before the Florence test yields positive results, some decomposition of semen occurs, and thus the test often fails where it concerns itself with fresh semen stains. Since lecithin also can occur in other biologic material the reaction is not specific. In this connection it is especially unfortunate that vaginal secretions now and then yield positive reactions.

Another chemical test that has been put to use is the formation of calcium sulfate crystals by addition of sulfuric acid to a stain extract. Now the calcium content in the semen is about double the quantity that is in blood; thus, one may already reject the test for this lessens its diagnostic worth.

Semen's characteristic smell that by itself can be a valuable guide in the search for stains, is due to a base, spermin, that has occurred in considerably large amounts only in other secretions and excretions.

In many tissues, the liver and pancreas, for example, spermin is found in a concentration approximately one eighth the concentration of normal semen. In such tissues spermin is hardly extracted by means of simple treatment with salt water.

Many methods for identification of semen stains consist of the development of saturated solution binding to spermin with different acids. In this manner *Barberio* (1911) used picric acid as the crystallizing agent, but experience has proven that the reaction is most doubtful. In a comparative investigation of *Ziemke,* the Florence reaction was found positive in 70%, and Barberio's reaction positive in 26%, of the cases investigated.

Great interest is attached to *Puranen's* crystallizing method (1936). He used naphthol yellow S (sodium-2, 4-dinitro-1-naphtholsulfonate; sodium flavianate) as the reagent. In the presence of spermin, characteristic crooked cross shapes are formed with short cross beams providing crystals of spermine flavianate. This test is undoubtedly the best of the existing chemical methods, but also it suffers from serious deficiencies. Many commonly occurring textiles, certain colors of artificial silk, leather or natural silk materials, for example, cause the reaction to be lacking, presumably because of strong absorption. Often one can get a positive reaction by itself on these textiles by using the sufficiently small extraction volume. *B. Jonsson* has observed spermatozoa in a good number of instances, where Puranen's reaction appeared negative.

The possibility of getting a positive reaction although the semen is not present is certainly possible, as the aforementioned spermin content in other tissues and secretions and in different biologic materials can by no means be ignored.

A simple physical method for identification of stains that involves a great deal of extensive diffusion is the investigation in ultraviolet light. Semen can contain substances that fluoresce strongly under light with wave lengths from 4200 to 4900 Å. However, the reaction is by no means specific because firstly, semen does not always contain the fluorescent substance; secondly, fluorescence is seen in practically all the secretions or excretions that can be counted among some interfering sources (vaginal secretions, nasal secretions, urine, feces, etc.); and, thirdly, the fluorescence disappears with the mixture of, for example, blood. However the investigation with ultraviolet light has meaning in that it is a valuable means for the discovery of the suspected stains.

The purpose of the histologic methods is of course to identify spermatozoa. One can either identify these cells on the isolated stained fibers of the fabric, or after a thorough extraction one can centrifuge the extract and attempt to observe the sperm in the deposit. Unstained preparations are used at some institutions; at others, preparations are stained

* Translation of: "Om forensisk paavisning of sperma og spermapletter." in *Nordisk Medicin* 28 (42): 2131 2132 (1945).
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according to more or less specific methods. Among the staining methods, *Baecchi's* is reputed to be the most widespread. /2132 The stain consists of acid fuchsin and methylene blue in 1% hydrochloric acid, and reacts with the successful result that the main body of the sperm is colored red, while the tail remains blue. The methods have proven themselves satisfactory through investigations over many years. Here at home, the *Ellermann* staining method is also used sometimes.

Microscopical identification of spermatozoa does not cause any difficulty in general. When one takes into consideration that a normal ejaculation consists of about 100-200 million sperm per mℓ one can understand that it is usually no problem to detect the outermost characteristic cells in the microscope. Nevertheless, the method is not ideal for the following reasons: 1) the composition of the ejaculate is not constant during the ejaculation; the first part contains almost exclusively of prostate secretion, while the spermatozoa first appear only in the later phases of the ejaculation. Because the forensically most important stains are often not formed of homogeneous semen, one can get stains for the investigation that do not contain spermatozoa, although they are made up of parts of a standard ejaculation. 2) Oligospermia and total aspermia are not unusual conditions and can yield semen stains that cannot be visualized; 3) Sterilization by vasectomy is now undertaken so frequently that especially among sex offenders one can expect to find such instances in forensic practice.

For this reason it would be valuable to have a specific method for the identification of semen that is independent of the presence of sperm. If one pays attention to the recent chemical and biochemical investigations on the composition of semen, one will find that most substances in semen are found in an increasingly greater concentration than in the body's remaining tissues and secretions. Acid-soluble phosphate is found in a quantity of over 100 m% (m% = 1/1,000% = mg%), while the concentration in, for example, blood is around 30 m%. Now the phosphate is such a diffuse material that it will hardly be worthwhile to try to use it for evidence of semen, particularly considering that urine can contain significant quantities of phosphate itself.

Ascorbic acid is certain to occur in semen in concentrations about 10 times as high as in blood plasma; but this material is, of course, so unstable and generally also so widely distributed that it cannot be considered either.

In 1936 *Schersten* proved that semen contains a large quantity of citric acid, about 0.5%. When one has sensitive, even if somewhat difficult, methods for determination of citric acid, there should be a possibility here for working out a procedure for specific evidence of semen, yet certainly only on the assumption that other stain-forming materials do not contain citric acid in quantities that can be compared with quantities in semen. Investigations on the proportion of citric acids are in process at the Institute.

A possibility that finally appears more promising is using the phosphatase content for the diagnosis of semen and semen stains. In the prostate secretion, there is a phosphatase with an acid pH optimum in colossal quantities. The enzyme was first found by *Kutscher & Wolberg* in 1935 and has been later investigated biochemically by *Kutscher* and collaborators, and from a clinical point of view in particular by American writers. *Gutman & Gutman* find 2,000–3,000 phosphatase units per mℓ semen; of that, the lowest value among 43 ejaculates is 540 units per mℓ. 24 aspermic individuals, with the exception of one, had values inside the normal range.

Even though the enzyme in semen is found in a large concentration in some other biologic material, and enzymes generally endure drying out well, there were reasons to expect that this method could lead to very specific evidence of semen stains independent of the presence of sperm cells, possibly even to a practical, valuable forensic method. When, therefore, it was time for the present director of the Institute in 1943 to propose the university's prize task in theoretical medicine, the author suggested to Prof. *Knud Sand* that the task be an experimental investigation on the use of phosphatase determination for identification of semen stains. The current examination papers, of which three have been rewarded with the University's gold medal, have shown that our expectations were justified. The three authors intend to publish the results of their investigations in the near future.

Dr. Paul Uhlenhuth 1870 1957
Courtesy National Library of Medicine

Section 3. Determination of Species of Origin

The oldest systematic species test is that of J.-P. Barruel. He said that concentrated sulfuric acid evoked an odiferous principle from blood and bloodstains, which could identify the source of the blood. The method was taken seriously for quite a number of years, although it seems absurd by present day standards. Casanti's proposal was not much more enlightened.

After the microscope gained popularity, there was considerable interest in, and controversy over, the micrometric method of determining species of origin. Even the illustrious Rudolf Virchow wrote a paper on the subject. The papers of Roussin, Masson and Vibert all deal with micrometry, and Masson gives an historical review as well.

The modern period began in 1901 with the first paper by Paul Uhlenhuth. There is no question that other fundamental immunological findings of the 1890's provided the groundwork for the immunological species tests, but Uhlenhuth's paper was the first to apply the immunological test to medico-legal species determination. Uhlenhuth (1870-1957) was an immunologist all his life. His obituary appeared in the *Zeitschrift für Immunitätsforschung und Experimentelle Therapie* **115** (4) for 1958, along with a list of his numerous publications. The 1900 paper on ovalbumin has been included, since the serum work so clearly evolved from it. A representative set of the 1901 papers has been included, as has an interesting account of his personal recollections of the early years which he was persuaded to write, and which appeared in 1949.

Wassermann and Schütze published the precipitin method almost simultaneously—about a week after Uhlenhuth's paper—and they are clearly to be regarded as joint originators of the technique. Neisser and Sachs introduced the complement fixation procedure for detecting the species antigen-antibody reaction in the medico-legal test.

Marx and Ehrnrooth's method was based on the agglutination of human cells by animal sera. It was actually a method for demonstrating the absence of human blood. Lattes in 1913 (see in Section 4) devoted a section of his paper to this procedure, which is closely related to isoagglutination, and hence, to blood grouping.

Memoir on the Existence of a Principle Peculiar to Blood to Characterize the Blood of Man and of Various Animal Species*

J. P. Barruel

267 Blood has been the object of the meditations of the most ancient philosophers; the imporant functions it fulfills, and the changes it undergoes in nutrition, have not ceased to occupy physiologists; finally, chemists make it the daily subject of their research. If it were left to me to express an opinion on the whole of these works, I would say that, numerous and important as they might be, they aren't sufficient for establishing satisfactory theories on "sanguification" or hematosis, the nutrition or organic composition, the warmth of or the secretions in blood. But it is not my object here to discuss, or even to recall the different theories, which try to explain the role blood plays in the phenomena of life. I find it sufficient to recall that chemists have already found a large number of principles in this liquid which participate not only in the composition of organs but also of the secreted products. Thus, it has long been known that the purified fibrin of blood is similar to muscle fiber, which differs only in its
268 organization; that *serum* is of the same nature as certain secretions destined for the mechanism and activity of various organs. We are all aware of the results of the research of the celebrated Vauquelin, who reported the existence of fat in blood, and of the experiments of Chevreul, who found in fibrin a fatty matter analogous to that found in cerebral matter. Finally, we have lately seen that, when the kidneys of an animal are removed, the blood contains urea a short time afterward. Nor are we ignorant of the existence in blood of phosphate and sodium carbonate, the bases of all bones.

Chyme, the immediate product of digestion, contains the elements of all known animal matter. Couldn't it be possible that, by the single act of "sanguification," the elements of air absorbed by respiration, in the course of circulation and under the influence of life, determine the reaction of these elements, their combinations in different proportions, from which would result the formation, in necessary quantity, of all the materials specific for constituting and renewing organs and furnishing the secreted fluids? I leave to the physiologists the difficult task of clarifying this scientific point. I should and will limit myself to what is related to the single fact, which is the subject of this memoir, and to the consequences derived from it.

Blood is divided into red or arterial blood and black or venous blood. The former blackens in a few hours when completely deprived of atmospheric air; the second reddens in a few seconds on immediate contact with oxygenated gas or atmospheric air. Hydrocarbon and carbon monoxide gas do not give venous blood a ruby color, as has been supposed. The action of oxygen gas on black blood demonstrated to me 269
a phenomenon worthy of note, and which strongly merits, I believe, the attention of physiologists; it happened that this liquid, preserved for several weeks, still possessed the property of becoming ruby colored even though some of its elements, especially fibrin and albumin, had already submitted to the immutable law attracting all things of which decomposition is only the result. It would appear that the coloring matter of blood, on which oxygen is preferentially carried, is endowed with a great assimilating or vital force, which is extinguished only a long time after the complete death of all the other immediate principles of the same liquid.

Moreover, whatever opinion can be expressed on the functions and nature of the coloring matter of blood, whether it comes from arterial blood, or whether it comes from venous blood a few minutes after extraction from the vessels where it circulates, I maintain that this substance has the same physical characteristics and that it is the only principle which distinguishes blood from all the other animal fluids. For the property of coagulating at rest and of dividing into a solid mass, or clot, and a liquid, or serum, is not exclusive to blood, but belongs also to chyme; likewise the property of hardening by the action of heat, acid or alcohol is not exclusive to it either, for all types of albumin possess it.

Brande, in England, and *Vauquelin,* in France, have already tried obtaining this coloring principle of blood separate from all other substances of this fluid. But with the procedures indicated by these chemists, it is never obtained 270
pure; it is always accompanied by a rather considerable proportion of albumin. Filtration does not give any better results because the coloring matter of blood is so fine it sifts through the tightest filters. It is also accompanied by serum of blood, from which it results that albumin cannot be entirely separated from it for the study of its chemical characteristics, but that is of little import.

It is sufficient to acknowledge that, either this coloring substance still enjoys the faculty of becoming ruby-colored on contact with air, or, deprived of this property, the manner in which it behaves under the influence of heat is the same, differing in one or the other of these conditions only in the following property: 1) in the first case, when blood is diluted with water, the solution takes on a ruby-red color and in the second it has a red-wine color; 2) blood dried in air takes on

* Translation of: "Mémoire sur l'existence d'un principe propre a caractériser le sang de l'homme et celui des diverses espèces d'animaux." in *Annales d'Hygiène Publique et de Médecine Légale* 1: 267-277 (1829).

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a red-wine color when treated with water, because the very act of dessication suffices to extinguish in blood the faculty of the coloring substance in changing to ruby red on contact with air.

It is in the very action of heat on the coloring substance of blood that resides the truly distinctive characteristic of this principle, which, as I have pointed out, is always accompanied by albumin.

I do not believe it necessary to recall here the details relative to this action because they have been faultlessly presented in the memoir published by Professor Orfila, in response to a work by a distinguished scientist, who alleged
271 it impossible, with our present level of knowledge, to decide in medico-legal cases if stains on linen were stains of blood or some other coloring substance. In this work, the scientist claimed to have composed a fluid which, even though containing no blood, possessed, however, all its properties. Orfila demonstrated beyond doubt that, up to the present, a liquid could not be composed, whose coloring matter exhibited the same chemical characteristics as those of the coloring matter of blood. It is important to acknowledge also that these characteristics are preserved in their integrity in blood dried in air, even after several years, fortunately permitting after a long lapse of time the immediate confirmation that stains are due to blood or another substance altogether. I will add that these characteristics are the same in the coloring principle of blood of all animal species.

Though chemists have been able for a long time now to pronounce and affirm before the magistrates in all tranquility of conscience that stains, provided they are extensive enough or at least numerous enough, are due to blood or some other coloring matter (three or four drops are sufficient to obtain this result), it is quite different when the authorities ask them if they can likewise say if these stains are formed by human blood, or the blood of another animal. I well know that already, in a few cases of this nature, fortunately very rare, some authorities have confirmed that the blood stains, which they were charged with examining, were produced by human blood, but they gave none of the grounds on which their opinion was based. It seems to me that when the life of
272 an innocent or the punishment of a guilty person might depend on the opinion of an expert, one cannot be too conservative, and that nothing should ever be affirmed in cases of this type without the support of positive proof, not hypothetical proof. One must never lose sight of the wise old adage: *When in doubt, refrain.*

As for myself, in a great number of instances, I have been charged by magistrates with determining if stains, perceived on clothing of people suspected of having committed homicide, were blood stains or stains of another nature. I never hesitated to pronounce the affirmative when I could find the principal characteristic of the coloring matter of blood in these stains, because I know of no other material possessing it; but when I was asked if these stains were of human blood, I never hesitated to reply it was impossible for me to express an opinion in this regard, because I knew of no trait in the blood of each animal species serving to characterize it.

Actually, the brilliant research with the microscope by Prévost and Dumas have demonstrated that blood is composed of serum in which float globules of form and dimension different in man and in animals. But besides the fact that these differences are only slightly marked, if not entirely non-existent, between individuals belonging to neighboring species, not everyone is familiar with observations with a microscope, an instrument not very widespread, and consequently at the disposal of a small number of people. Besides, the form of various globules can only be recognized inasmuch as the blood has not lost its liquid form. For as soon as it has been dried on any object whatever, if this blood 273
is diluted in water, the resulting solution presents nothing distinctive, and it is almost always dried blood stains that chemists are called upon to test. Thus, the discovery of Prévost and Dumas can be only very rarely applied to cases of homicide and legal medicine.

For many years, in seeking to obtain the coloring matter of blood by the procedure given to us by Vauquelin, which consists of boiling the blood clot for a while in fairly concentrated sulfuric acid, and having employed in this context a clot of beef blood, I was struck by the strong odor of a cattle barn which emanated from it. This fact remained engraved in my memory, without my looking to derive any consequences from it until, lately, a very peculiar circumstance permitted me to observe an analogous fact: an individual decided to commit suicide after a considerable gambling loss and swallowed for this purpose a considerable quantity of opium. This deadly design was known about almost as soon as executed, Orfila was called on, arriving just in time to save the patient; and because among the means employed in fighting the effect of the poison was a profuse bleeding, Orfila profited from this circumstance to look into whether blood from persons under the influence of a large quantity of opium didn't contain traces of morphine. With this intention in mind, he brought me this blood and invited me to do the necessary research.

I began by coagulating the blood in a water-bath, to be 274
able to more easily divide it by crushing, which I did without perceiving any odor. I then heated the divided blood to the boiling point with an ample quantity of sulfuric acid diluted with water, and there immediately escaped from the round-bottom flask which I was using an odor of human sweat so intense that it permeated the laboratory to the point where I was forced to abandon it for a few moments. This reminded me of the odor which manifested itself when I was extracting the coloring principle of blood by the procedure of Vauquelin, and from that moment I imagined the possibility of arriving at distinguishing the blood of various animals from that of man. It was with this in mind that I took up numerous experiments, of which the principal results are:

1) That blood of each animal species contains a principle peculiar to each of them.

2) That this principle, which is very volatile, has an odor similar to that of sweat, or cutaneous and pulmonary ex-

halation, of the animal from which the blood comes.

3) That this volatile principle is bound in the blood and, inasmuch as this combination exists, it is not discernible.

4) That when this combination is ruptured, the principle of blood which gives off the odor volatilizes and, from then on, it is not only possible but even rather easy to recognize the animal to whom it belongs.

5) That in each animal species the principle of odor of blood is much more pronounced, or, in other terms, has more of an intensity in the blood of the male than that of the female, and that in man hair color brings nuances to the odor of this principle.

275 6) That the binding of this principle of odor is in a state of solution in the blood, which permits its development, either in whole blood, or in blood deprived of fibrin, or in blood serum.

7) Lastly, that of all the means I employed to liberate the principle of odor of blood, concentrated sulfuric acid gave best results.

It suffices, to obtain these results, to pour a few drops of blood or blood serum in a glass; then to pour a slight excess of concentrated sulfuric acid into it, about a third or a half of the volume of blood, and to stir with a glass rod: the aromatic principle immediately manifests itself. It is by these means that I easily distinguish all the bloods which I am going to name in designating the odor peculiar to each of them.

1) That of man releases a strong odor of the sweat of man, which is impossible to confuse with any other.

2) That of woman, an analogous odor, but much less strong, in short, that of the sweat of woman.

3) That of beef, a strong odor of cattle barn or of beef manure.

4) That of horse, a strong odor of horse sweat or of horse droppings.

5) That of sheep, a vivid odor of wool impregnated with its sweat.

6) That of ewe, an odor analogous to that of sheep mixed with a strong odor of billy goat.

276 7) That of dog, the odor of perspiration of dog.

8) That of hog, a disagreeable odor of a pig sty.

9) That of rat diffuses a disagreeable odor of rat.

Analogous results are obtained with the blood of various birds: thus the blood of hen, of turkey, of duck and of pigeon release a particular odor peculiar to each of them. Finally, I just recently experimented on frog blood. It released a strongly pronounced odor of marsh reeds, and the blood of carp furnished an odor principle similar to that of mucus covering the body of fresh-water fish.

It was important to experiment to see if it were still possible to distinguish the aromatic principle of each blood with blood stains applied to linen and dried. I assured myself by direct experiments that, provided the stain was of a certain size, it was easy to recognize with what blood it had been produced, even after more than two weeks. For this, it suffices to cut out a portion of the stained linen, to put it in a watch glass, to pour a small amount of water on it and to leave it to rest for a while. When the stain is well-moistened, concentrated sulfuric acid is poured on it, it is stirred with a rod and sniffed. I don't know if after a more considerable lapse of time the species of blood on the linen might still be characterized. When in doubt, I believe it necessary to recommend to the examining magistrates, when they are charged with investigating a person accused of homicide, to delay as little as possible the experiments which the experts must do to determine not only if the stains observed on the 277
clothing are due to blood, but particularly to designate their species.

I believe it necessary to urge the physicians and pharmacists who, by their status, are ordinarily requested by the magistrates in these instances, to repeat my experiments to educate their sense of smell, so to speak. For, if the odor of the aromatic principle of certain blood is so strong that it suffices to have smelled it once never to forget it; if it is, so to speak, impossible to confuse human blood with that of other animals, it is only after having experimented a certain number of times with human blood that the blood of man can be differentiated from that of woman, and important services might then be rendered to the magistracy in the case of a suspicion of homicide, in certain cases of actual and alleged rape, and especially cases of pretended defloration.

I will stop myself here. What I have said here suffices, I believe, for everything in relation to legal medicine. But I have not yet satisfied science, for she will ask me of what nature is the aromatic principle of blood. I reply that this will be the subject of the continuation of my research; but that, at this moment, I have strong reasons to think it a very peculiar acid substance and that it exists in blood as a salt.

A New Way of Distinguishing Human Blood from that of Other Mammals*

Casanti

673 The attempts by means of sulfuric acid with this purpose in mind are known. Casanti employed phosphoric acid of a density of 1.18, but following principles and with the intention of what actually constituted a new method.

A first necessity was that of finding means of distinguishing blood of a mammal from that of another vertebrate, of *Gallinaceae,* for example. For this, after collection of the blood and its reduction by evaporation into a dry substance, it is treated with excess phosphoric acid. It is noted that mammalian blood enjoys the property of agglutinating in a brilliant, homogeneous, coherent, somewhat stiff mass, whereas that of the Gallinaceae is entirely lacking in this characteristic. This state of agglutination is distinct from coagulation in that in the first case the accumulated blood not only does not soften and no longer liquefies when left under the same conditions, but on the contrary, contracts, hardens and becomes almost tough, does not adhere at all to solid bodies, and does not change its characteristics even when heated up to 100°.

This having been determined, the author sought a more specific differentiation for the blood of man. Six grains of this blood, reduced to a fine powder, then 9 grains of phosphoric acid were placed in a glass. On stirring with a glass rod, the blood was observed to swell and soften; its particles attracted each other and adhered together, then united in a very brilliant mass of the color of liver, of the consistency of a very dense, non-glutinous extract, very coherent and having a lot of plasticity. Compression with the glass rod causes it to yield to the pressure without dividing, becoming, on the
674 contrary, more homogeneous, more coherent as it is pressed for a longer time. Left to itself, it becomes harder, more difficult to break, without losing its brilliance.

Performing the same experiment with horse blood, the phenomena were entirely different. Blood molecules penetrated by the acid at first swelled and softened. But, instead of uniting to form a single homogeneous mass, they formed diverse lumps the color of liver, very hard and brilliant, obstinately refusing to adhere to each other. Pressed by the glass rod, they did not appear very coherent or very hard and were almost entirely lacking in plasticity, explaining their division into several parts, and of these into successively smaller parts; the more one tries to unite them, the more they separate into fine particles which lose their sheen quite rapidly.

Casanti experimented on blood of ox, calf, mule, mare, pig, roebuck and waterhog, and the results were always the same as those of horse. The blood of cat presents a few differences. It becomes a single homogeneous mass at first, like that of man; but it shows a lesser density, coherence and toughness, and it suffices to compress or fold it to see it instantly divide into several parts.

The author repeated these experiments numerous times, always with identical results. He also remarked that human blood presents the same properties despite differences in age, sex, health or various diseases.

The applications of this discovery in legal medicine, and especially in those cases where the purpose is to shed light on the investigations of criminal justice, are self-evident. However, human blood presents a different aspect in a particular case: that of menstruation. The author has twice seen the reaction of menstrual blood. Addition of phosphoric acid 675
provoked a homogeneous mass, yielding to pressure; but it was so lacking in coherence that it sufficed to compress it for an instant or to fold it, to reduce it to a mass of dry, swollen particles no longer able to be united into a *whole.* These characteristics will surely differentiate menstrual blood from that coming from any other part of the vacular system.

Editors note. We are contemplating, along with Lassaigne, doing the experiments to discover the value of the procedure reported by Casanti.

* Translation of: "Nouvelle Manière de Distinguer le Sang Humain de Celui des Autres Mammifères."

in *Journal de Chimie Médicale de Pharmacie et de Toxicologie* 4 (3rd series): 673-675 (1848).

On the Forensic Investigation of Dried Bloodstains*

Rudolf Virchow

334 The various methods of testing dried blood stains were recently submitted to earnest criticism by Brücke (*Wiener medic. Wochenschrift,* 1857, no. 23), and they were, at the same time, substantially expanded by this perceptive observer. By chance I was recently in a position to conduct several such examinations for forensic purposes. In these two cases, following closely upon one another, a large wooden pole and two very dirty coats were handed over to me to determine whether there were stains of human blood on these objects. I took this opportunity to make a few different examinations to test the usefulness of these various methods.

As has been recognized, the first question which the researcher must solve is always whether blood is even involved; then come the more specific questions whether mammal, and finally, whether human blood is present. For the first question, we can find an approximately certain solution by means of a strictly chemical method; for an answer to both of the other questions, I believe, as do all the more sober research-
335 ers, that we can rely only on the microscope. Even for determining whether blood is present at all, the microscope can provide a much more dependable decision than the purely chemical examination.

Here I believe I must point out from the outset that we have totally neglected one morphological component of the blood, namely, the colorless blood corpuscles. It was not really the case that I came to this idea after first having busied myself with these elements, but rather I came to it through simple experience. As I treated dried blood drops with the media usually suggested (water, salt water, iodine water, sulphuric acid and acetic acid-containing water), it turned out that I obtained every time very clear bodies which resembled completely the colorless corpuscles in form, size, content, and nucleus, and which, more than any other part of the blood, resisted the various effects of being dried and then dissolved again in solution. I was able to measure very easily not just the whole corpuscles but also the nuclei, and was able to compare them with other known, colorless corpuscles. The value of this experience is obvious. The colorless blood corpuscles do not have any characteristics specific enough that their discovery would be sufficient, in itself, to prove that any organic substance whatsoever is blood or contains blood, but their discovery, along with the other determining signs, strengthens substantially the probability of the diagnosis; indeed, one could say that their absence very much lowers the probability that one is dealing with blood. Although stains from pus could contain the same corpuscles, this objection has not much validity, since pus often is mixed with enough blood that the usual blood tests will also not be correct in this case. Here, however, is a fact of very great importance, namely, the determination of the number of colorless corpuscles in comparision to the size of the stain under examination. If a great many such corpuscles are present, then it is likely that one is dealing with pus, a purulent mucus, or some similar, pathological product. If relatively few are present, then it is probable that these are colorless blood corpuscles. The possibility of leukemia should be kept in mind, but in view of the rarity of the disease, this problem recedes into the background. In one forensic case I counted in a particle of dried blood of $\frac{1}{300}$ inch (Par.)† 7, in one of $\frac{1}{200}$ inch (Par.)† 5 of such corpuscles which on the average measured 0.004 to 0.006 lines across. Not all of these, however, were located in the same place; some were found in different layers of the drop.

It would be incomparably more important if one could find red corpuscles in a suspicious stain and could also measure these corpuscles. If these proved not to have nuclei, one could declare these the corpuscles of mammals or humans, and measuring would finally determine whether one was dealing with one or the other species. The latter is now in fact recommended and maintained by C. Schmidt. I know, however, of only one single case—and that from an uncertain description (*Med. Times and Gaz.* 1857, April, No. 354, p. 365)—in which such an assertion, based on measurement, 336
was involved in a judicial decision; namely a case at Taunton in which Herapath had used the microscopical examination. In evaluating this method, I can only agree with the damning judgment of Brüke, and I do not believe that any microscopist would consider himself justified in placing a man's life in question as the result of the uncertain estimation of the drying coefficient of a blood corpuscle. Blood, of course, at times dries so that one can still clearly recognize the individual corpuscles, if one moistens the dried blood with oil. In some cases turpentine oil is still better suited for this task, while glycerine has almost always failed in my tests. The drying process, alone, is subject to so many conditions, and, after drying, the blood can be exposed to so many unfavorable influences, that a judgment concerning the size of individual components can make no claim to be reliable.

* Translation of: "Ueber die forensische Untersuchung von trockenen Blutflecken."

in *Archiv für pathologische Anatomie und Physiologie und für Klinische Medizin* [Virchow's Archiv] 12: 334-338 (1857).

† [Note: The term in the original article was "Zoll Par.," and may be a reference to an old micrometric measure called a Paris Line, which was equal to 0.0888 inch. In present day German, "Zoll" would be translated as "inch" in this context.]

In the cases which I examined, apparently moisture had had an effect; mold had formed, and neither fatty nor volatile oil enabled me to perceive anything of the blood corpuscles. Nevertheless, it is certainly justified in every case to submit the substances to testing.

Among the substances for moistening the blood and separating the individual blood corpuscles, most researchers use only a very limited number in those cases where the blood has been preserved under unfavorable circumstances. I, however, have found one substance to be very valuable, a substance which Donders mentioned casually some time ago, namely, concentrated potassium hydroxide. Kölliker also mentions this special characteristic, that the blood corpuscles preserve well in potassium hydroxide, if that chemical is concentrated, while they break up when it is diluted. If, to the dried blood which has been divided into smaller fragments, one adds directly the concentrated reagent, one can see after a short time the individual red-colored little globules outlined clearly on the surface of the fragment; not infrequently, one sees individual corpuscles separate themselves from the main body, bodies which reveal their nature as red blood corpuscles by their mobility, their more flatly-rounded shape, and their gold-green hue. Thus, the dichroism of the hemoglobin, emphasized by Brücke, is validated in this fashion. At times, it is also possible to carry out specific measurements.

There now remains a third morphological component of the blood, namely, fibrin, the identification of which completes the microscopical diagnosis. One recognizes it clearly as the binding agent of blood fragments if one treats these fragments for some time with water. Its character, now fibrous and now more pleated and homogeneous, makes it stand out. The most likely source of error in identifying fibrin is mucus, but mucus has a much greater swelling capacity. It also has the characteristic that it coagulates with acetic acid, while fibrin contracts greatly at first but then swells up and becomes transparent. These differences make distinguishing the two easy. Moreover, while examining the above-mentioned pole which had blood-red, gleaming spots on it, marks very similar to blood stains, I happened to meet with a colorless substance which very much resembled fibrin. This, however, produced with iodine very beautiful blue colorations, and it seemed that a pasty substance, apparently of 137
plant amylases, formed all of the efflorescence, whose apparent coloration was caused only by the underlying brown bark of the wood.

Although it was possible to identify successfully the three morphological components of the blood (red and colorless corpuscles along with fibrin) both with the microscope (and with microchemistry), it is still obvious that the chemical identification is not very successful with albumin, salt, and extractive substances, especially when one has only very small and impure particles to test. The chief task is the identification of hematin. The older methods are well-known, although one also knows that they were not very dependable, and that many iron substances produced a positive reaction, which were, however, not hematin. Even the Brücke method, which tests the behavior of solutions of the pigment with alkali, proved unreliable as far as I was concerned in the two cases in which I had to examine coats made of dyed fabric. On the other hand, I was perfectly successful in carrying out the method, first recommended by Teichmann (*Zeitschr. für rat. Med.* N. F. Bd. III, p. 375) and in producing the hemin crystals which he discovered. In this work, I adhered closely to the process he described, while I was not satisfied with the modification suggested by Brücke. I would like to recommend the first method all the more, because very small drops of blood (for example, drops from ⅓ to ½ lines across were sufficient) were capable of producing a more certain result. I collected carefully the quantity of dried blood on a slide; in this process it is of no concern whether individual foreign particles (e.g., vegetable fiber) are mixed in. Then, I added dry, finely pulverized salt, amounting to about half of the mass of blood, and covered the whole with a cover slide in such a way that this rested loosely on the lower slide. Then I put as much acetic acid on the slide as it takes to fill the entire space under the cover slide, and then evaporated this over an alcohol lamp by slowly heating it. After cooling, one adds to the dried mass some distilled water; now one looks through a microscope at the place where the blood fragments were before, and he sees everything completely filled with hemin crystallizations, easily recognized by their black-brown or gold-brown color, their rhombic crystal form, and their indifference to reagents.

During one experiment, it happened that I obtained through this treatment blue crystals from a fiber, apparently dyed with indigo, crystals which displayed a distant similarity to hemin crystals. Besides the facts that the color was very different and that the crystal form also showed a recognizably different structure, I obtained these blue crystals by treating the fiber simply with acetic acid, without needing to add the salt, an ingredient which was absolutely indispensable for the production of hemin crystals.

In this way, I believe the forensic blood test has been made significantly more certain than was previously the case. To determine whether human blood is present seems to me to be a demand that can scarcely be met. On the other hand, if the presence of blood has been established, one can state with certainty that either mammal or human blood must be present if in the fibrin mass, extracted with water and acetic 138
acid, no other nuclei can be seen besides those of the colorless corpuscles. If one has enough material, he may try also the older methods of testing, but he should always begin with the ones presented here.

Medico-legal Examination of Blood Stains*

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The law discovers that a murder has just been committed 139
and eagerly gathers information about it. Following information of every kind procured for itself or spontaneously furnished by public opinion, a man is arrested. A meticulous house search, performed as much on the accused himself as on his home, uncovers some clothing or various objects soiled 140
with reddish stains suspected of being blood. The accused denies this or claims that these stains come from some other source and particularly from the blood of various domestic animals. Due to the impossiblility of discovering the truth by investigation by ordinary interrogation, the examining magistrates commit one or several experts to the examination of the above-mentioned stains.

Apart from secondary questions which are variables according to the details of the affair itself, the magistrate generally asks the experts to express clearly their opinion on the two following points.

1) Are the observed stains produced by blood?

2) In the case of the affirmative, is the blood human blood?

Such is, in a few words, the summary of the usual course in this type of inquiry. For the past several years, since the Public Prosecutor's Office on the Seine has been confiding these assessments to us, we have never seen another course of procedure.

Is it always possible for the experts to express an opinion as explicitly as the case demands? This report has precisely as its purpose to find out if our present level of knowledge permits a response to these two questions in every case, and what degree of certainty the various means recommended and used up to the present entail. These means are of two kinds: 1) Examination of stains exclusively by chemical procedures; 2) examination by microscope.

§I. Chemical reactions. To appreciate the value of these reactions, bearing on the various elements of blood, it is fitting to recall in a few words the composition of this liquid.

Human blood can be considered as a solution of albumin and fibrin, in which float two types of blood cells. The first 141
cells, red and very small, compose a considerable proportion; they are called red blood cells. They are formed essentially of a special albuminous matter; iron is contained among their elements. The other blood cells are much larger, not very numerous, uncolored and of a singular transparence; they are called white blood cells. Let us add that blood also contains sea salt, sodium phosphate, etc. etc., and undoubtedly special diverse elements still little known, for they are present only in small quantity and they vary in composition according to the part of the body from which they are drawn.

None of the organic substances enumerated above possess well-defined chemical characteristics such that they might at least permit the certain identification of *traces*.

Blood albumin resembles albumin of egg or any other origin. It presents the property of coagulation upon heating or the addition of nitric acid when in solution. Other than the oft-encountered difficulty, when confronted with only one droplet of blood dried on the surface of a knifeblade or buried in the thickness of a fabric, of determining coagulation by heat or nitric acid, it is certainly impossible for the expert to affirm if the coagulum belongs to albumin of blood or some other animal or vegetable matter containing this last substance. Besides, a solution of albumin too diluted with water no longer precipitates either by heat or nitric acid, and the very nature of the stains to be examined does not often permit testing under the best conditions for the expert. Let us even allow that with great care and ability, all the soluble portions of a suspicious red stain can be concentrated in four or five drops of clear liquid. Introduced into a small tube closed at one end and heated to boiling, the reddish liquid precipitates a small coagulum. How to prove that this co- 142
agulum is due to albumin and, *a fortiori*, comes from a bloodstain?

The search for fibrin is also illusory. Deposited on the surface of cloth, as is the case in general, a drop of blood dries by evaporation and very rapidly coagulates. The fibrin, rendered insoluble, becomes entangled in the thousands of fibrils of wool, cotton, or hemp, strongly adheres to each anfractuosity, and is detached only with the greatest difficulty. In this last case, the proportion of fibrin is infinitely small, often hardly visible, completely amorphous, sometimes mixed with debris of the fabric which it imprisons, and does not lend itself to chemical determination. Moreover, insoluble fibrin presents none of the special properties sufficient to characterize a substance when present in only small amount; it exhibits only the properties common to all the nitrogenous matters called proteinaceous matters.

We will say nothing of the many special chemical reactions devised to reveal blood stains. They have all been successively abandoned by those seriously interested in toxicology.

* Translation of "Examen Médico-légal des Taches de Sang", in *Annales d'Hygiène Publique et de Médecine Légale* 23 (2nd series): 139–157 (1865).

We will not exclude from this rightful proscription those crystals called *blood crystals*, whose formation some chemists and physiologists have observed in several instances and with certain blood samples. Other than that the composition of these microscopic crystalline corpuscles is still problematic, and that their form is neither precise nor consistent, it is admitted today that they cannot be produced in every case, especially with human blood, that the appearance of these crystals is, instead, an accident, an almost fortuitous case of a delicate reaction, and a fortunate evaporation, rather than a consistent fact, easy to observe and to reproduce on every occasion.This method of investigation
143 lacks, then, the two qualities indispensable to every scientific procedure, rigor and consistency. Although it can undoubtedly provide useful information in certain instances, it would be more than imprudent, in our opinion, to apply it exclusively to the legal investigation of blood stains.

The successive elimination of each of the preceding properties, which are specifically chemical, naturally leads us to the examination of the value of observation by microscope in medico-legal investigation of blood stains.

§ II. Observation by microscope. A fresh droplet of human blood, deposited between two glass slides, and examined with a good microscope, offering a magnification of at least 350 diameters, presents in the field of the instrument the following objects which we will describe with care.

In the middle of a uniformly lighted circular space a considerable number of small red discs, of a perfectly circular form and a uniform diameter, are seen floating in an uncolored or scarcely rose liquid. Their form was not actually known until a few years ago. It was, on the one hand, by very prolonged observation by microscope, and notably by the differences in focusing according to the different parts of the surface of the blood cells, and on the other, by observation by the binocular microscope, producing the usual stereoscopic effect, permitting the appreciation of the reliefs and depressions there where they exist, that the exact form of red blood cells was definitively determined.

From these observations, these last-mentioned discs are none other than small water-skins closed on all sides, extremely flat, formed by a very thin, elastic, transparent
144 membrane, containing a red liquid in its interior. Their exact form is a circular disc concave on both sides. To give a better idea, imagine a checker piece, slightly hollow on each of its large surfaces in such a way as to determine two large concavities, but rounded at the angles. It results that red blood cells are thicker toward the circumference than at the center, and are quite similar to small biconcave lenses with rounded edges. When one of these blood cells being examined by microscope presents itself by chance in a three-quarter view, the concavities are splendidly apparent. This biconcave lenticular form permits explanation of the two following facts: 1) if, after focusing on a red blood cell lying flat on one of its surfaces, the body of the microscope is slightly raised, a shadow imperceptibly forms at the center of the blood cell, increases, and soon takes on the form of a concentric, round spot; 2) if, on the contrary, the same blood cell is in focus and one lowers the body of the microscope, the periphery gradually dims, whereas the center appears more luminous. These two phenomena, easy to observe and reproduce on human blood cells, if not deformed, in every type of circumstance, constitute, along with the form itself, the color and especially the invariable diameter of the blood cells, the best criterion for identification of blood cells today.

The diameter of blood cells of man and woman is $\frac{1}{126}$ of a millimeter. Though the determination of this diameter presents no difficulties, we feel it useful to discuss a few practical details in this regard.

There exist several methods for measuring microscopic objects. We will mention only the following for it is the simplest and most rigorous.

Good microscopes, those of Nachet in particular, are fur- 145
nished with two types of micrometers, i.e. two glass slides divided into equal parts by lines engraved with diamonds. The first is the ocular micrometer (thus called because it is introduced into the ocular itself), in which the millimeter is divided into ten parts; the second is the objective micrometer (placed below the objective and on the stage itself of the microscope) in which the millimeter is generally divided into a hundred parts.

If the objective micrometer is used as the true microscopic object, the divisions engraved on the glass slide can be exactly focused and, after introduction of the ocular micrometer into the ocular, a little trial and error will bring any two of the divisions of these two micrometers to coincide exactly. This done, another similar coincidence, either to the right or to the left can easily be found. From here, it is easy to count with precision how many divisions of the ocular micrometer between the two coincidences are needed to cover the subjacent divisions of the objective micrometer.

Let us suppose that for these observations we are using objective no. 3 and ocular no. 2, a combination which gives an average magnification of 390 diameters (we are intentionally choosing these numbers considering that they are perfectly suitable for medico-legal research of blood stains). After focusing of the divisions of the objective micrometer (the millimeter divided into a hundred parts), the ocular micrometer (millimeter divided into ten parts) is introduced into the body of the ocular. After the two superimposed divisions coincide, it is determined by scrupulous counting that twenty divisions of the objective micrometer are exactly 146
covered by sixty-six divisions of the ocular micrometer.

The divisions of the objective micrometer equaling $\frac{1}{100}$ of a millimeter, it results that $\frac{20}{100}$ of a millimeter equal sixty-six divisions of the ocular micrometer, or what comes to the same thing, $\frac{1}{100}$ of a millimeter corresponds to three and three-tenths divisions of the ocular micrometer. Any object of $\frac{1}{100}$ of a millimeter in diameter, seen with a microscope furnished with an objective no. 3 and ocualr no. 2, will necessarily occupy three and three-tenths divisions of the ocular micrometer; reciprocally, any object of unknown diameter,

seen in the same microscope, occupying three and three-tenths of a division of the ocular micrometer will necessarily have a diameter of $\frac{1}{100}$ of a millimeter.

Since three and three-tenths divisions of the ocular micrometer represent $\frac{1}{100}$ of a millimeter, one of the divisions of this micrometer represents $\frac{1}{330}$ of a millimeter. For the measurement of microscopic objects, only this last figure is suitable for retention. Let us measure the diameter of a red blood cell with its help.

After arrangement of the preparation intended for microscopical examination in a suitable manner (we will indicate the procedure further on), the various blood cells observed are meticulously focused. The above-mentioned ocular micrometer is then introduced into ocular no. 2, and the number of divisions and fractions of divisions occupied by a blood cell lying flat is determined. In measuring various blood cells in different sites, it is found that a blood cell occupies on the average two and six-tenths divisions of the ocular micrometer.

147 Each division of the ocular micrometer representing $\frac{1}{330}$ of a millimeter, two and six-tenths divisions represent $\frac{2.6}{330}$ millimeters. This value is simplified in dividing 330 into 2.6 and $\frac{1}{126}$ of a millimeter is obtained as the exact measurement of the blood cell of man.

The natural conclusion of these observations is self-evident: "To measure the diameter of any microscopic object whatever, it suffices to furnish the microscope with objective no. 3 and ocular no. 2, to focus it exactly, to see how many divisions of the ocular micrometer the diameter of the object being examined occupies. The number of these divisions substituted for the numerator of the fraction $\frac{1}{330}$ will give in fractions of a millimeter the exact diameter of the object in question".

The diameter of the normal blood cell is almost invariable. The maximum it varies is between $\frac{1}{123}$ and $\frac{1}{128}$ of a millimeter. It is undertandable from this of what importance is the exactness with which this measurement is performed, from a medico-legal viewpoint.

It is fitting here to present some explanation concerning endosmosis in blood corpuscles and the difficulty often encountered in observing them intact. Each of these little water-skins, called blood cells or blood corpuscles, is filled with a reddish liquid denser than pure water. As soon as water is added to a droplet of blood a rapid endosmosis is established between the contents of the blood cell and the external liquid. The biconcave disc gradually deforms under the continual influx of liquid into its interior; it swells, takes the form of a small sphere, pales considerably, breaks up and disappears. There remains only some formless, scarcely visible debris of the external transluscent memberane. If the blood cells are placed in contact with a liquid denser than the
148 contents of the blood cell, the inverse phenomenon is produced; each blood cell gradually empties itself of liquid it contains, its surface wrinkles and shortens. After a little while, if the difference in density is rather considerable, the blood cell finds itself reduced to a small corpuscle, externally crenated, and greatly diminished in volume.

The biconcave form and the diameter of $\frac{1}{126}$ of a millimeter can only be found, then, in blood cells which have not been subjected to either of these two phenomena, and consequently have not undergone any deformation.

If the blood is fresh, nothing is simpler than to determine the presence, form and diameter of blood cells. It is otherwise for blood dried on the surface of a fabric or of any other object. This case must be recognized as the most frequent and most difficult for the expert. In the small red stain submitted for examination, blood cells exist; they can regain their form and their diameter. The only difficulty lies in dilution of solid blood such that there is no appreciable endosmosis or exosmosis for the blood cells, and consequently, no deformation to be feared.

The best liquid which could be used would be the serum itself of a blood-letting, used after filtration, if it were not often a bit difficult to procure and conserve it, and if the origin of this liquid itself didn't tend, regardless of what is done, to cast doubt on medico-legal experiments. It is preferable to employ artificial liquids whose density is intentionally brought to approximate that of serum, so as to avoid all endosmosis of liquids; for example, a solution of sodium sulfate, gum or sugar made in proportions such that the density is about 1.028. These liquids have only one inconvenience, which is often serious; they spontaneously concentrate by evaporation, and taking on a higher density, deforming and 149
shrinking the blood cells they are supposed to conserve.

As well as many micrographs, we have had in our possession a liquid remarkable for the facility with which it conserves blood cells[1]. It concentrates very little by spontaneous evaporation at the surface of the bottom slide, and keeps indefinitely, with no clouding or any alteration whatever.

The following mixture, which we have been using for the past five years, and whose formula we present, offers the same advantages:

Liquid specific for the preservation of blood cells

Ordinary glycerin of pharmacies . . . 3 parts by weight
Concentrated, pure sulfuric acid . . . 1 part
Distilled water in sufficient quantity to obtain a solution which gives a density of 1.028 at a temperature of +15°.

The presence of sulfuric acid does not alter the form or color of the red corpuscles in any way. The mixture of this acid and glycerin with water largely delays the evaporation and concentration of the liquid. It is necessary to avoid touching it with a metallic instrument and to restrict drawing from the flask to glass tubes.

Operating procedure. After a long and meticulous examination in broad daylight, a single stain is chosen, clearly limited and very distinct and having escaped, as much as is possible, any serious traction or rubbing. A fragment of stained material, with a surface area the size of a 20-centime piece

[1] This liquid is sold by a manufacturer of microscopical objects.

is removed with sharp scissors, or the point of a scalpel, and deposited on the bottom slide. A few drops of preserving liquid
150 are drawn from the flask by means of a tapered tube and dropped on the material, and imbibition is allowed to occur for about three hours. At the end of this time, the fragment of material is rubbed, turned over several times, and finally unravelled on the surface of the glass slide by means of two small solid tubes tapered at their extremities so that the insoluble substances are detached and placed in suspension. After removal of the fabric, there remains on the slide a droplet of liquid somewhat clear, somewhat colored, which is immediately covered by a very thin glass slide and placed on the stage of the microscope. As we pointed out previously, the magnification which appears most favorable to these observations is obtained by the combination of objective no. 3 and ocular no. 2 (Nachet microscope). Apart from the red blood cells which can be discovered in this examination, a certain number of foreign bodies are generally seen whose origin is self-explanatory: 1) debris of cotton, hemp or wool fibers immediately recognizable by their considerable size and length; 2) cells and debris of epithelial cells if the fabric being examined comes from a shirt, trousers, handkerchief or any other article of clothing in contact with skin or mucus; 3) amorphous bodies of very diverse origin, which are instinctively ignored, because there are never two of the same form and they are extraneous to the purpose of the research. If, on the contrary, the preparation contains red blood cells, they are immdiately perceived in considerable number, sometimes several hundred at once, presenting a uniformity of diameter and color. It is then that the exact measurement of a few of the least deformed of these blood cells is taken. The average of these various observations approximating the
151 true diameter of these blood cells ($\frac{1}{126}$ of a millimeter) suffices to settle the medico-legal question. It often happens that endosmosis or exosmosis cannot be completely avoided, and the bloodcells do not present a diameter of exactly $\frac{1}{126}$ of a millimeter. The divergence in this case is not very considerable, and the general form, the color, as well as the large number of blood cells observed, suffice to demonstrate that the stains are of blood.

It is unnecessary to add that despite all imaginable precautions, blood cells of this kind never present the sharpness of unaltered blood cells. Experience in observation by microscope and in this sort of examination are indispensable for an expert charged with these determinations.

It is good in these assessments to have constantly on hand and to observe from time to time a glass slide covered with some blood. This slide is easily prepared in the following manner: a fine droplet of blood is placed on a very clean glass slide, and immediately spread over a large area by a small tube or the feather of a plume. This blood dries in a few moments and constitutes a very convenient, unalterable preparation in which blood cells preserve their form, their color, and their true diameter.

In the observation of white blood cells many micrographers have looked for a method surer than the preceding to determine the presence of blood stains. We can not agree with this opinion and here are our reasons: it is without doubt that white blood cells resist washings and various deformations accompanying the action of aqueous liquid on dried blood more than red blood cells. We readily add that this latter character should tend to render them preferable to red blood cells themselves in many cases in medico-legal research, ex- 152
cept for the following fact which just about equals an absolute rejection. In the opinion of every micrographer, white blood cells completely resemble mucus and pus corpuscles in their form, color and diameter. It would suffice to have some nasal, urethral, or other mucus, some pus from a pimple, boil or superficial abcess present to lead an expert into error. Simple enunciation of this fact condemns this method of investigation. It is suitable to add here that white blood cells, in relation to red blood cells, are present in an extremely small proportion, and that they easily escape observation, due to their singular transparency. Although the determination by itself of these white blood cells does not prove very much, we are obliged to add that the simultaneous presence in the same stain of red blood cells and white blood cells, however, constitutes an additional proof in favor of the existence of blood. The expert will not be neglecting anything in observing it and pointing it out when the occasion presents itself.

To sum up, there exists today in science only one sure means of expressing an opinion on the presence of a blood stain on the surface of a fabric or any object whatever. This means is observation by microscope of the form, color and diameter of red blood cells. In all the cases where this observation does not reveal anything positive, we conclude in the negative, no matter what the external appearance of the stains submitted to our examination.

If it is of importance in the preliminary examination to determine the presence of a blood stain; it is sometimes just as imporant to know if the blood is human blood. This second part of the expert's task is always the trickier.

The solution of this problem is still to be found: no one today takes seriously the indications furnished by a Parisian chemist, who formerly claimed that by only the odor devel- 153
oped by contact of such and such a stain with sulfuric acid, or the series of bizarre chemical reactions devised by an Italian chemist, it could be determined if blood comes from such and such an animal.

At our present level of knowledge, human blood differs from other mammalian blood only in the diameter of its red blood cells. It is only micrometry, then, which would be able to furnish the solution of this desideratum, if the diameter of red blood cells of the principal mammals did not, unfortunately, closely approximate the diameter of blood cells of man. The following table presents the results:

Animals	*Diameter of red blood cells*
Man	$\frac{1}{126}$
Dog	$\frac{1}{130}$
Hare	$\frac{1}{142}$
Pig	$\frac{1}{166}$

Ox	$\frac{1}{178}$
Horse	$\frac{1}{182}$
Sheep	$\frac{1}{200}$

If the red blood cells of man are compared to those of the other animals presented in the preceding table, it is evident that the diameter of the former are larger. *A priori,* it would seem easy, then, to express an opinion on the origin of blood stains submitted to assessment by an exact measurement. This is not entirely so. Other than the fact that it is often difficult in micrometric measurements performed by microscope to be answerable for an error of $\frac{1}{500}$ or $\frac{1}{600}$ of a millimeter, the changes of dryness and humidity, to which the blood could have been exposed, the more or less rapid endosmosis produced during moistening, and the deformation which can result, are so many incentives for hesitation on the part of the
154 expert. Such a circumstance is possible giving in the observation, and the micrometric measurement, a diameter a bit larger or smaller than human blood cells and consequently, cause them to resemble blood cells of another animal. The inverse would be more serious. When the difference in diameter with the blood cells observed is considerable, if, for example, a series of executed measurements gives an average of $\frac{1}{300}$ of a millimeter, if the blood cells do not present any appreciable deformations, tears, folding or crenation, so that it appears evident that their external volume has not been appreciably modified by dessication or moistening, the expert can claim that the examined stain does not appear to come from human blood.

The most delicate case is the following given that the expert has determined in the most evident manner the presence of red blood cells in considerable number, and that the average of all the measurements is precisely $\frac{1}{126}$ of a millimeter, should he conclude in the affirmative as to the presence of human blood? Enlightened today by the experience of several years, especially dominated by the fear of a chance coincidence and the awesome responsibility of a conclusion which sometimes draws capital punishment, we do not hesitate to reply: *Even in this case, the expert should remain doubtful and be wary of affirming that blood is human blood.*

Not all red-blooded animals have circular blood cells. Without citing the few exceptions in the mammals, it has been demonstrated that all fish, birds, batrachians, ophidians, etc. have elliptical blood cells and an interior nucleus. It is superfluous to add that the single determination of this type of blood cell suffices for rejection of the possibility of human blood. This is the most favorable case for the accused, given that, as a result, there can be no doubt in the mind of the jury of the exact nature and origin of the blood stains, if the expert correctly exposes these facts.

155 Observation I. In the month of October, 1860, a man was found murdered in the neighborhood of B . . . , stabbed twice with a knife, which must have caused a rapid death. There were traces of a struggle around the corpse. Legal machinations immediately intervened and directed the chase in several directions. Two days after the crime was discovered, a man was arrested and his premises meticulously searched. Among other objects were seized a blue smock, as well as a cotton handkerchief, both covered with blood stains. The defendant denied this, but could not find an explanation for the above-mentioned stains, which he attributed sometimes to a nosebleed, sometimes to an old wound incurred on his hand and of which there remained a slight scar. He is, moreover, of a very limited intelligence and does not seem to understand the importance of the questions very well. On the execution of a rogatory commission of the Public Prosecutor's Office of B . . . . , these two stained objects are submitted for our examination. We have to answer the following questions:

1) *Have the red stains soiling the smock and the handkerchief of Mr. X . . . been produced by blood?*

2) *In the case of the affirmative, is this blood human?*

A careful inspection of these two objects reveals at first the following facts: 1) the smock was stained at the opening and in the interior of one of the two pockets; 2) the handkerchief was stained in two places, the stains being large and very stiff.

Examination by microscope shows us we are dealing with elliptical blood cells. The largest diameter was $\frac{1}{90}$ and the smallest $\frac{1}{176}$ of a millimeter. We were quite happy, in addition, to discover, buried in the middle of one of the large stains of the handkerchief, three shiny scales, whose form and sparkling color, as well as the presence of sinuous parellel striations sufficently characterized them as scales of fish. As a result of our research, which we present here only in summary, we adopted the following conclusions:

"1) The red stains soiling the smock and handkerchief of Mr. X . . . have certainly been produced by blood.

2) The red blood cells observed in the preceding stains, being of elliptical form, can only belong to blood of fish, bird or reptile. Due to the presence of three fish scales found by us in the middle of one of these stains, it is highly probable that the blood soiling the smock and handkerchief of Mr. X . . . is blood of fish. It is certain, in any case, that these stains were not produced by human blood."

As a result of our report, there was a dismissal of the charge.

Forty days after these events, the real murderer was discovered and convicted as a result of thorough confessions to 156
this last crime and several others.

It was hardly a couple of days ago (November 1864) that Ambroise Tardieu and myself received the following rogatory commission:

Observation II. "We, Louis-August Parmentier, examining magistrate of the district of Sancerre,

"Owing to the proceedings conducted at the request of the Public Prosecutor against Marie D. . ., wife of Louis F. . ., proprietor and farmer, residing at Garigny, accused of the double crime of castration and poisoning:

disclose the following facts:

§1. Marie D. . ., thirty-five years old, married to the said F. . ., well-to-do farmer, but of limited intelligence.

Given to profligacy these past several years, this woman was most recently the lover of the man named Simon J. . ., who customarily worked at her home as a thresher in the barn; this liaison, however, was terminated last June as a result of the marriage of Simon J. . . . The woman F. . ., seeing herself abandoned by her lover, gave rein to an intense resentment and resolved to exact from him a terrible vengeance. She entices him to her home on the evening of October 23 last, under the pretext of remitting to him a sum of money she owed him, and makes toward him the most provocative advances and overwhelms him with caresses which were received with utmost indifference. She went so far as to unbutton his trousers and take his sexual parts in her hands. Finally, at the moment when J. . . made a movement to escape her grip, she severed his member with a razor. Despite the damning testimony of the injured, who survived this hideous mutilation, despite other charges useless to mention here, the accused persists in denying the charges. A petticoat spotted with red stains was seized at her home. Those stains, presumed to be the blood of her victim, were, according to her, the blood of a goose which had fallen on her petticoat while she was bleeding a fowl. It would be useful, then, to submit these stains to a serious examination and to verify if they were produced by human blood or the blood of a fowl."

§II. This second chapter of the rogatory commission deals with a poisoning executed by the woman F. . . on the person of her child of ten months. It is useless to relate it here.

Following this rogatory commission, Tardieu and myself
157 were designated by Chopin, examining magistrate attached to the court of first instance of the Seine, to proceed with the various experiments indicated above.

It is understandable of what importance it is in this serious affair to determine precisely if the petticoat stains are formed by the blood of a mammal or the blood of a bird. Now, at this very moment when we were writing these lines, this determination has just been accomplished. Here is how we have proceeded.

We cut out a strip of material from the petticoat with scissors, in a part completely free of all stains, and we dropped several droplets of goose blood which we have expressly pricked in the neck. Four days later, when the stains were completely dried and brittle, we proceeded to examine them by microscope comparatively with the stains under suspicion on the petticoat itself. The two types of stains were simultaneously treated according to the procedure indicated above, submitted to the same time of imbibition, examined under the same conditions of time and temperature, and finally, submitted to the same magnification. The two results are as dissimilar as possible. The stains produced by the blood of goose yield to the preserving liquid, and permit the observation in the field of the instrument of a considerable number of elliptical red blood cells, with an evident central nucleus and with the following average for microscopic measurements: largest diameter, $\frac{1}{80}$ of a millimeter; smallest diameter, $\frac{1}{160}$ of a millimeter. The observation by microscope of the stains under suspicion present nothing comparable; as a matter of fact, a considerable number of reddish corpuscles are indeed discovered, but *all of them* are perfectly circular, and despite the most sustained attention, we could not discover any elliptical forms. The average of twelve measurements executed on these circular red blood cells, chosen, of course, from the most intact and the least deformed, gave $\frac{1}{157}$ of a millimeter in diameter.

The conclusion of these facts is self-evident:

1) It is incontestable that the stains observed on the petticoat of the woman F. . . are stains of blood,

2) Although the red blood cells observed and measured by microscope approximate by their dimensions human blood cells more than the blood of any other domestic animal, it is *impossible for us to affirm* that the stains of the petticoat are produced by human blood.

3) It is quite certain, in any case, that the stains of the petticoat do not and could not have as their origin the blood of a goose or any other bird.

The Source of Blood in Legal Medicine*

Dr. Charles Masson

Pharmacist-Medical Officer of the Army

385 During our stay in Algeria, we were called upon many times to give an opinion on this serious question of the source of blood.

Our research, with imperfect equipment and a very limited library, had no other result than to inspire in us a great uncertainty and a judicious reserve: sentiments which agreed poorly with the affirmative character of certain model reports, and instilled an intense desire in us to better enlighten ourselves on this important question.

At Lyon, where the scientific resources are so generously put at the disposal of everyone, the opportunity presented itself and we eagerly seized upon it.

Historical Review

The history of the methods used to characterize blood stains is divided into two periods. During the first, recapitu-
386 lated by the works of Orfila around 1848, the chemical characteristics of certain immediate principles formed the basis of every assessment whose purpose was the identification of blood. Albumin, fibrin, a particular coloring substance, nitrogen and iron were sought: isolated, these characteristics were without value; together, they were diagnostic.

Until 1829, no one was occupied with finding if it were possible to distinguish human blood from that of other animals. At that time, Barruel published an interesting memoir on this question whose conclusions, very coldly received by chemists, in particular, Raspail and Orfila, are unconditionally rejected today. Barruel wanted to identify the source of blood by appreciation of the odor of a particular principle liberated by sulfuric acid. Every nose cannot serve as a reagent, said Raspail; a comparable proof, we will say, does not have sufficient scientific character for legitimately winning a conviction.

The discovery of Prevost and Dumas, of blood cells of different form and dimension, and the work of Mandl[1] who demonstrated the possibility of distinguishing oviparous from mammalian blood, made little impression on Orfila, who preferred chemical experiments to the microscope, and wrote on this subject: "After having repeatedly examined by means of excellent microscopes human blood and pigeon blood, detached from fabric, not only was it impossible to distinguish them, but sometimes even to recognize that it was blood".

In 1857 the second period abruptly arrived; from the most complete obscurity came a most intense enlightenment, without transition. Ch. Robin, the eminent histologist, on the occasion of a medico-legal assessment, published a remarkable memoir[2] in which he presented his research, his oper- 387
ating procedures and his results. The chemical experiments of Orfila were, from this moment, relegated to second place. The microscope reigned as master: only the morphologic elements of blood were invoked to decide the nature and source of the stain.

Let us quote from this work, the passages most relevant to our investigations:

> After maceration of small superficial crusts for twelve hours in liquid of Bourgogne.
>
> Each blood cell [says Robin] had just about recaptured its flat, biconcave, circular form. All were 6 to 7 μ, rarely a bit bigger.
>
> After twelve hours of immersion in the same liquid, the dissociated filaments of fabric impregnated with blood. . . .
>
> It was easier yet, he claims, here than in the first series of operations, to isolate the blood cells, to separate them from the cotton filaments. . . .
>
> Conclusions: 5) the elements of blood forming the stains on the smock are elements of blood belonging to the human species. The red blood cells found here have the volume, etc. . . . but at the present stage of science it is impossible . . . to determine either the sex or age of the individual from whom it comes.

This was indeed a big step forward! Such a brilliant beginning inspired the greatest hopes for the next solution of desiderata, which the master seemed grudgingly to confess. This, however, was not to be the case: this intense light would soon be dimmed.

This same year, Virchow wrote[3]:

> I don't believe that a micrograph can ever be authorized to determine that the life of a man depend on the yet so uncertain appreciation of the coefficient of dessication of blood cells. Blood undoubtedly dessicates sometimes in such a way that isolated globules can clearly be identified; but the dessication is under the control of so many varied conditions. And blood, once dried, can be exposed to such unfavorable circumstances, that a judgment on the importance of its constituent parts cannot be formulated with certainty.

* Translation of: "De l'Origine du Sang en Médecine Légale."
in *Annales d'Hygiène Publique et de Médecine Légale* 13 (3rd series) 385–402 and 530–549 (1885).

In 1865 Roussin, a military pharmacist, and one of the
368 greatest authorities, ended one of the most knowledgeable and wise communications done on this subject:

> In addition to the difficulty of being responsible for an error of $\frac{1}{500}$ to $\frac{1}{600}$ of a millimeter in micrometric measurements at the microscope, the alternations between dryness and humidity to which blood cells have been exposed and the more or less rapid endosmosis produced during moistening are just as much incentives for hesitation on the part of the expert: even in the case where the average of all the measurements give $\frac{1}{126}$, the expert must be in doubt and keep himself from affirming that the blood is human blood[4].

For Blondlot, then professor at Nancy:

> If the blood has been dried, the corpuscles change, deform, and according to the density of the liquid used to dilute them, diminish or swell in such a way as to be most often unrecognizable even for the most practiced eye.[5]

In 1873 a report of the Society of Legal Medicine, drawn up by a committee composed of Mayet, Mialhe, Cornil and Lefort[6], returned us to affirmative ground:

> When the dessication is not carried too far and the stain not washed in water, especially hot water, red blood cells in a sufficient state of preservation are always found after after careful, lengthy search.
>
> Conclusion: the expert measures the blood cells and can thereby determine if it consists of human blood or not.

The following year, Rabuteau replied[7]:

> In our opinion the authors of the report of the Society of Legal Medicine go beyond present-day science, saying that measurement of blood cells permits determination of whether blood is human or from another mammal. The diameter of human blood cells is 0.0075, that of the dog 0.0073; moreover, blood cells are always more or less distorted; the conclusion that blood can be diagnosed as being human or not is inadmissible.

389 In 1876, Malinin, of Tiflis, in a remarkable work where measurement of blood cells was the object of a profound study, does not find it possible at this present stage of science, to distinguish blood of man from blood of dog, rabbit, or pig.[8]

In 1877, Professor Cauvet said in a report on blood stains:[9]

> Research on blood cells is easy. It consists of cutting out from one of the points of the stain a piece of material, which is impregnated with a few drops of Roussin preserving liquid.
>
> Conclusions: the stains are due to blood. The size of the corpuscles observed determines whether the blood can be regarded as human blood.

In 1879 Malassez published a very interesting report from two points of view. He had been able to find characteristic blood cells where it was impossible to obtain hemin crystals; in addition, the blood cells being altered, he tried to evaluate what Virchow calls the coefficient of dessication, without, however, arriving at any affirmative conclusion,

> The circular form of the blood cells, [he points out] the absence of nuclei, show they are mammalian blood cells. Their dimensions give less certain results. These blood cells have all become spherical and lost their diameter. In addition, they have dried and their volume diminished. Taking these facts into consideration, it is seen that in their primitive state they should have had dimensions very near those of human blood cells, and of those of several of our domestic animals. Conclusions: it is mammalian blood. It is impossible to identify the blood as human or of one of our domestic animals.

In 1880, Professor Morache, principal physician, in a most instructive medico-legal report[10] presents meticulous research, to which he must have devoted himself, to assert that blood submitted for his examination was blood from a bird. Some very interesting plates accompany this work, 390
which is stamped with the most sensible reserve.

Finally, in 1882 Vibert published a study of the possibility of distinguishing blood of man from that of mammals[11]. The study was essentially practical, of great originality, where certain points are brought out and elaborated on, they being the most delicate points of this difficult question. Here are his conclusions:

> It is always impossible to assert that a stain is formed by human blood. It can only be said, in certain cases, that it could have come from human blood.

Such are the most noteworthy original works published on this question in the last sixty years: the classical works are the most often more or less faithful reflections of this. In Briand and Chaudé, the memoir of Robin is reviewed more or less favorably. The ideas arising from particular facts are sometimes generalized without experimental control. The author, for example, formulates the erroneous proposition that blood stains on flax, hemp and cotton fabrics are most favorable for the investigation of blood cells. Furthermore, no conclusions can be drawn unless certain restrictive notes are considered, which singularly diminish the affirmative character of the exemplary report which follows them.

In Dragendorff, there is a summary of the work of Roussin.

Taylor[12] does not concede that it is possible to distinguish the blood of man from that of dog, hare or rabbit.

Hoffman[13] concludes: "It is difficult to decide if blood cells, contracted by dessication and later rendered visible by use of liquids, come from the blood of man or other mammals."

For Clément[14], the data furnished by measurement of elements of blood can be considered only indicative, except 391
in the case where it's a question of distinguishing blood of man from that of animals with elliptical blood cells.

On the subject of the species of blood, Tourdes says[15]:

> The diagnostic signs are furnished by the form and dimensions of blood cells. The measurements have great value with fresh blood; but with blood cells altered by dessication and by the liquid used for extraction, the diagnosis is difficult.

Vibert[16] ends an excellent article, entirely his own work, an article we have frequently consulted, and whose essential data have been most often confirmed by our personal experiments:

> To conform with scientific data, the expert must conclude thusly: Such and such a stain is not constituted by blood from such and such an animal (beef, mutton, goat, according to the accused), it comes from man or a mammal of blood cells of similar dimensions (dog, rabbit).

In bringing to light opinions of the most authoritative experts, so contradictory, this review proves how uncertain the question of the source of blood, with regard to species, still is. Studies being conducted most often during a case assessment, each experimenter conceptualized from the point of view of the particular case submitted to him, supporting his conclusions on experiments similarly performed, under conditions as identical as possible, much more than from scientific principles.

The composition of liquids used varies with each expert: sometimes neutral, sometimes acidic, they are sometimes of an alkalinity making it at first difficult to understand how blood cells resist their destructive action. Some of these liquids seem to have no action on the blood cell itself, others dilating or contracting it.

This variability in reagents as in the methods, besides,
392 explains a bit the comparable variability in the results. Some attribute to certain liquids the property of rendering to blood cells their natural softness, with their circular, flat, biconcave form: others recognize only the property of dissociating, isolating the blood cells as they are. Some regenerate blood cells in great number; others find two or three in one preparation, and this, with difficulty. For some, the blood cells regain their previous form, presenting for some a preparation like that of fresh blood; for others, they remain irregular, deformed, unrecognizable. To recognize human blood and to affirm its presence is an easy thing for some authors, impossible for the greater number of authors.

Most often, the nature of the stain substratum, wood, various fabrics, paper, iron, etc., seem without importance: if their influence is appreciated, it is different for different authors. Little is known of the causes of alteration of blood cells, what Virchow called the coefficient of dessication; there is no definition of the consequences. As for measurements, the difficulty of which Roussin had already shown in 1865, not everyone seemed to concede that the diameter of blood cells, variable even in the same animal, as well as the imperfection of our apparatus and senses, necessarily limit the scope of our evaluations.

How much this uncertainty must weigh on the expert, called on to give an opinion on a question so serious, who seeks in vain for a guide to provide him with limits in which he can and must enlighten justice!

What previous work could not provide, we looked for in experimentation. Completely immersed in the reading of these works, but operating, however, without preconceived ideas, confirming the brutal facts and reporting, as they gradually passed under our eyes, the different methods of operation which we will summarize further on, we were led to formulate certain conclusions. Without resolving a problem out of reach of our resources, it should at least result in rendering the task of the expert easier and, especially more 393
certain, in defining better the principle elements of this very complex question.

Our research essentially tends to clear up the three following points, which seem to us to sum up the desiderata of the question;

1) To determine under the conditions which present themselves to the expert, the liquid which gives the best preparations with human blood, the most favorable to a good measurement;

2) To study the causes of the alterations of blood cells and to define the consequences, as much as possible;

3) To conduct experiments on the blood of animals in the same way as on that of man and to find out within what limits the expert can give an opinion as to the source of blood.

FIRST PART

A. Action of the Principal Liquids on Fresh Human Blood

Liquid of Virchow: *Composition*. Solution of potassium hydroxide of 30 parts per 100; potassium hydroxide 30, distilled water 70.

Microscopical Examination [hereinafter: "M.E."]: Blood cells quite regular, but immediately contracted to about $\frac{1}{176}$ of a millimeter; variable destruction after twenty-four to thrity-six hours.

Liquid of Bourgogne: *Composition*. Unknown.

M.E. The discoid form of blood cells is exaggerated. The very thick circular brim often shows traces of tears on its internal border at the limit of the center which appears more depressed. The blood cell, thickened and dilated on the whole, has, however, a lesser diameter, about $\frac{1}{139}$ millimeter.

Liquid of Roussin: *Composition*. Glycerine, 3; Sulfuric acid, 1; water, quantity sufficient for obtaining a liquid of 1.028 at +15°.

M.E. Blood cells are discolored, spherical and dilated to $\frac{1}{97}$ of a millimeter.

Iodated Serum of Ranvier: *Composition*. Potassium iodide, 2 grams; iodine, sufficient quantity for saturation; water, 100 cc.

M.E. Blood cells very colored and strongly contracted.

Liquid of Vibert: *Composition*. Mercury bichloride, 0.5; 394
Sodium chloride, 2.0; water, 100.

M.E. Many blood cells of normal form and dimensions; average, after sixty measurements, after two and four days, $\frac{1}{136}$ millimeter; some blood cells crumpled.

Liquid of Paccini: *Composition*. Water 300, glycerine 100, Sodium chloride 2, Mercury bichloride 1.

M.E. Results comparable to preceding, but not as good.

Solution of Sodium Sulfate at 5 or 6%.

M.E. From the beginning, mixture of blood cells dilated to $\frac{1}{100}$ millimeter and contracted in the form of blackberries; then some regular, spherical globules appear, contracted to about $\frac{1}{193}$; these latter become more and more numerous and all the blood cells look this way after forty-eight hours.

Artificial Serum of Malassez and Potain: *Composition.* Solution of gum at 1.020, 1 volume; solution of sodium sulfate and sodium chloride, equal parts, at 1.020, two volumes.

M.E. Same action as the solution of sodium sulfate.

Of all of them the liquid of Vibert alters the blood cells of fresh blood the least, the greater part of them conserving their characteristic form, with a normal diameter. The expert must use this in preference to all the others when he has to examine blood not yet dried, liquid, clotted or saturating a fabric.

But, whenever practical, the procedure of Welcker, which seemed to us to have been quite successfully used in this circumstance, must be preferred to the liquid of Vibert, despite this liquid's qualities. This procedure, to be treated in the second part of this work, surpasses all the others: simple and rapid, it delivers to the expert all the diagnostic elements, definitively reunited and fixed, and at the same time material evidence of considerable importance.

If the blood is liquid, the operation is one of the simplest; if it is clotted, it suffices to take a fragment from the most fluid part, bring it to the bottom slide, and push it before the needle held horizontally, This leaves a smear of blood cells which are immediately fixed by dessication, and can later be measured.

395 With this procedure we have obtained excellent results with clots of beef, mutton and chicken blood.

B. Action of Liquids on Dried Human Blood on Varied, More or Less Permeable Substances

Liquid of Virchow: *O.P.*† With a scraper, small scales are separated and macerated in liquid, protected from all evaporation, for a time varying from 1½ to 4 hours. A portion is then placed on the bottom slide with a very small drop of liquid; the slide is covered, and subjected to light sliding movements. If the maceration is sufficient, the dissociation is easy. If too prolonged, the small crust can take on an elastic consistency, the dissociation becomes difficult, and the blood cells undergo a light contraction.

M.E. Numerous characteristic blood cells, well isolated, more or less regularly circular, flat, biconcave. Average of 100 measurements, $\frac{1}{127}$ of a millimeter.

†For each liquid, we indicate the operating procedure (*O.P.*) which gives the most favorable result.

Liquid of Bourgogne: *O.P.* Place a drop of liquid on the stain, leave for five minutes, then brush with a fine paintbrush and transport the drop to the microscope.

M.E. Characteristic blood cells, more or less regular; the discoid form is slightly accentuated and the thickened blood cell has a diameter reduced to about $\frac{1}{134}$.

Liquid of Roussin: *O.P.* More or less prolonged maceration. Dissociation difficult.

M.E. Irregular plaques, formed by a mass of discolored blood cells, in the middle of which very clear, very colored white blood cells are distinguished; a few rare, isolated, irregular, transparent, dilated blood cells; abundant granular debris.

Liquid of Ranvier: *O.P.* More or less prolonged maceration. Dissociation difficult, hard, breaking masses.

M.E. Blood cells with very clear contour, very colored and very contracted.

Solution of Sodium Sulfate: *O.P.* Maceration; hemoglobin dissolves, the liquid colors.

M.E. Irregular plaques, uniformly pale yellow, on which 396
very apparent white blood cells stand out; attempts to dissociate the blood cells result only in irregular debris, not very colored and granular.

Artificial Serum: Result similar to the preceding.

Liquid of Vibert: *O.P.* Maceration for about an hour.

M.E. Characteristic globules, but with no flexibility and not very clear contours; the preparation is congested with debris.

Liquid of Paccini: Results about the same as the preceding.

From these experiments it is evident that the liquid of Virchow certainly gives the best preparations under these conditions, most favorable to the diagnosis of the source of blood. Blood cells, which resist its action so poorly while in fresh blood, seem to have acquired sufficient resistance from dessication to undergo its action without alteration during all the time necessary for their dissociation and after. With a thin blood stain, dried on a knife blade two and a half months before, we obtained a splendid preparation, which we edged in paraffin, after three hours of maceration in liquid of Virchow. Fifty measurements done immediately gave us an average of $\frac{1}{128}$ of a millimeter; after forty-eight hours $\frac{1}{142}$, and after eight days $\frac{1}{133}$.

This liquid liberates blood cells by dissolving the matrix uniting them. The preparation is splendid, with no debris which can alter the clarity: all is dissolved but the blood cell! In the middle of the blood cells, isolated in infinite number, characteristic, but most often more or less deformed by reciprocal compression, are always found those that escaped any deformation, and can serve as a basis for serious measurement.

After the liquid of Virchow comes the liquid of Bourgogne, which, by the procedure recommended to us by the inventor, a procedure which marvelously suits the properties of this liquid, also gives very good preparations, but in general inferior to the preceding.

Besides, the liquid of Bourgogne, in the presence of dried

397 blood cells, has, although to a lesser degree, this property of dilating fresh blood cells, in exaggerating the discoid form, and diminishing, however, the diameter. This action appeared appreciable to us in blood from beef and pig, whose globules seemed contracted to $\frac{1}{162}$ and $\frac{1}{180}$ millimeters. Let us now add that, in contrast to the liquid of Virchow, the liquid of Bourgogne gives poor preparations with dried blood from animals with elliptic blood cells.

To sum up, when called upon to examine dried blood stains forming a crust, thin though it might be, on wood, iron or any not very permeable substance on a weapon, flooring, woodwork, paving-stone, on straw and on certain papers, as well as on pieces of bone or fiber of clothing, the expert must not hesitate to use the liquid of Virchow, following the procedure which has worked so well for us. It is under these circumstances that he will obtain, with dried blood, the most convincing results and the most affirmative conclusions.

It is not necessary that the stain be thick or extensive. Would that every examining magistrate were well aware of this principle. It is true, if not probable, that a very small stain, as big as the eye of a needle, under the conditions we have just studied, is more amenable to yielding useful information to the expert than a shirt of cloth or calico stained all over with blood.

C. The Action of Liquids on Human Blood Dried on Fabrics

Whether the fabric is wool, fur, flax, cotton or hemp, the precise distinction as to its influence on the medico-legal investigation of blood has not been established until now. This is a gap which can explain, in a certain measure, the contradictions as well as the skepticism of certain experimenters.

398 The following experiments permit a grouping of the different fabrics into two essentially different classes: 1) material whose natural element is not dampened by blood; 2) material whose element is dampened by this liquid.

The laws of capillarity justify this differentiation. When a drop of blood falls on a fabric, the capillary phenomena vary with the nature of the material. If this fabric is formed by elements which dampen with blood with difficulty, like wool and fur of certain animals, the liquid is almost as if pressed down at the points of contact; the blood dries in a mass more or less independent of its support. If a drop of blood is projected onto cloth or felt, the mutual attraction of the liquid molecules prevailing over that exerted between these molecules and the material, the drop tends to conserve its spherical form and to dry on the surface of the material without penetrating it.

If, in contrast, the material is susceptible to dampening by blood, the capillary phenomena are altogether different: they draw and indefinitely disperse the blood cells, which penetrate far into the central part of the material and deposit around the filament, (a barrier they cannot overcome, a veritable filter) a more or less regular sheath, constituted by blood cells strongly united, applied and drawn in every direction by the force of capillarity, without intervention from plasma, which goes beyond, and penetrates the filament itself. Does not the drop of blood which falls on linen instantaneously make an oil stain?

1) **Fabrics not dampened by blood.** With the exception of liquid of Bourgogne, with which it is suitable to follow the procedure already indicated, the procedure which gives the best results consists in scraping the surface of the stain, when it forms a crust or light coating on closely-woven linen or felt; or, if the fabric is loose and aerated like certain linens, like the fabric of Arab burnoose or of a knit, in cutting out the stain, and unravelling the subjacent material with needles on a watch glass. In both cases, a more or less coarse 399
powder is obtained, which is subjected to the action of the liquids.

In proceeding as we have done previously, the results are perhaps less favorable, but substantially the same as those obtained from blood forming a crust on impermeable bodies: the liquid of Virchow again gives the most splendid preparations and the most favorable to a good measurement.

We will not summarize the results of our experiments in order not to be redundant. These results show nothing which should be surprising! Are not the blood cells here in conditions analogous to those in which they find themselves in dried blood on an impermeable body? Are they not independent of support and united by plasma which forms, on drying, a matrix which dissolves so easily in the liquid of Virchow, which thus liberates them without altering them?

2) **Fabric dampened by blood—Liquid of Virchow:** *O.P.* — The imperceptible powder obtained in unraveling the dry material again gives better results than direct maceration of the stain.

M.E. Very irregular blood cells, contracted from $\frac{1}{160}$ to $\frac{1}{240}$ of a millimeter, rarely isolated; more or less complete envelopes, fragmented, formed from distorted blood cells, contracted and intimately united.

Liquid of Bourgogne: *O.P.* More or less prolonged maceration. *M.E.* Results similar to preceding.

Liquid of Roussin: *O.P.* More or less prolonged maceration or absorption.

M.E. Identical results, but clearer. The limits of each blood cell are more easily appreciable. Not many blood cells isolated, and all are contracted and irregular.

Solution of Sodium Sulfate: same results, less clear.

Artificial Serum: similar to preceding.

Liquid of Ranvier: does not seem to contract the blood cells more than they already are.

Liquid of Vibert: *O.P.* More or less prolonged maceration. 400
M.E. contracted, granulous blood cells, quite clear mosaic casings, but formed by granulous elements.

Liquid of Paccini: analogous to the preceding.

What is amazing here is the consistent uniformity of results and their imperfection. Whether the stain is few days or months old, whether the powder obtained in unraveling the dry material stained with blood is used, or whether the material is moistened by absorption or more or less pro-

longed maceration, the liquids, no matter whether they are dilators or not, are impotent in regenerating distorted and contracted blood cells. In every case, microscopical examination reveals the same phenomena of which these are the essential ones: filaments of cotton, flax or hemp appear covered in a yellowish varnish, forming a casing more or less complete, which the maneuvers were able to remove and sometimes to separate completely and fragment into irregular plaques. This casing, like its debris, under careful examination with the liquid of Roussin rather than any other, appears very clearly constituted by the immediate juxtaposition of irregular blood cells, contracted and intimately cemented together: a veritable mosaic at the surface of which a few independent blood cells, more or less regular, but contracted, are sometimes distinguished on a higher plane. Between the filaments, in the spaces which they circumscribe, debris of variable form is seen and in the middle of it some rare, isolated blood cells, but always distorted and contracted. An isolated blood cell is exceptionally found which has escaped the common fate by who knows what luck, and has conserved its form and perhaps its normal dimensions. But, not counting that this good fortune is very rare, what expert would dare establish a diagnosis on such a narrow basis?

Furthermore, the dissociation of blood cells in the present case is not an easy thing to do. The blood cells being intimately cemented together, the mosaic quite often fragments outside the lines separating the various elements, due to the sliding movements, and if the observer is not careful,
401 he runs a strong risk of mistaking fragments, constituted by debris from neighboring blood cells, for isolated blood cells.

In the realm of the ideas we are pursuing, the most important phenomenon, along with deformation, is the contraction of the blood cells, an enormous contraction which resists the action of all liquids.

Why are the blood cells contracted whose dessication was very rapid, more rapid than in any other circumstance, since capillarity has dispersed them on a greater surface? Does the filament, swollen by plasma and returning to a lesser volume by evaporation of water, form its casing, drawing together the elements composing it, in this reaction? This is an hypothesis to which the following experiment seems to lend some weight. If blood drops are deposited on a strip of cloth or calico which is immediately placed under a bell-jar, in an atmosphere saturated with humidity, the blood does not dry, and, if dissociated in the liquid of Vibert, after one, two, three and even six hours, blood cells more or less distorted, but not contracted, can be noted.

In summary, as for blood stains on fabric, the expert, called upon to give an opinion on their origin, cannot ignore the fact that the results which he reports will be most conclusive if the fabric is wool or fur, but very limited, by contrast, if it is flax, cotton, or hemp. In the last case, he would search in vain for blood cells of characteristic form and dimension: he might be able to confirm that it is blood and distinguish mammal from oviparious blood, but that is all! And again, it is the presence of nuclei, more than the dimensions and the form of the blood cells, which permits him to express an opinion. However, a restriction must be made in favor of certain new, very compact material, whose finish might inhibit the destructive action of capillary phenomena; it must be also made for the case where the blood is in such abun- 402
dance that it dries in clots.

SECOND PART
Causes of the Alteration of Blood Cells

As causes of the alteration of blood cells, without deter- 530
mining the role of each one of them, it is generally admitted: the nature of the substratum of the stain, the thickness of the stain and its age, water, dessication, temperature and the humidity of the environment.

From the experiments summarized in the first part of this work, the influence of the supporting substratum can be deduced.

The age of the stain, within limits of one day to ten months, did not appear to us to have appreciable influence. Once fixed by dessication, the blood cell no longer tends to change, if no cause of alteration intervenes.

The experiments which follow will permit judgment of the 531
importance of the thickness of the stain, the rapidity of dessication being inversely proportional to this thickness.

Under the influence of water, the blood cells swell, become spherical, and diminish in diameter. Hemoglobin is dissolved, but the cellular stroma is not completely dissolved. This last phenomenon explains how, in certain cases, blood cells could be found without having obtained the reaction of Teichmann. When water acts on fresh blood, not yet dried, only contracted, distorted blood cells, unfit for the diagnosis of the origin of blood, are obtained after dessication. If, by contrast, water acts on dried blood, on a stain forming a more or less thick crust, the deepest parts can escape all alteration.

As for dessication, its influence is poorly interpreted, in attributing to it a destructive action not belonging to it. When dessication is rapid, it suddenly fixes the blood cells in their primeval form; comparative examination has demonstrated that, under these conditions, their dimensions have not been modified. Supporting this principle, Velcker has done measurements, whose evaluations have been recognized as perfectly exact by all hematologists. Renaut[17] recommends for the measure of blood cells the procedure of Velcker, which consists in depositing a droplet of blood on the bottom slide, heated to 60°, and spreading it out immediately in a thin layer with a needle held horizontally.

If the dessication does not occur rapidly, by contrast, within limits which the experiments which follow will permit one to appreciate, the blood cell remains a very fragile element, and soon alters in form and dimension.

It is evidently not the dessication, but rather the causes tending to delay it, which wreak destruction on the blood cells: the most powerful, which seems to sum up all of them, 532

is the degree of humidity of the environment. Here, the element of temperature intervenes, for with a low temperature, there can be a lot of humidity with little water vapor; with a high temperature, little humidity with a lot of water vapor. No matter what other variable conditions combine to prevent the dessication (such as an article of clothing, folded up, imprisoning still humid stains and let this article of clothing be concealed, hidden in a basement, or left to the night air for nights during which the air chills below the saturation point), it is always the humidity of the environment, confined or not, which opposes the evaporation of the water of plasma during a time more or less prolonged, in a continual or intermittent fashion, and thus favors alteration of the blood cell.

To study this influence, and to define its consequences as much as possible, we deposited drops of blood on different objects (a plate of glass, a knife blade, wooden planks, pebbles, and cloth), which we immediately placed under a glass bell-jar, over a saucer filled with water. After a specific time, this blood was examined, on its removal from the humid chamber before dessication, and later, after dessication. When the blood drop was still fluid, sometimes separated as a small clot bathed in serum, we made a preparation according to the procedure of Velcker, with a droplet of this serum in which we had diluted a piece of clot. If the blood were thick, we would dissociate blood cells from it in liquid of Vibert; after dessication, in liquid of Virchow.

In order not to repeat ourselves, let us now say that, with a similar stain, after a stay of the same duration, we observed essentially the same phenomena on removal from the bell-jar, or after dessication. This is a consequence of and a new proof of the influence attributed to dessication, which suspends all alteration, and fixes the blood cells in the form they have at the moment it begins to affect them.

533 ## Summary of Results Obtained

1) Influence of humidity on fresh human blood:

After one hour in the humid chamber: no appreciable alteration.

After two hours: blood cells generally healthy; some rare blood cells with wavy contours.

After three hours: Twenty measurements done on a preparation (Velcker procedure) give an average of $\frac{1}{130}$ millimeters; the blood cells are, in general, regular.

After four hours: regular blood cells; one in ten show buddings. Some rare contracted blood cells have become spherical.

After eight hours: healthy blood cells mixed with wavy, angular, jagged, more or less contracted blood cells and spherical blood cells.

After twelve hours: still some regular blood cells; altered blood cells predominate.

After twenty-four hours: rare regular blood cells, the others more or less altered.

After forty-eight hours: in certain cases, as a consequence of more or less great changes in humidity, due to variations in temperature, there are no more blood cells, but granulous debris; in others, morphologic alteration continues, thorny, berry-like blood cells, more or less contracted, and spherical blood cells.

After four days: all the blood cells are regular, spherical, contracted to $\frac{1}{200}$ millimeters, not changing form in passing under the microscope and more colored.

After eight days: same state; the blood of this preparation, left to dessicate, gives with the liquid of Virchow,after a month, blood cells absolutely simulating those of sheep blood, less the discoid form.

2) Influence of humidity on fresh rabbit blood:

After an hour and a half: numerous regular blood cells (average of thirty measurements, $\frac{1}{145}$ millimeters); some wavy, thorny blood cells, more or less contracted and sometimes spherical.

After three hours: three in four blood cells were altered.

After six hours: nine in ten were altered.

After twenty-four hours: all the blood cells are contracted, more or less spherical, thorny or regular.

After four days: all are spherical, contracted to about $\frac{1}{230}$. 534

After eight days: same.

3) Influence of humidity on fresh quail blood:

After one hour: healthy blood cells, a few circular globules, tending toward the spherical form.

After six hours: some circular blood cells cracked around their edges.

After twelve hours: healthy blood cells in a ratio of 1 to 3, the others circular, more or less spherical.

After twenty-four hours: sometimes granulous debris or alteration, continuing to take on the spherical form, with an avarage diameter of $\frac{1}{160}$ millimeters. Elliptic blood cells do not take the berry-like, notched appearance of discoid blood cells; they pass directly to the circular form, then become spherical[18].

The different phases of alteration of blood cells in fresh blood, sheltered from dessication, can easily be followed step 535
by step, by making a preparation of fresh blood which is immediately bordered with paraffin.

4) Influence of humidity on human blood dried beforehand:

Blood stains twenty days old are placed in a humic chamber for twenty-four hours, then left to the atmosphere for forty-eight hours. Under the microscope, after dissociation in the liquid of Virchow, the blood cells appear paler, smoother, their contours less well defined, but do not seem contracted; there is a lot of blood cell debris.

The same stains are placed once again into the humid chamber and left for four days. The blood cells seem almost dissolved, effaced in part. They are more transparent, the contours more blurred; but a certain number still have their normal form and dimensions.

Though it varies slightly, according to the source of the blood and the diverse conditions of temperature and humidity, successive alterations of mammal blood cells can be summarized thus: as early as the first, second or third hour, slight alteration or contours, which, wavy in the beginning,

soon become angular, sometimes giving a very clearly hexagonal or octagonal form to the blood cell; later, the sides of these geometric figures depress, the angles become more protruding, and their area is sprinkled with small projections, a kind of budding; finally, the blood cells, contracting more and more, become berry-like, thorny and reach their ultimate state from the first hour to the fourth day, the spherical form, which they can conserve a long time, and which dessication does not modify.

No liquid can regenerate altered blood cells. All that can
536 be expected from the best of liquids is that it isolate the blood cells as they are, altered or not, without acting on the blood cells themselves. Causes of error are thus diminished, in that those which might result from alterations produced by the liquid itself are not added. Besides, would it not be mere fancy to expect a dilator liquid to exert this action with a variable intensity, proportional to the degree of alteration of the blood cell?

The contraction of the blood cell is the essential characteristic of its alteration under the influence of humidity: a contraction accompanied by diverse distortions before getting to the regularly spherical form. This ultimate state, to which hardly any attention has been drawn, is, however, the most durable. Always consistent in the same animal, it is represented by a spherical blood cell, having $\frac{1}{250}$ millimeter in man, $\frac{1}{270}$ in the rabbit, $\frac{1}{160}$ in the quail. These figures adequately indicate that the diameters of blood cells, having become spherical, are appreciably proportional to the diameters of healthy blood cells.

The essential data resulting from the preceding experiments can be summarized thusly:

1) If the support substratum of the stain is not one which would alter them, most of the blood cells conserve their characteristic form and their normal diameter if dessication of the blood occurs within the first two hours.

2) If any cause whatever delays dessication beyond this period, the blood cells are altered; the alteration becomes more profound as the dessication is delayed more, and as the blood, as a function of its source, is more rapidly alterable. Human blood seems to be the one whose blood cells present the greatest resistance to various destructive influences.

3) Dessication holds spontaneous alteration of blood cells in abeyance, and fixes them in the form they have at the moment they start to dry.

537 4) In dried blood, the blood cell resists the causes of alteration for a much longer time.

5) No liquid is capable of rendering the primeval form and dimensions to an altered blood cell.

6) No element permits evaluation of the coefficient of dessication of Virchow, which should be more correctly called the coefficient of non-dessication; once distorted and contracted, all mammalian blood cells can resemble each other at a certain moment in their destructive evolution. In the last phase, however, the diameter of the spherical blood cells seems proportional to the diameter of normal blood cells.

7) The contraction of blood cells accompanies characteristic distortions, which are very easy to distinguish from those resulting from reciprocal compression; from the moment a blood cell conserves its flat, biconcave form with clear contours, it can be considered as healthy, and serve as a basis of serious measurement. In the opposite case, the expert must abstain: where the blood cell is altered, all diagnosis, already very uncertain, soon becomes impossible with the beginning of the alteration.[19]

THIRD PART
Measurement of Blood Cells—Source of Blood

The diameter of blood cells constituting the essential distinctive characteristic, its exact evaluation would necessarily 538
lead to the diagnosis of the source of mammalian blood. Unfortunately, the measurement of a blood cell is a delicate operation in itself, which, furthermore, occurs here under conditions and on a basis which render the data that can result from it imprecise.

Vibert, in his work[20], studied the diverse causes which oppose an exact measurement and limit the scope of our evaluations. Let us sum them up in a few lines:

> The diameter of blood cells, even in the absence of every pathological state, varies in relatively considerable limits, not only for a given animal species, but even for a given individual
>
> The most competent authors are far from alloting the same average diameter, or the same extreme limits, to blood cells of an animal.

Using this table (Table I), to what species would the 539
expert attribute the blood cells measuring between 0.006 and 0.008?

In admitting that blood cells have absolutely fixed dimensions, it is not possible to distinguish with certitude a blood cell of 0.0075 (man) from another having 0.0073 (dog) or even 0.0069 (rabbit).

Even on perfectly immobile blood cells, a measurement cannot be made within $\frac{1}{10}$ μ.

With the ocular micrometer, likewise even with the camera lucida, it is impossible, as says Vibert, to arrive at a rigorously exact evaluation of blood cells; according to our experience an approximation of 1 to $\frac{3}{10}$ of a thousandth of a millimeter can be attained.

But, if one considers that these $\frac{3}{10}$ μ intervals sometimes diminish, and sometimes increase the actual diameter, it seems to us it can be admitted that the average of the measurements will not be appreciably far from reality, in any case, within $\frac{3}{10}$ μ.

As for attributing to such and such an animal a blood cell of a determined diameter, no prudent expert shold consider it. It's not the diameter of a blood cell, but the average diameter of 50, or of 100 blood cells, which must serve as the basis of a serious diagnosis. Do not the large blood cells of pig and even of beef have a diameter near the average diameter of blood cells of man?

TABLE I

DESIGNATION	FREY	WELCKER	GUIDELINES OF THE SOCIETY OF LEGAL MEDICINE	TOURDES	DRAGENDORFF
Man	0.0046 to 0.0069	0.0045 to 0.0097	0.0075	0.0074 to 0.0080	0.0077
Dog	"	0.0073	0.0073	0.0066 to 0.0074	0.0070
Rabbit	0.00713	0.0069	0.0069	0.0060 to 0.0070	0.0064
Cat	"	0.0065	0.0065	0.0053 to 0.0060	0.0056
Horse	0.00575	"	0.0056	0.0055	0.0057
Ox	"	"	0.0056	0.0056 to 0.006	0.0058
Sheep	"	0.005	0.005	0.0047 to 0.0050	0.0045
Pig	"	"	0.006	0.0060 to 0.0065	0.0062
Goat	"	0.0041	0.0046	0.0040 to 0.0046	0.0062

The most obvious cause of error results from a variable diameter of blood cells in the same individual. Indeed, from this variability stems a very great difficulty, that of obtaining averages representing different blood cells in a normal proportion. Hayem admits that, of 100 blood cells of human blood, 75 are of average size, 12 are big and 12 are small. As a consequence, if the average obtained does not represent them exactly in these proportions, it will be too high or too low, according to whether the large or the small blood cells will have been measured in greater number. Such is the
540 cause of divergence which is inevitably produced between averages of measurement done on the same preparation under identical conditions. The expert would not know how to correct for this effect; he can only attenuate the effect by multiplying the measurements.

To this cause of permanent error is added another, exceptional it is true, but which must be taken into consideration, however, to inspire in the expert the sentiments of reserve which must always preside over the establishment of his conclusions. It results from the possible alteration of the diameter of blood cells under various pathological influences. Kelsch determined an increase in the volume of red blood cells under the influence of malaria, and Malassez demonstrated that healthy blood cells of a man which are 7.6, are 8.29 in chlorosis and 6.64 in cancer. In the presence of such considerable differences, is it not permissible to concede that, in animals, certain afflictions can likewise modify the diameter of blood cells in such a way as to impose them on the expert?

Such are the principal causes of error which oppose an exact evaluation of the diameter of blood cells, and remove from the results obtained all characteristics of absolute verity. Let us now look at the data of the experiments.

We performed our experiments with an ocular micrometer, adapted for a Nachet microscope, giving a magnification of 800 diameters.

The blood cells were immobilized by bordering the preparation with paraffin; currents resulting from evaporation of the liquid at the limit of the top slide are avoided.

1) **Measurement of fresh blood cells by the procedure of Welcker.** This operation permitted us to appreciate the degree of precision that can be attained under these eminently favorable conditions, quite exceptional in legal medicine, it is true! It gave us at the same time, an evaluation of 541
differences resulting, all other things being equal, from the variability of the diameter of globules, a basis for comparison, permitting us to better judge the results obtained with dried blood by the same observer and with the same apparatus.

The following table (Table II) represents the averages of measurements carried out on preparations of known and unknown origin:

Table II

Origin of Blood	Average of 25 measurements Preparations of *known* origin					Average of 125 measurements		Average of 100 measurements Preparations of *unknown* origin
Man	1/129	1/125	1/130	1/127	1/127	1/127.8	0.0078	1/127
Guinea pig	1/129	1/130	1/129	1/126	1/128	1/128,4	0.0077	1/126
Dog	1/138	1/140	1/135	1/141	1/141	1/139	0.0071	1/140
Rabbit	1/145	1/139	1/140	1/142	1/144	1/142	0.0070	1/144
Pig	1/159	1/160	1/164	1/161	1/165	1/161	0.0062	1/144
Beef	1/162	1/167	1/162	1/163	1/166	1/164	0.0060	1/144
Cat	1/174	1/176	1/172	1/173	1/175	1/174	0.0057	1/144
Chicken	1/86	1/176	1/140	1/173	1/175	1/174	0.0057	1/144
Carp	1/79	1/176	1/106	1/173	1/175	1/174	0.0057	1/144

From the analysis of figures, it results that:

1) To the blood cells of dog and rabbit, which were considered by different authors as approaching closest to those of man, must be added those of the guinea pig, whose aver-

age diameter, more considerable, is so close to those of the
542 blood cells of man, it cannot be determined if they are larger or smaller.

2) Divergences existing between the different averages of 25 measurements done on the same preparation of known origin, divergences which could likely be attributed to the variability of the diameter of blood cells in the same individual, are 3 to $\frac{4}{10}\ \mu$. Then, even under conditions as favorable as this, if the difference of the average diameter of the blood cells of two animals is not over 3 or $\frac{4}{10}\ \mu$, it will be impossible to distinguish them with certainty. It will be impossible to distinguish the blood of man from that of guinea pig, the blood of dog from that of rabbit, the blood of pig from that of ox; but one can distinguish with certainty blood of man and guinea pig from that of dog and rabbit and blood of the latter from that of pig, ox and cat.

If the figures given in the Guidelines of the Society of Legal Medicine are taken, if human blood cells are admitted to be 0.0075 and those of dog 0.0073, it will be deemed that distinction of the blood of these two animals is impossible; if, on the contrary, figures are accepted from Roussin, Tourdes and Dragendorff, 0.0077 and 0.0070, figures which closely approximate our own, it is permissible to consider that a distinction can be established. In fact, working with preparations of unknown origin, we have always obtained figures such that we could give an opinion with certainty.

3) The difference existing between the large diameters of the elliptic blood cells of chicken and carp is such that the distinction of blood from these two oviparı is an easy thing.

2) **Measurement of dried blood cells, isolated with liquid of Virchow.** To measure blood cells of known origin is an operation of little value, from which serious information
543 cannot be drawn. Indeed, no matter what one does to maintain objectivity, one is involuntarily dominated and led too easily to preconceived results. So, neglecting the results of numerous measurements we had done under these conditions, we entrusted to our colleague and friend, regimental adjutant pharmacist Péré, fifty varied objects stained with blood asking him to remove small crusts, and to return them to us furnished with a serial number that we might determine the unknown origin.

These stains, from different sources, were one to six months old. Their support was of variable nature: wood of ash, walnut and oak (used in the manufacture of axe handles, rifle butts and floor boards), paper, knife-blade, plates of glass, straw, material of wool or fur.

We had absolutely excluded from this study material of flax, cotton or hemp, after having acquired the conviction that blood cells of different animals undergo the same destructive influences on contact with them as blood cells of man; and that the principles propounded on this subject in the first part of this work are fully confirmed by these new experiments. These principles seemed to invalidate the conclusions of the memoir of Professor Ch. Robin[21], who was able not only to find numerous intact blood cells on a smock of blue cotton, but could affirm their origin. This contradiction is only apparent, for it must be considered, as Robin wrote himself in his memoir, that he was able to remove small superficial crusts from each stain. Contrary to the opinion of Briand and Chaudé, one is led to believe that the eminent histologist found himself confronted with one of the exceptional cases noted above. In ordinary conditions, when blood drops fall on material of cotton, they do not form a
544 crust at the surface, but are absorbed by the material so as to give a stain the same appearance and equal diameter on both sides.

To finish with these materials, it remains for us to sum up their influence on nucleated elliptic blood cells. Under the conditions recommended in the first part of this work, examination of oviparious blood, dried and absorbed onto material of cotton, flax or hemp, treated with liquid of Virchow or Roussin, shows the following:

The filaments are covered in a yellowish coat seeded with more or less brilliant nuclei, of a pale rose, that much more apparent when the thickness of the coat, separated from the filament, is less. The edges of different blood cells are discerned only with difficulty: it is a smooth varnish. The elliptical form is gone. The isolated blood cells are contracted and their very irregular form recalls very little of their primeval form.

The nuclei are then the essential, the only durable characteristic, that which permits easy, certain distinction, even in these unfavorable circumstances, of oviparous from mammal blood.

Let us return to blood stains forming crust, to the measurement of their blood cells and the diagnosis of their origin. In the following table (Table III) the results of twenty assessments done on blood of absolutely unknown origin are summarized:

Table III

SERIAL NUMBER	STATE OF BLOOD CELLS	NUMBER OF MEASUREMENTS	AVERAGE DIAMETER	ATTRIBUTED TO	ACTUAL ORIGIN	NATURE OF SUBSTRATUM	AGE OF STAIN (MONTHS)
1	rather well conserved	120	1/142	dog or rabbit	dog	wood	1
2	well conserved	120	1/128	man or guinea pig	man	glass	1
3	altered in general	75	1/172	ox or cat	ox	glass	1
4	"	75	1/162	pig, ox or cat	pig	cloth	1
5	"	75	1/166	"	cat	wood	1
6	well conserved	150	1/129	man or guinea pig	man	straw	5
7	altered in general	75	1/172	ox or cat	ox	knife-blade	2

Table III—Continued

SERIAL NUMBER	STATE OF BLOOD CELLS	NUMBER OF MEASUREMENTS	AVERAGE DIAMETER	ATTRIBUTED TO	ACTUAL ORIGIN	NATURE OF SUBSTRATUM	AGE OF STAIN (MONTHS)
8	distorted at nucleus	75	1/172	oviparious	carp	cloth	4
9	rather well conserved	120	1/143	dog or rabbit	dog	wood	2
10	"	100	1/130	man or guinea pig	man	"	5
11	"	150	1/136	dog or rabbit	rabbit	"	5
12	passable	100	1/157	pig or ox	pig	cloth	2
13	"	75	1/169	pig, ox, cat	cat	wood	2
14	rather well conserved	120	1/129	guinea pig or man	guinea pig	cloth	2
15	"	150	1/138	rabbit or dog	rabbit	wood	5
16	"	25	1/233	goat	goat	"	3
17	elliptic, clear	25	1/135	(large diameter) chicken	chicken	"	4
18	passable	200	1/137	rabbit, dog	rabbit	paper	4
19	"	75	1/165	pig, ox, cat	cat	knife-blade	2
20	well conserved	75	1/129	man, guinea pig	man	wood	6

544 From these figures, and from the numerous observations we have performed, the following information can be drawn:

Blood cells of man preserve the best of all. After them, come, in order of resistance, those of dog, goat, rabbit, cat, guinea pig, ox and pig; the blood cells of these last are always more or less profoundly altered, even in the most favorable circumstances.

The blood cells of man and dog are most colored; the others, those of the rabbit in particular, are paler, more transparent and more difficult to measure Here is an element which, in certain cases, can be taken into account; but it must not be forgotten that man's blood cells discolor and become paler under certain influences.

546 The blood cells of chicken and carp give excellent preparations with liquid of Virchow. The blood cells, a bit dilated it is true, conserve their elliptic form with clear contours and a very apparent nucleus. Under these conditions, it is easy to distinguish not only oviparous blood from that of mammals, but again, in measuring the large diameter of the blood cells, the blood of chicken from that of carp.

As for the diagnosis of mammalian blood, the preceding table (Table III) shows that, in short, the results we have obtained obviously approximate, in appearance at least, those given us by the procedure of Velcker. We have been able to distinguish the blood of man and guinea pig from that of dog and rabbit, and blood of the latter from that of pig, ox and cat. But, it must be admitted, this diagnosis, easy and sure in certain cases, was quite difficult and unsure in others. If, after fifty measurments, we could give an opinion with certainty when it was a question of blood from pig, ox or cat; if the diagnosis of blood of dog appeared to us, though very delicate, to have, however, a fair degree of certainty, it was only in performing one hundred fifty to two hundred measurements that we could arrive at a simple probability in favor of blood of rabbit.

The different averages of thirty measurements, done on rabbit blood from the same source, presented considerable differences so as to mislead the expert: $\frac{1}{132}$ *and* $\frac{1}{143}$ *mm.* for example. Moreover, these averages have always been greater than those we obtained by the procedure of Velcker, whereas, 547 for other animals, the cat excepted, they have been, in general, essentially equal or less. To what can these differences be attributed? The measurement of blood cells of rabbit, paler and more transparent, is certainly more difficult; but that does not explain a constant increase in the average diameter of these blood cells.

If the dimensions of the largest blood cells of rabbit blood prepared by the procedure of Velcker are compared to that of these same blood cells isolated by liquid of Virchow, it is noted that these have a diameter appreciably greater than that of the former. These blood cells, whose diameter is exaggerated, have a peculiar aspect: they seem spread out, larger and flatter. This is not a dilation; the liquid of Virchow, after twenty-four hours, contracts them from $\frac{1}{132}$ to $\frac{1}{168}$ mm! This is a collapse of the less resistant globular stroma, which has the consequence of flattening the blood cell and slightly increasing its diameter, to the detriment of its biconcave form.

This phenomenon especially evident in large blood cells, proves that the best of liquids is not perfect! None of them can adapt equally to the variable qualities of blood cells coming from different species and even to those of different blood cells from the same animal. However that might be, experience shows us that, relative to human blood, the diagnosis of blood of pig, ox and cat are easy, that of blood of dog, delicate, that of blood of rabbit, uncertain, that of blood of guinea pig, impossible.

In light of these facts, instilling in us controlling sentiments of reserve and the gravity of the subject and the varied causes of uncertainty and error which we have pinted out, as well as the responsibilities of the expert who owes to justice as much enlightenment as possible; for the future, when called upon to give our opinion on the origin of blood stains, we will proceed and form conclusions in the following manner.

The expert is only very exceptionally called upon to give 548

his opinion on the source of fresh blood, this particular case having been sufficiently studied in the course of this study; moreover, blood absorbed by material of cotton, flax or hemp, limits the diagnosis to the identification of oviparous or mammalian blood; the conclusions which follow apply exclusively to blood stains forming a crust, thin as it might be, on any object whatever, more or less impermeable.

These conclusions aside, as for interpretation of averages which, in an assessment, do not exactly fit into one of the models we have formulated, or, for example, more or less clearly approximate the first or the third, while belonging to the second, these conclusions cannot be considered as absolute. They express a direction, which remains subordinate to the ensemble of characteristics peculiar to each assessment.

General Conclusions.

To isolate blood cells we use liquid of Virchow.

We do a minimum of five series of thirty measurements, at five different times and on five different preparations.

We indicate in our report the number of measurements done; we confirm that the blood cells, serving as basis for these measurements, are healthy.

All the measurements completed, the averages of each series are established and included in the report.

If these averages are found to be *between* $\frac{1}{735}$ *and* $\frac{1}{730}$ *mm* we conclude thusly:

The average diameter of blood corpuscles being greater than $\frac{1}{730}$ *mm*, the blood can belong to man or one of the animals (guinea pig, dog, rabbit) who, in our environment,
549 possess with him the largest circular blood cells: these dimensions are closer, however, to those of blood cells of man and guinea pig.

Between $\frac{1}{730}$ *and* $\frac{1}{735}$ *mm:* The average diameter of blood corpuscles, less than $\frac{1}{730}$ and greater than $\frac{1}{735}$ mm, the blood can belong to man or one of the animals (guinea pig, dog, rabbit) which possess with him the largest blood cells.

Between $\frac{1}{735}$ *and* $\frac{1}{740}$ *mm:* the average diameter of blood corpuscles being less than $\frac{1}{735}$, the blood probably does not belong to man, but to one of the animals who, after him and the guinea pig, possess the largest blood cells.

Over $\frac{1}{740}$ *mm:* the blood does not belong to man, but to one of the animals whose blood cells have a diameter which evidently approaches the average diameter of the observed blood cells.

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18. In studying quail blood, we had the good fortune of observing in the blood of two quails, out of eight examined, a microscopic hematozoan in the embryonic state, belonging to the genus filaria, and having a close analogy with the human parasite *filaria sanguinis hominis.* There were 10 or 12 per drop of blood, measuring 180 μ in length, by 4 μ in diameter, smooth and transparent, etc . . . . . [Editorial Note: The remainder of this lengthy footnote deals strictly with Masson's description of the parasites in the quail blood, and has nothing to do with the main subject of the paper. It has not been translated].
19. We had ended this part of our work when we became acquainted with the research of Mayet on the spontaneous alterations of blood cells in plasma sheltered from air (*Archives de physiologie,* 1882).

    Our research on blood cells in blood protected from dessication shows that, contrary to the opinion of this hematologist, the spontaneous alterations of blood cells are produced equally well in blood in bulk, and in presence of air, and that they depend exclusively on the conservation of blood in a more or less liquid state favoring the evolution of the blood cell to the spherical form, whereas dessication holds this change in abeyance.

    As for spherical blood cells of blood, everything leads us to believe that they are products of transformation of discoid blood cells: a more or less rapid transformation which takes place from the first hour to the fourth day.
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On the Possibility of Distinguishing Human Blood from that of Mammals. (Medico-legal Study)*

Ch. Vibert

I

548 When medical experts have determined that stains found on clothing, weapons or other objects are composed of blood, it is not uncommon that they are asked if the blood comes from man or a domestic animal. When the species of the animal has been specified in the question and the animal does not belong to the mammalian class, the problem is relatively simple. The form and dimensions of the red blood cells, the presence or absence of internal nuclei, form very distinct characteristics, clearly differential, and which generally permit a certain reply after a well-conducted examination by microscope. But when it is a matter of differentiating the blood of man from that of another mammal, the problem becomes more difficult, for the only distinctive discernible characteristic consists of the differences in dimensions which are most often minimal. Moreover, the difficulty of an assessment such as this has long since been noted. Already in 1857, Virchow remarked: ". . . I do not believe a micrographer should ever be allowed to let the life of a man depend on the yet so uncertain evaluation of the coefficient of dessication of blood cells. Blood undoubtedly dries sometimes in a way so as to clearly recognize individual blood cells . . . but dessication occurs under so many variable conditions, and blood, once dried, can be exposed to such unfavorable circumstances, that a judgment on the size
549 of its constituent parts cannot be exercised with certainty."[1]

Most histologists share this viewpoint. However, treatises of legal medicine admit or seem to admit the possibility of recognizing from which mammal blood stains derive, and they limit themselves to recommending reserve, without explaining the numerous motives of such wise counsel. It is regrettable that the guidelines for blood stains drawn up by the Society of Legal Medicine, guidelines which merit being the official guide of experts in every other regard, limits itself to simply giving the dimensions of red blood cells of various domestic animals and then to comment laconically in its conclusions: "He (the expert) will measure the blood cells and can thus affirm if it is a matter of human blood or not."[2]

As a result of the incomplete manner of presenting the question in classical works, experts have, several times, not hesitated to affirm in court that stains submitted for their examination were produced by human blood. Now, in general, and without taking into account the circumstances specific to each assessment, such an absolute affirmation would never appear to us to be permissible. This is what we are going to try to demonstrate at the same time as we make an effort to specify the difficulties of the question.

II

At our present level of knowledge, the only characteristic which can be invoked for the differentiation of blood of various mammals consists in the difference of the diameter of the blood cells. An important remark must now be made; it is that this diameter, even outside of any pathological state, varies in relatively considerable limits, not only for the same animal species, but also for the same individual. It is thus that in blood preparations, Malassez observed about 150 blood cells in the same microscopic field with the following dimensions:[3]

	Max.	*Minim.*	*Average*
Man	0.009	0.007	0.0074
Dog	0.0087	0.0062	0.0074
Dog	0.0095	0.0065	0.0074
Rabbit	0.0085	0.006	0.0072

For man, Welcker assigns as limits 0.0045 to 0.0097, and Frey, 0.0046 to 0.0069.

It might be claimed that these extreme figures represent exceptions which should not be taken into consideration. It is possible, though not likely, that an expert, who often experiences difficulties in isolating one or two blood cells, might come across precisely these dwarf or giant blood cells. But, in any case, the intermediate blood cells vary enough between themselves so that it is impossible to attribute a blood cell with a diameter of 0.007, for example, to man rather than to dog or rabbit.

These differences in dimensions are so accentuated that very competent authors are nowhere near assigning the same average diameter or the same limits to blood cells of the same animal. Consultation of the table below is convincing:

* Translation of: "De la Possibilité de Distinguer le Sang de l'Homme de Celui des Mammifères (Étude Médico-légale)."

in *Archives de Physiologie Normale et Pathologique* 14 (2nd series 9): 48-58 (1882).

Designation	Frey[4]	Welcker[5]	Guidelines of the Society of Legal Medicine	Tourdes[6]	Dragendorff[7]
Man	0.0046 to 0.0069	0.0045 to 0.0097	0.0075	0.0074 to 0.0080	0.5077
Dog	"	0.0073	0.0073	0.0066 to 0.0074	0.8070
Rabbit	0.00713	0.0069	0.0069	0.0060 to 0.0070	0.0064
Cat	"	0.0065	0.0065	0.0053 to 0.0060	0.0056
Horse	0.00575	"	0.0056	0.0055	0.0057
Ox	"	"	0.0056	0.0056 to 0.006	0.0058
Sheep	"	0.005	0.005	0.0047 to 0.005	0.0045
Pig	"	"	0.006	0.0060 to 0.0065	0.0062
Goat	"	0.0041	0.0046	0.0040 to 0.0046	"

51 With this table at hand, what will the expert choose as the standard figure serving as the reference point for his research; to return to the preceding example, to what species would be attributed blood cells included between 0.006 and 0.008? Such blood cells might belong to a dog or rabbit, as much as to man, and it appears evident to us that a differential diagnosis of this kind is absolutely impossible, especially with the absolute certainty required in legal medicine.

But let us disregard this difficulty, however considerable it might be. Let us allow that for each animal the red blood cells have an absolutely fixed diameter, and let us take the figures given by the Guideline of the Society of Legal Medicine. Even working with fresh blood just drawn from a vessel, is it possible to differentiate with certainty a blood cell of 0.0075 (man) from another of 0.0073 (dog), or even 0.0069 (rabbit)? All who have performed measurements on blood elements know that such precision is almost impossible. Other than the fact that the evaluation of such minimal differences is always extremely delicate, the difficulty is singularly increased in the particular case where the blood cells are always subject to variations on whatever vehicle they might find themselves. In taking care to let the preparation lie still, to avoid even the slightest movement on the table on which the work is being performed, and to refrain from breathing near the slide or bringing your hand near it, it is sometimes, though quite rarely, possible to make out a blood cell immobile enough so that its contour can be exactly delineated with a camera lucida. But even with a perfectly immobile blood cell, we wonder how a measurement within $^1/_{10}$ of a μ can be done, if an ocular micrometer is used, as recommended by the Guidelines of the Society of Legal Medicine.

The preceding considerations appear to us to justify considerably our earlier assertion: that it is unthinkable for an expert to assert that these stains originate from human blood. If a very capable histologist is not in a position to
52 determine whether fresh blood, just drawn from a vessel, and prepared with all suitable precautions, belongs to man rather than to a dog or a rabbit, then all the more reason will the question be unanswerable for an expert almost always investigating dried blood. Indeed, here appear difficulties of another order which most often render the problem impossible to resolve, even if the blood to be examined comes from an animal whose blood cells are appreciably smaller than those of man.

III

It is known that in drying, blood cells lose their characteristic form to become characteristically spherical or polyhedric, to form spikes, etc. At the same time, their diameter appreciably diminishes. The conditions of heat and humidity in which the dessication took place, the nature of the substance on which the blood had been deposited, the size and thickness of the stain, and the time which has passed, play a considerable role in the degree of these deformations. But it would be nothing but day-dreaming to hope that, by taking all these factors into consideration, an estimation can be made of what Virchow calls the "coefficient of dessication" or that calculation can be made of the primeval dimensions of blood cells isolated from the preparation. On the other hand, these changes are definitive, and no reagent can restore the primeval form or dimensions to dried blood cells. All that can be asked of the various liquids used for the examination of blood stains is that they promote the dissociation and isolation of blood cells. The imperfection of this dissociation constitutes a very frequent cause of error against which it is important to be on guard. Indeed, the blood cells most often break at the same time as they separate. Either a blood cell missing a part or, on the contrary, an entire blood cell to which a fragment of a neighboring blood cell remains fixed, without any clear limit of separation, are seen under the microscope. The naturally irregular form and jagged contour then render difficult the 53
determination of whether it is actually a matter of an intact, perfectly isolated, globule. Finally, the different diameters of the same blood cells are most often unequal, leaving it doubtful as to which it is convenient to adopt.

The reality of all these difficulties is put into perspective by a perusal of the figures below.[1] They are reproduced from an observation by camera lucida, at a magnification of 1000 diameters, of blood cells from blood stains of varied dates and origins. These stains were made by us under well-defined conditions or were of absolutely certain origin. The examination concerned either the small bloody crusts often found on the surface of these stains or solely on the impregnated material. In this latter case, the stained linen or fabric was divided into small pieces; each piece was wetted with a few drops of one of the following solutions:

[1] [The figures have not been reproduced in the translation.]

A. Mercury bichloride 0.50
Sodium chloride 2
Water 100

B. Solution of sodium sulfate of a density of 1.020

C. Solution of sodium sulfate of
1.020 ..... 100 g
Mercury bichloride ..... 0.50 g

After a prolonged maceration for ½ hour or an hour, the material was unravelled by fine glass needles, then the red or reddish liquid thus obtained was covered with a slide and brought into the field of the microscope. The blood cells appearing the least isolated and the most clearly delimited were then outlined. The figures do not represent a single unique field but a collection of blood cells chosen from within the preparation. Besides, it is less difficult to immobilize blood cells under these conditions than in a preparation of fresh blood, for here, they are often stopped and maintained by undissociated threads or fragments of the stain which are found in the preparation. For the design, we placed the paper
54 on the plate invented by Malassez,[8] a plate which can be inclined exactly along the same angle as that of the prism of the camera lucida, so as to eliminate all deformation of the image. Use of this procedure facilitates the assurance these deformations actually do not exist. It suffices to delineate the divisions of an objective micrometer, being sure they are of rigorously equal distance from each other.

Figure 1 represents a preparation obtained from small crusts of dried blood found on the shirt of a murdered infant. Examination was performed one month after the murder. Liquid A was used.

Figure 2 was obtained from a non-scaly stain found on the same garment and examined with liquid B after 45 days.

Figures 3 and 4 represent preparations made from the blood of a rabbit, deposited on linen, and placed under conditions as identical as possible with those to which the preceding shirt had been subjected. In figure 3 the examined blood was in small crusts; it was treated with liquid A after a month. In figure 4, the blood impregnated the linen without formation of crusts. After 43 days the stain was treated with liquid B.

55 Comparison of these four figures clearly demonstrates that it is impossible to differentiate blood cells coming from man from those coming from rabbit. It is evident how difficult it is, with such irregular forms and such unequal diameters for the same blood cell, to compare these elements either among themselves, or with typical blood cells whose exact dimensions are known.

Figure 5 is more instructive in that it demonstrates that even blood coming from an animal whose blood cells are relatively very small, such as the sheep, cannot easily be distinguished from human blood when dessicated. This figure is a reproduction of a preparation obtained from sheep blood, deposited on linen 10 days before, and treated with liquid C. It can be seen that many of the blood cells have dimensions that are equal to and even greater than those of figures 1 and 2. In *a*, ‡ a blood cell is seen to which a portion of another blood cell is adhering; there was not found, however, the line of demarcation or traces of fusion proving it is actually so. It might be objected that the stain is recent, that the liquid employed was not the same as that used for the child's blood, etc., but these objections support precisely the hypothesis we are putting forth.

We don't maintain, however, that searching for blood cells 56
in blood stains always gives results as incomplete and dangerous with regard to interpretation. Recently, we had the opportunity of examining blood deposited two months before on a woolen garment, and we could find blood cells, the greater part of which were perfectly isolated and had preserved their normal form almost intact. This observation depends on a particular combination of numerous factors enumerated above and whose mode of action, we repeat, is yet unknown. It can be said only that, when blood is protected from evaporation, the blood cells preserve their morphological characteristics very clearly for a long time. This circumstance is not as rare in legal medicine as might be believed. It is enough that a linen or fabric be folded several times immediately after being stained for the blood to remain liquid between the folds for several days. We dipped linen into blood of a kid. After having let the linen drip a bit, it was folded in half several times, then wrapped in paper and carried to the laboratory. Only after five days was the package opened. The blood was still in the liquid state in the center of the piece of linen. A small piece was removed with 57
a scalpel and placed on a slide without the addition of any reagent. Figure 6 represents blood cells, delineated rather haphazardly, without choosing those which were clearest as in the preceding investigation. It is evident that, in this case, it could be concluded that the blood did not come from a human being and it is the same in every case where the assessment can be done under favorable conditions, and that the blood belongs to a species whose blood cells are relatively very small. We can easily believe that Richardson[9] was able to differentiate successively the blood of calf and of sheep, which he had someone else deposit on white paper from human blood. But, even while operating under such exceptionally good conditions, the diagnosis would not have been made if the sheep or the calf were animals whose blood cells normally offered dimensions more closely approximating those of human blood cells.

It is here that it cannot be repeated enough to warn experts against rash assertions, profoundly regrettable from every point of view. The limits within which an affirmation is permitted can be indicated in the following conclusions:

1) It is always impossible to assert that a stain is formed 58
by human blood. One can only say, in certain cases, that it *could* have come from human blood.

‡ refers to a label on one of the cells in Figure 5, which is not reproduced in the translation.

2) It can sometimes be asserted that a stain comes from a mammal other than man. But for this, it is necessary that the animal whose blood produced the stain belong to a species whose blood cells are much smaller than those of man, and that the investigation be able to be executed under very favorable conditions.

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A Simple Method for the Forensic Differentiation of Human and Mammal Blood*

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First Communication

293 In the biological process of Wassermann-Schütze and Uhlenhuth, we possess such an excellent method for the forensic identification of human blood that it could appear almost superfluous to publicize a new method in addition to it. If we dare to do this, however, we do it because our method is easy and because it can be a useful preliminary or auxiliary test along with the other process.

The preparatory studies were begun by one of us (Marx) in February 1903; they will be published in the April issue of this year's volume of the *Vierteljahrschrift für gerichtliche Medizin.* The principle of the method relies on distinguishing, with the aid of a microscope, the difference between the effects of homologous and heterologous sera on fresh human blood. The human blood corpuscles are quickly agglutinated by a foreign serum in such a way that, under the right conditions, the erythrocytes flatten out immediately after the addition of serum and stick together in small piles. If the foreign serum is less concentrated and older, the agglutination takes place less dramatically; in all cases, though, the differences, when compared to the effects of a homologous serum on fresh blood, are unusually clear. Figures 1 and 2 [not reproduced in the translation] show a reaction of medium strength. In Figure 1, human serum has acted upon human serum; in Figure 1, pig serum has acted on human blood. The human blood serum came from twelve-month old dried human blood, the pig blood serum from a ten-month old dried sample. The photomicrographs were taken with a Leitz objective no. 5 and ocular no. 3, which corresponds to a magnification of 1:250.

In Figure 1 (human blood with human serum), the blood corpuscles lie next to each other, though clearly separate and not gathered together into piles; in Figure 2 (human blood with pig serum), there is a most complete agglutination, in some places agglutination of the erythrocytes so that individual blood cells become unrecognizable. The difference, therefore, is that the erythrocytes of fresh human blood are influenced by homologous serum only in that they reach a state where they lie close to one another, arranging themselves next to each other, an effect which one can scarcely call agglutination compared to the reaction of heterologous serum. Here, the individual erythrocytes remain recognizable, each one clearly isolated from the other. On the other hand, the human blood corpuscles are quickly agglutinated by heterologous serum, are tightly bound together in little piles, and are finally no longer recognizable as individual cells (hemolysis).

It interested us greatly to find out that ape blood serum produced an effect similar to homologous serum; we were able to recognize a difference, in that the human erythrocytes usually took on the shape of thorn apples when acted on by human blood; when ape blood was added, on the other hand, they shrank, became polygonal, and did not show the thorn apple shape. We had at our disposal ten-month old, dried blood from an Indian ape.

The technique used in our process is the following. From dried blood in some substratum, or on linen, wood, sand, blotting paper, or similar objects, a brown to black-brown-red solution, as concentrated as possible, is produced on a slide by adding one or more drops of 0.6% saline solution. One then extracts a small drop of blood from one's fingertip with a glowing hot needle and mixes it for five to six seconds into the blood solution on the slide with a glass rod. This is covered with a cover slip and observed under higher and lower magnification for the next fifteen minutes. The fresher the heterologous blood and the stronger the concentration, the quicker the reaction is finished. With blood only a few months old, it takes place for the most part in a few seconds but becomes still more pronounced from minute to minute; with blood only a few weeks old it takes place quite drastically, almost immediately after the initial mixing. Instead of covering the preparation immediately with the cover slip, one can smooth it out on the slide and let it dry for two or three minutes. One thus obtains neat, long-lasting demonstrative preparations.

We have examined the following types of blood with definite success:

* Translation of: "Eine einfache Methode zur forensischen Unterscheidung von Menschen- und Säugetierblut," I and II Mitteilung,

in *Muenchener Medizinische Wochenschrift* **51** (7): 293 and **51** (16): 696–697 (1904).

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fluid blood		*dried blood*
human	horse dog calf	3 years old, dried on a piece of linen
dog horse cattle	human	dried on linen, blotting paper, wood, and in substrata two weeks to two years old
pig mutton rabbit	rabbit pig	dried for a period of two weeks to one year on linen, sand, blotting paper, wood, and on substrata
white mouse	cattle	
	sheep	

If one wishes to proceed to a follow-up test of these experiments, we recommend that one begin by studying the action of fresh, defibrinated animal blood, diluted to half strength with saline solution, on human blood, while observing our technical prescriptions. In order to determine with certainty the variations, a certain amount of practice is necessary, practice which can be quickly acquired. Our colleagues in the institute were very soon in a position to recognize from among the preparations which we put before them whether we had allowed homologous or heterologous blood to act upon the human blood. For practical reasons, one might want to allow the term "serum" for blood solution, though it is not completely correct.

We hope that we will soon be able to report on further successful experiments with our method. We have expressly refrained from reporting whether it was possible to distinguish animal tissue sections from those of humans by our process.

In our second communication we wish to examine more closely the works of Lansteiner, Ascoli, von Decastello and Sturli, and Landsteiner and Richter concerning isoagglutinins. We wish to point out once again with special emphasis that the agglutination, caused by heterologous serum, is always accompanied by hemolysis (by a progressive decay and dissolution of the cells)†, while in the case of occasional clumping formation caused by homologous serum the erythrocytes remain visible to the last as individual, well preserved and colored cells.

We need scarcely affirm that this communication does not concern the publication of new facts but rather represents an attempt to render well-known facts useful for forensic purposes.

Second Communication[1]

696 Since Landois[2], we have known that blood serum has the characteristic that it agglutinates and dissolves the blood corpuscles of other animal types. Our procedure is based on this phenomenon. We would have to spell out here an outline of all of our knowledge about transfusion if we wanted to indicate the foundation of our work in all its aspects. It is self-evident that we must be satisfied here with this reference to the fundamental experiments of Landois.

† Modification in stroma fibrin (Landois)

We are obligated, on the other hand, to deal with a series of more recent works since they are very closely related to our theme. Indeed, Landsteiner[3] was the first to point out that under certain circumstances the blood corpuscles of one species are agglutinated by the serum of another individual of the same species. An attempt was then made to determine the causal connection between the appearance of these so-called isoagglutinins and the pathological conditions of the individuals from whom the agglutinating serum originated.[4] Further experiments by Landsteiner[5] himself, by Ascoli[6], by von Decastello and Sturli,[7] by Langer[8], and others, however, have shown that any normal human serum can possess the characteristic that it agglutinates the blood corpuscles of another human being. In any case this characteristic is not constant; it varies in each individual. Landsteiner and Richter[9] attempted to devise a method based on such individual blood differences that would enable them to assert with certainty that a given blood stain did or did not come from a specific person. Both authors, however, came to the conclusion that, when agglutination fails to take place, one 697
cannot exclude the possibility that the blood stain under examination could have come just as well from some other individual as from the one who provided the blood corpuscles for the test, precisely because the isoagglutinins are not present in every serum. On the other hand, there are human blood corpuscles which apparently are influenced by no other human serum (compare the tables of Landsteiner and Richter, l. c.). In any case the existence of isoagglutinins is of decisive importance for our process. We will have to come to terms with them in what follows.

First, we were in the fortunate situation for our purposes that the blood corpuscles of one of us (Marx) belonged to the insensitive group which were influenced by none of the many fresh and old human blood types which we tested,[10] while Ehrnrooth's blood corpuscles belonged to the group whose blood corpuscles were easily influenced, i.e., easily agglutinated. Our blood corpuscles thus represented two opposite types of erythrocytes with regard to isoagglutination. Besides our own blood corpuscles, we tested, in defibrinated blood, the relative susceptibility of the blood corpuscles from other persons by means of homologous and heterologous sera. Having established this in advance, we achieved the following results.

Agglutination by means of homologous[11] serum never appears so markedly as does that produced by a heterologous serum of the same age. In the case of isoagglutination the erythrocytes arrange themselves next to each other or in rouleaux forms (pseudo-agglutination; compare the article, cited above, by Decastello and Sturli); the individual blood corpuscles as such remain clearly recognizable; their pigment does not dissipate or disintegrate; there is no formation of "stroma fibrin' (Landois). Dried, homologous blood looses its isoagglutinins relatively quickly so that, after a few weeks (two to four), there are left only traces of recognizable isoagglutinating action. Dried blood from mammals shows the liveliest agglutination and hemocytolytic action to all

human blood corpuscles even years later (more than three years later according to our experience to date). If, accordingly, the isoagglutinins are only to be seriously considered in the case of relatively fresh blood (up to one month old), then in every case a reaction with a trace of animal blood, known to be of the same age, must be carried out by way of comparison in order to clear up for the practical observer whether he is observing the forces just mentioned. To aid us in cases which still remain doubtful, we use in every case the following reactions which we recommend be carried out in a specific order.

The experiments of Malkoff[12] have demonstrated that a serum of type A, which will agglutinate the blood corpuscles of type B, looses this characteristic when treated with serum B. On the other hand, we have noticed in repeated experiments that human serum C, which will agglutinate human blood corpuscles D, undergoes a strengthening of its agglutinating action against blood corpuscles D, when it is treated with any other non-agglutinating human serum or with serum D itself. Accordingly, we observe the following. In a two cc. test tube, we produce a 20-25% blood solution from our own blood, taken from the fingertip, in 0.6% saline solution; after 24 hours a layer of clear, diluted serum had formed at room temperature. We introduce a drop of our serum into the blood solution to be tested, a solution which is produced according to the technique presented in our first communication.[13] The drop of our serum is at least the same size as that of the solution to be tested; the sera are thoroughly mixed. If the agglutinating serum is heterologous, then the agglutination effect is weakened, or it is completely halted. If the agglutinating serum is homologous, then the agglutination effect is considerably strengthened. One can also carry out the experiment in the following simple fashion. One sets up our experiment according to the method indicated in *Communication I,* once with animal blood and once with human blood. If one now adds to each preparation just one drop of the particular serum, the erythrocyte clumps, formed by the heterologous blood, break up again into individual blood corpuscles, which then only gradually arrange themselves anew into loose associations. In a preparation, made with homologous blood, on the other hand, clear piles or rouleaux formations appear, or the piles and rouleaux formations grow stronger, if they were already present. We are fully aware that the introduction of this experiment in doubtful cases, where the blood traces are very fresh, means a complication of the process; we can, however, assert that we have put our method on a surer foundation by it.

The indications for using our process follow. In a case where there is a relatively large quantity of dried blood available, one will, of course, set up first the Wassermann-Uhlenhuth reaction, as one does in all other cases. From the material which is left over, one produces with a very small amount of physiological saline solution a highly concentrated blood solution of a somewhat reddish, brown-black hue; then he sets up our reaction in the manner described. In the case of blood traces which are over a month old, one can make a diagnosis without any further hesitation. If agglutination does not occur throughout the concentrated solution, then one is surely dealing with human blood (or ape blood?[14]). If very strong agglutination appears immediately and is followed by cytolysis and finally by the formation of stroma-fibrin, the blood definitely comes from an animal. If, in the case of a blood trace less than a month old, no agglutination follows, then we clearly have before us human or ape blood. If, a short time after the beginning of the reaction, agglutination appears without clear cytolysis, it could be human blood; in this case, our auxiliary reaction, mentioned above, will soon clarify the situation for us. It goes without saying that one sets up, in every case, comparative reactions with dried human and animal blood of known provenance and age, if possible of the same age as that of the blood trace under scrutiny. Last but not least, the *alpha* and *omega* of our reaction will always be the comparison with the result of the Wassermann-Uhlenhuth reaction. We believe, however, that under certain conditions the conclusion of our test can be a valuable support to the results of the biological process.

To close, a few technical observations. Our reaction takes place most clearly at room temperature. Its results must be evaluated fifteen minutes after the blood has been introduced. One tests the effect of fresh sera best in a dilution with physiological saline solution in the ratio of one part serum to two of NaCl. Serum, preserved over chloroform, soon looses its potency. Moreover, the action of sera, in a forensic context, is naturally not of the same significance which attaches to the action of old, dried blood. It can be observed, furthermore, that fresh, homologous sera can call forth an intensive rouleaux formation which has nothing to do with agglutination.

Footnote made during the correction: In cases of older and less concentrated blood solutions, the following modifications of technique are recommended. A drop of blood solution is placed on the microscope slide; a small drop of blood from the finger is placed on the cover-slide. The two slides are put together. The changes, then, make their appearance most clearly on the edges of the preparation. At the same time, such preparations make it very easy to recognize hemolysis caused by the heterologous sera by means of numerous blood-corpuscle shadows.

The blood of a different species of monkey (Meerkatze), which we were able to test in the meantime, behaved as a homologous blood. On the other hand, we did not see the polygonal form of erythrocytes which we noticed in our first communication.

Notes and References

1. I Mitteilung in No. 7, this journal, 1904.
2. Landois: *Die Transfusion des Blutes.* Leipzig 1875. *Beiträge zur Transfusion des Blutes.* Leipzig 1878. Article "Transfusion" in *Eulenburgs Realenzyklopädie* 1890.
3. *Zentralbl. f. Bakt.* 1900 **XXVII.** page 357.
4. Compare the works of Lo Monaco and Panichi, *Rif. Med.*, 1902; referred to in this journal, 1902, No. 25.
5. This journal 1903, and *Wiener Klin. Wochenschr.* 1901

6. This journal. 1901. 31
7. This journal. 1902. 26
8. *Zeitschr. f. Heilk.* **XXIV.** 1903
9. *Zeitschr. f. Med.-Beamte* 1903, No. 3
10. Compare the blood of Hübler, Mealy, Mechauk, Eiff. in the Tables of Landsteiner and Richter in *Zeitschr. für Med.-Beamte,* l.c.
11. Since we allow the different sera to work only on corpuscles of human blood, then homologous serum is always to be understood to mean human blood serum.
12. *Deutsche Med. Wochenschr.* 1900. No. 14
13. One must extract older blood spots by processing them for about two to three hours in order to obtain very concentrated solutions. In the case of fiber materials, we recommend that they be moistened in some saline solution and then be pressed vigorously between the pincers of a tweezers.
14. We hope soon to be able to continue our experiments concerning ape blood.

A New Contribution to the Specific Identification of Egg Protein Using the Biological Method*

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/734 Our experiments concerning immunity have placed before our eyes in a striking fashion how the serum of animals which are pretreated with increasing doses with various poisonous substrates, whether of an organic or inorganic nature, is able to react in a very specific way. Thus the animal body reacts to the injection of toxins of diphtheria, tetanus, or of snake or eel poison, etc., by forming antitoxins which neutralize the poison. When the animal body is inoculated with cholera, typhus, or plague bacteria, it answers by producing substances which agglutinate these bacteria in the test-tube, and which break up the bacteria in the stomach cavity of the guinea pig.

These facts, established in the field of immunity against bacteria and their metabolites, have their analogues in a similar area, as the latest research has demonstrated. Thus Bordet could detect in the serum of animals pretreated with repeated injections of blood corpuscles, agglutinating and hemolytic qualities developed against these blood corpuscles.

Ehrlich and Morgenroth then provided an explanation for these specific, hemolytic qualities of sera when, by following exactly the explanation of R. Pfeiffer for the serum which destroys typhus and cholera bacteria, they attributed the effect to two substances which form in the body of the immune animal, the so-called immune body and the activating enzymes, the so-called addiment. Through the agency of the immune body, the addiment is bound to the substance of the red-blood corpuscles, through which process their dissolution is achieved.

The immune body is very stable and can bear a one-hour heating to 60°, whereas the addiment, which is also present in normal serum, is extremely unstable. Similar specific substances as these also appear in the serum of animals pretreated with injections of other animal cells.

Von Dungern was able to produce an antibody, by repeated injections with ciliated epithelium, which destroyed these cells in the stomach cavity of the guinea pig. Metschnikoff experimented with rat spleen and lymph glands from rabbits and produced, with repeated injections of these substances, a substance which agglutinated and destroyed leukocytes. In an analogous manner were found immune sera against spermatozoa (Metschnikoff, Moxter, Landsteiner), liver epithelia, etc. (Lindemann). The next step was to examine the products of animal cells with reference to their capacity to produce antibodies. Such experiments were then carried out with rennin (Briot) and trypsin (v. Dungern). Further, Bordet confirmed that substances formed in the serum of animals pretreated with repeated injections of cow's milk, substances which precipitated protein bodies when added to milk. According to Wassermann's experiments these substances of the lactosera are specific, in that the serum of animals pretreated with cow's milk precipitated only the protein bodies of cow's milk, and that of animals pretreated with goat or human milk similarly reacted only to the protein substances of these types of milk.

I was interested now in determining whether specific antibodies developed in the serum of animals pretreated with egg protein and whether the protein substances of various birds' eggs could be distinguished from one another in this fashion. For my experiment I chose first hen's egg protein.[1] I let this protein flow out of a cleaned and carefully cracked egg into a sterile beaker of sterile physiological saline solution. By beating this solution with a sterile glass rod I made it thin enough that it was suitable for injection. In this way I injected each time the whites of two to three hens, eggs into the stomach cavity of a rabbit at intervals of several days. Despite the rather high quantity of liquid, which at times reached 100 cc, the animals withstood the injections very well and appeared to be in good health with this animal nutriment. When one has administered a certain amount of albumin—the albumin from five or six eggs is enough—a few drops of the serum from these animals demonstrates definite turbidity when added to a solution of 5–10% hen egg albumin made up with physiological NaCl solution. This occurs at the bottom of the test tube because the serum, which has a greater specific gravity, sinks downward and then spreads gradually throughout the rest of the liquid. If one observes these tubes further, one can observe how the turbidity settles and a flocculated sediment forms.

This reaction becomes all the more striking the more egg albumin the animal receives intraperitoneally.

One can thus confirm that no chemical reaction can com-

*Translation of: "Neuer Beitrag zum spezifischen Nachweis von Eiereiweiss auf biologischem Wege."

in *Deutsche Medizinische Wochenschrift* **26** (46): 734–735 (1900).

pete with the exactness of these biological reactions.

In a comparable way I tested the most common protein reagents regarding their effectiveness vis-a-vis the biological reaction. I was able to obtain a clear reaction with a few drops of my serum even in a protein solution diluted to 1:100,000, while the chemical agents, concentrated potassium nitrate, acetic acid, potassium ferrocyanide, a mixture of magnesium sulfate and potassium nitrate, are no longer capable of calling forth a reaction in a dilution of over 1:1000. I am confident that the titer of my serum can be raised even higher.

Obviously, a great number of control experiments were also set up. Normal rabbit serum of a great number of rabbits never produced this reaction. At the beginning of each treatment of the rabbits their serum was also checked. It never showed the reaction described

At the next step this serum was added to a great many different solutions of protein preparations. I selected nutrose, somatose, Deyche's alkalialbuminate, Heyden nutrients, peptone, Riedel, casein, and horse, cattle, mutton, and donkey serum. Not once was the reaction positive. Moreover, no reaction took place with a serum albumin preparations obtained from various sources.

Now, to test the reaction with egg albumin from other birds' eggs, I set up the same experiment using pigeon egg albumin. Here, too, the results were clearly positive, although they were decidedly weaker than in the hen's egg albumin solution. From this it follows that this reaction is not specific for hen egg albumin.

Moreover, the serum of a rabbit repeatedly injected intraperitoneally with pigeon egg albumin produces, when added to a solution of hen egg albumin, a definite turbidity, which is, however, not as strong as in the solution of pigeon egg albumin. From this observation it seems safe to conclude that the same albumin substances are contained in hens' and pigeons' eggs.

Unfortunately, it has not yet been possible for me to expand my experiments to other birds' eggs, since in this season those are impossible to obtain. I remain determined to continue as soon as possible these very interesting experiments with other eggs, and I will report on them later. Likewise, I will busy myself further with the very difficult chemical aspect of these reactions in order to clarify the
735 process of this reaction. The important question arises here as to whether the precipitation takes place in the protein solution or in the serum added. So far I am able to report only that the serum still causes as clear a reaction as before after being heated to 60°C for an hour.

It was of further interest to discover whether, after repeated intrastomach doses of hen egg protein, these bodies formed in the serum of such rabbits. In order to determine this, I administered with a probang for several weeks a daily dose of hen egg protein, beaten, and diluted with a physiological saline solution. The serum of these animals was tested every eight days; the reaction remained at first negative. After 24 days a positive reaction took place. In order to multiply the antibodies as fast as possible in the serum of the animal. I gave it a hen's egg white both morning and evening for twenty-four days. It turned out that the reaction produced by the serum was essentially not more definite even though the animal had up to that day received forty-three egg whites *per os* (orally).

The observation seems to me to be of special significance because it proves that, despite the effects of stomach acids in the case of intrastomach application over a long period of time, specific antibodies can form in the animal's body, a fact that is also to be considered in immunization experiments *per os*.

As we see, the results, briefly sketched here, encouraged us to approach the question of the biological differentiation of protein substances; all the more so since we have not come very far with the purely chemical approach to this problem in the last few years. The biological method is so much more full of promise because the reaction far exceeds all the chemical methods in exactness.

Following this line of thought, I set up, among other things, an important problem for myself: to prove whether it would be possible to distinguish by means of the biological method a great variety of blood types. One observation, which I had made with the serum of a rabbit pretreated with hen's blood, especially inspired me to go on. Such serum produces a definite, rapidly developing turbidity when added to a laked hen's blood solution, which was extremely diluted (a weak red color). The turbidity gradually settled as a flocculated sediment. This same serum produces no turbidity in similarly prepared solutions of horse, donkey, cattle, mutton, and pigeon blood. I wish also to mention that this serum has not as yet called forth any turbidity in a solution of hen's egg albumin. Rabbit serum also does not produce turbidity in any of these blood solutions.

If we summarize briefly the chief results of our experiments, we obtain the following:

1. When rabbits are repeatedly injected intraperitoneally, as well as in the stomach, with a solution of hen's egg albumin, substances form in the serum of these animals which produce a turbidity, i.e. a precipitation, when added to a solution of hen's egg albumin. This reaction occurred also in a solution of pigeon's egg albumin.

2. The serum of a rabbit pretreated intraperitoneally with pigeon's egg albumin contains substances which produce turbidity, i.e. precipitation, both in a solution of hen's egg albumin and in one of pigeon's egg albumin.

3. The reaction, caused by the serum of rabbits pretreated in this way, occurs only in egg albumin, not in the many other sorts of protein which I tested.

4. This biological method of protein identification surpasses in accuracy the chemical reactions and is suited, most likely to a high degree, for differentiating the different varieties of protein substances.

5. The serum can stand a one-hour heating to 60° without losing its reactive capacity.

To close, allow me to express my most humble thanks to Professor Dr. Loeffler for the kind interest he took in my experiments.

References

1. After I determined the facts mentioned here a preliminary communication of Myers concerning immunity against proteins appeared in the *Centralblatt für Bacteriologie* (vol. 28, no. 819). He experimented with crystalline egg albumin, serum globulin, and Witte's peptone, and he came to similar results as mine.

A Method for the Differentiation of Various Specific Blood Types, in Particular for the Differential Diagnosis of Human Blood*

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82 In my study entitled "A New Contribution to the Specific Identification of Egg Protein Using the Biological Method" (this Journal, 1900, No. 46), I reported an observation in the serum of a rabbit which had been intraperitoneally pretreated whith chicken blood. When a chicken blood solution, laked by adding water, was mixed with the serum of this animal, definite clouding developed rapidly. The clouding gradually formed a flaky precipitate at the bottom of the container. On the other hand, horse, cattle, ram and pigeon blood serum solutions showed no clouding when treated in the identical manner. The above finding induced me to undertake further investigations, so as to determine whether it would be possible to discriminate between the blood of various animal species with the aid of this biological method. I considered these studies important, the more so since the problem could not be solved so far with any procedure. My principal aim was to answer the forensically significant question: how to discriminate between human blood and other specific blood types.

Before dealing with this interesting problem, however, I conducted a few orientation experiments with cattle blood.

At intervals of 6 to 8 days, I injected 10 cc defibrinated cattle blood into the abdominal cavity of rabbits.

After five of these injections the animals already yielded an active serum, as shown by the experiment described below.

I first prepared solutions of the various specific blood types
83 with ordinary tap water; I added enough water to these solutions to obtain a pale red color (dilution 1:100). I eliminated interfering stroma residue either by letting them deposit in the test tube, or by means of filtration. I removed approximately 2 cc from the resulting clear solution and placed it into a small test tube with a diameter of 6 mm, mixing an identical volume of a double concentrated physiological salt solution (1.6%) with it. It is extremely important to put the blood solution in a physiological salt solution for these experiments, since normal rabbit serum will cloud when water is added, and could interfere with the determination of a specific clouding. No clouding occurs in the normal rabbit serum when physiological salt solution is used.

The absolutely clear, reddish blood solutions, prepared as indicated, originated from the following animals: cattle, horse, donkey, hog, ram, dog, cat, stag, fallow deer, hare, guinea pig, rat, mouse, rabbit, chicken, goose, turkey, pigeon. Human blood was included in the experiment as well.

When I then added to each of my small test tubes 6 to 8 drops of the rabbit serum, pretreated with cattle blood, using a capillary tube with elongated point, clouding developed quite soon in the cattle blood solution only; it was especially conspicuous in penetrating sunlight. The rest of the test tubes showed completely clear coments. Prolonged observation subsequently revealed that the clouding intensifies and finally drops to the bottom as a definite, flaky precipitate. Normal rabbit serum causes no clouding in cattle blood solutions.

Privy Councillor Loeffler asked me to select the test tube containing the cattle blood among the above-mentioned 19 test tubes containing blood, which were unmarked and arbitrarily aligned.

After adding a few drops of my serum, I was immediately able to determine which test tube contained cattle blood.

Encouraged by the specificity of the above reaction, I used the identical method when injecting human blood intraperitoneally into rabbits. When added to each of the aforementioned series of 19 blood solutions, the serum of these animals developed clouding and precipitated only in the human blood solution. All other solutions remained absolutely clear. I wish to stress once more that normal rabbit serum causes no clouding in human blood solutions. Accordingly, I was able with this reaction, to differentiate reliably between human blood and the rest of the specific blood types listed.

It seems reasonable to assume that the specificity of said reaction applies, appropriate changes having been made, to other specific blood types as well. I am at this time engaged in studies concerning this problem; I wish to determine in particular whether the specificity exists also in closely related animal species such as the horse and donkey, for example, or whether the relationship between thse animals becomes evident in the reaction as well. It should be investigated in this

*Translation of: "Eine Methode zur Unterscheidung der verschiedenen Blutarten, im besonderen zum differential-diagnostischen Nachweise des Menschenblutes."

in *Deutsche Medizinische Wochenschrift* 27 (6): 82–83 (1901).

context, for example, whether the serum of rabbits pretreated with human blood causes clouding in monkey blood, which I was regrettably unable to obtain so far.

The reaction is extremely sensitive and, therefore, traces of blood are sufficient to determine from which species the blood originates. Accordingly, the verification of each specific blood type requires pretreatment of the animals with the various blood samples, so as to obtain a serum usable for diagnostic purposes in suitable cases. The pretreatment of the animals should be continued until the serum shows rapid clouding and produces a precipitate.

It is of particular interest that I was also able to determine with the aid of my serum the human blood among samples of human, horse and cattle blood; these blood samples had dried for four weeks on a plank and were then dissolved in a physiological NaCl solution. This is certainly a fact of special significance.

As for the nature of the reaction in question: the process presumably involves the formation of "coagulinen" in the animal organism, as defined by Ehrlich, similar to those resulting from the injection of various milk caseins, as performed by Bordet and Wassermann, and as observed by me with egg albumin; Myers observed the same phenomenon simultaneously and independently from my own findings.

In conclusion, I wish to thank Privy Councillor Prof. Dr. Loeffler for his interest in my investigations.

Additional Reports on my Method for the Identification of Human Blood*

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260 My investigations on the differentiation of human blood from other specific blood types with the aid of a certain serum[1] were fully confirmed by the detailed work of Wassermann and Schütze[2] published shortly after my own report, as well as by the reports of Stern[3] and Mertens.[4] My method is based on the fact that the blood serum of rabbits, and of some other animal species, which were pretreated intraperitoneally with defibrinated human blood or with the defibrinated blood of certain other animal species, produces a precipitate in the laked solution of the blood in question.

I determined the above fact in the course of my work on the biological differentiation of various egg albumins,[5] while investigating whether the albumins of chicken eggs and those of chicken blood are identical. For this purpose, I had injected larger quantities of defibrinated chicken blood into a rabbit. I found that the serum of the pretreated animal, as indicated by the standard serum titer at that time, formed a precipitate in a chicken egg albumin solution, while no such precipitate resulted in a laked chicken blood solution.

I wish to refer in particular to the course of my investigations here because as I subsequently determined Bordet and Tschistovitsch[6] described, with reference to another matter, a similar precipitating effect in the serum of a rabbit pretreated with blood; the serum reacted with the corresponding blood despite the fact that this blood was not laked.

I continued to elaborate the aforementioned experiments, related to my studies on egg albumin; the experiment is fundamental for my subsequent investigations aimed at the discrimination between various specific blood types, as will be shown below. It became evident that the serum of a rabbit pretreated with a chicken egg albumin solution can be induced to show definite clouding and to form a precipitate subsequently, in a considerable diluted laked chicken blood solution. A less intensive clouding occurs in a goose blood solution. The serum was so effective that it produced a precipitate within seconds when a 2.5% chicken egg albumin solution was added. The effect was almost as powerful when the serum was mixed with goose, duck or guinea fowl egg albumin solutions; the reaction was weaker with pigeon egg albumin. Moreover, a rabbit pretreated with goose egg albumin solution yielded a serum which caused significant clouding in a laked goose blood solution, while the clouding was less pronounced in a chicken blood solution. When the same serum was added to goose or duck egg albumin solutions, a substantial flaky precipitate formed within seconds, while only clouding was observable in guinea fowl, chicken and pigeon egg albumin solutions. I will not elaborate on these interesting studies here; instead, I intend to report on them later in connection with my planned studies which should include as many various bird eggs as possible.

The investigations performed so far nevertheless indicate that chicken, goose, duck, guinea fowl and pigeon eggs contain albumins, some of which are found in the blood of the above-mentioned birds as well. However, the albumins of the various bird eggs cannot be as reliably differentiated with the reaction as the albumins in blood.

The fact that the serum of rabbits pretreated with human blood has a precipitating effect on laked blood solutions indicates the forensic usefulness of the phenomenon. However, the following finding was decisive for application in practice: even old blood, desiccated for a prolonged period, retains its reactivity, since material of this type is presumably under examination in most cases by forensic experts. My blood samples, dried for three months, still react efficiently. I therefore feel entitled to assume that this blood could tolerate even much longer periods of desiccation. But the human blood to be examined by the expert is not always 261
desiccated; for example, blood which putrefied some time ago could be involved.

Accordingly, the important question must be asked as to whether such material is still suitable for the reaction. To solve the problem, I let the various blood samples decompose at room temperature in the laboratory; some of the blood samples were obtained from cadavers in an advanced stage of decomposition and from anatomical preparations.

The following putrefied blood samples were used for the experiment performed on March 19:

1. Blood from a cadaver dissected on January 22 of the current year (phthisis pulmonum).
2. Blood from a cadaver dissected on January 22 (uremia).
3. Blood from an infant stillborn on January 20.

* Translation of "Weitere Mittheilungen über meine Methode zum Nachweise von Menschenblut"

in *Deutsche Medizinische Wochenschrift* 27 (17): 260–261 (1901).

4. Blood from the anatomy mortuary; the blood was already significantly putrefied when the sample was collected on March 1.
5. Blood from a subject whose death was caused by military tuberculosis.
6. Blood samples from healthy persons, which were subjected to putrefaction a) since February 20, b) since March 4, and c) since March 10.

The following served as controls: blood samples from the ram, hog, horse, donkey, cattle, cat, dog, goose, chicken, hare, rabbit and stag, which underwent putrefaction for the same length of time.

All these samples were discolored, reddish to blackish-brown, with a penetrating odor, partially indicating the presence of H_2S. When a glass rod immersed into HCl was held over the samples, ample volumes of ammonium chloride vapors were released by the putrefied fluid. The reaction was weakly alkaline.

These putrefied blood fluids were then diluted considerably with a physiological salt solution, according to the method described by me. Filtration through a sterilized Berkefeld's filter, which retains all bacteria and other corpuscles, followed. A filtration of this type can be rapidly performed with the aid of a water jet suction device fitted to any water system. The filtration is definitely necessary because an absolutely clear blood solution for the reaction is obtainable only with this method. The filtration also sterilizes the blood solution, which can then be stored for a prolonged period of time. Subsequently, approximately 4 cc of the resulting, partially yellowish-brown and partially reddish fluid is mixed with 12 drops of my serum. All test tubes containing human blood[7] showed clouding; the fluid in all other test tubes remained clear.

The above experiment shows that the reactivity of human blood was not eliminated by up to three months of intensive, odorous putrefaction. This fact is presumably of general biologic interest. Further tests should be carried out to determine whether a still longer period of putrefaction alters the blood, thus preventing a specific reaction. Such an effect, however, seems highly unlikely because putrefaction, like fermentation, stops after a certain period of time, before all substances subject to putrefaction or fermentation, respectively, are completely converted.

I included still other questions, important in practice, in my studies. Since the blood to be evaluated can be suspended in a wide variety of liquids, the forensically significant question arises as to whether blood in such liquids is eventually still reliably determinable. Among various blood wash waters prepared with weakly alkaline soap, the water containing human blood could be readily verified. Human blood in menstrual urine could likewise be successfully determined; all other urine samples mixed with cattle, hog, ram, chicken, horse and cat blood failed to react.

Moreover, I was able to diagnose traces of human blood immediately among various blood traces frozen in snow at −10°C for 14 days.

As expected, the reaction was likewise definitely positive in human blood solutions in which hemoglobin had been converted into carbon monoxide-hemoglobin.

Stern[3] and Mertens[4] recently pointed out that rabbit serum, formed after the injection of human blood, also causes a precipitate in human urine containing albumin. I can fully confirm their finding: several urine samples with high albumin content showed a characteristic reaction. The reaction was especially intensive in a urine sample containing fetid pus, originating from a cystitis and pyelonephritis case. The serums from rabbits pretreated with chicken and goose egg albumin caused no clouding in such urine samples containing albumin.

As for the serum used for the reaction: it will tolerate heating to 60° for 1 hour without loss of its precipitating property. The specifically coagulating substances seem also quite resistant to preservatives, such as carbolic acid (Carbol), for example. Admittedly, my findings in this respect are not yet conclusive. It is certain as of now, however, that serum mixed with 0.5% Carbol remained reactive for three months. But, whenever feasible, I always prefer to use quite fresh serum for the reaction. It is suggested that five to six large and vigorous rabbits be subjected to pretreatment; the serum volume needed for the examination can then be obtained at any time by taking blood samples from the ear vein, without killing the animals as a result of exsanguination. When the blood has clotted, the serum is removed and centrifugation is performed, so as to obtain a clear serum. I collect the blood needed for the pretreatment with a sterilized Heurteloup cupping device, like that used for therapeutic blood elimination in ophthalmology. With this method, 10 to 20 cc fluid blood is readily obtainable; defibrination and injection into the rabbits can follow immediately. Accordingly, blood is obtainable without difficulties; the required volumes are readily available at any time from healthy persons as well. As for the chemical nature of the reaction, I am engaged in the study of the same at this time. I wish to state now merely that the precipitate originating from the serum is soluble in excess NH_3 as well as in H_3PO_4.

In conclusion, I wish to thank Privy Councillor Professor Dr. Loeffler for his interest in my investigations.

Notes

1. *Dtsch. Med. Wochenschr.*, 1901, No. 6
2. *Berl. Klin. Wochenschr.*, 1901, No. 7
3. *Dtsch. Med. Wochenschr.*, 1901, No. 9
4. *Dtsch. Med. Wochenschr.*, 1901, No. 11
5. *Dtsch. Med. Wochenschr.*, 1900, No. 46
6. *Ann. Inst. Pasteur*, Paris, 1899
7. I used small test tubes with a diameter of approx. 8 mm. I added the serum drop by drop, from a capillary tube, the point of which had been elongated over a flame. When the reaction is to be accelerated, the test tube is placed near a hot oven or into the incubator at 37°.

Additional Reports on the Practical Application of My Forensic Method for the Identification of Human and Animal Blood*

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499 I stated in earlier publications that the identification of human blood—under a wide variety of practically important conditions, such as blood dried for a prolonged period, putrefied for several months, or frozen—will definitely succeed with the method initially indicated by me.[1] The observations made somewhat later by Wassermann and Schütze[2] have by now been fully confirmed by Stern,[3] Mertens,[4] Dieudonné,[5] and quite recently by medical examiners, including the reports of Ogier[6] from the Toxicology Laboratory in Paris, and on the quite extensive material of the State Medical Institute at Berlin through the investigations carried out by Ziemke.[7]

All these studies prove the forensic usefulness of my method brilliantly. Its value will become most evident when investigating as large a number as possible bloodstained *corpora delicti,* such as those submitted in forensic practice to judges and experts. I had several recent opportunities to examine such objects, kindly made available by the State Prosecutors, in particular by First State Prosecutor Mr. Hübschmann at Greifswald, as well as by the Director of the Local Institute for Legal Medicine, Dr. Beumer. Sentence had already been passed on some of the cases at issue here; the origin of the blood adhering to the submitted *corpora delicta* was not in doubt; however, at my request the information was initially withheld by the aforementioned gentlemen, so as to control the accuracy of my diagnosis. Some of the cases were new, and the specific blood type was subject to doubt, either from the start, or during the legal proceedings. Since the last-mentioned cases are not yet *res judicatae,* I am unable to report on them at this time, but will do so later.

As for the old (*res judicatae*) cases: I will briefly summarize the results of my investigations.

1. 1 meter long, ridged club, with a few faded brownish, stains, from the year 1900.

Some of the suspicious material was scraped off and diluted in a physiological salt solution. The resulting liquid shows no definite color; it is clear and foams slightly when shaken. Five drops of serum from a rabbit pretreated with human blood (Serum E) are added to 4 cc of the above liquid. Clouding results almost immediately; it soon deposits in the form of a precipitate.

Diagnosis: human blood.

Subsequent information: a case of serious bodily injury; blow on the head. Bleeding lesion.

2. Reddish sand, from the year 1896.

The sand is placed into a physiological salt solution. A pale, yellowish, clear liquid results. Serum is added as in Case 1. Precipitation occurs almost immediately.

Diagnosis: human blood.

Subsequent information: track of blood, originating from a murder committed in the vicinity of Greifswald.

3. Cotton cloth with a few reddish stains, from the year 1897.

The suspicious stains were rinsed out with a physiological salt solution. Admixture to the yellowish liquid as above. Almost immediate clouding, which soon drops to the bottom as a precipitate.

Diagnosis: human blood.

Subsequent information: the cloth was found near a strangled person.

4. Trousers with reddish, faded, small stains on the trouser fly and on the lining in the area of the genitals.

Procedure as above.

Diagnosis: human blood.

Subsequent information: suspected rape; in fact, intercourse with a menstruating person.

5. Hatchet with a few blood traces on the handle. From the year 1900.

Procedure as above.

Diagnosis: human blood.

Subsequent information: case of serious bodily injury.

Accordingly, the accuracy of my diagnosis was confirmed in all cases. The procedure used by me seems indeed to be the simplest and fastest way to demonstrate my method's forensic usefulness. Privy Councillor Dr. Loeffler therefore kindly proposed to his Excellency, the Minister of Justice, that

*Translation of: "Weitere Mittheilungen über die praktische Anwendung meiner forensischen Methode zum Nachweis von Menschen- und Thierblut".

in *Deutsche Medizinische Wochenschrift* **27** (30): 499–501 (1901).

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bloodstained objects submitted to the Courts as evidence be forwarded to the Hygienics Institute at Greifswald, where the *corpora delicti* in question could be examined by me without additional information, so as to compare my diagnosis with the pertinent documentation.

The Minister of Justice then ruled that all *corpora delicti* of the above type, from the sphere of jurisdiction of the Breslau State and Supreme Courts be handed over to me; accordingly, I expect that a larger amount of material will soon be put at my disposal.

Thanks to the good offices of Prof. Beumer, I also had the opportunity to investigate the following bloodstained objects and blood samples, respectively, without receiving preliminary data on their origin:

1. Blood-soaked linen cloth. Procedure as above. Serum E added. Negative reaction.

Admixture of serum from a rabbit pretreated with ram blood in the same test tube: negative reaction.

Admixture of serum from a rabbit pretreated with horse blood in the same test tube: negative reaction.

Admixture of serum from a rabbit pretreated with hog blood; strongly positive reaction.

Diagnosis: hog blood.

When I notified Prof. Beumer of the diagnosis, he stated that the cloth had been soaked with hog blood several years ago, for use in a demonstration.

2. Dried blood from the year 1897. Procedure as in Case 1.

Diagnosis: hog blood. Confirmed by Prof. Beumer.

3. Dried blood from the year 1900.

Diagnosis: human blood. Confirmed by Prof. Beumer.

4. Dried mixture of blood from various mammals, from the year 1889.

Diagnosis: hog and ram blood. According to information received from Prof. Beumer: hog and ram blood.

Moreover, I wish to state that I was able to identify hog blood on a bloodstained and singed music sheet found in a large puddle of blood on the Gützkower highway; this excluded any suspected crime from the start. I was also able to determine hog blood in an extract from hog organs dried for 1-½ years. I likewise identified human blood – a fact which could be added to my earlier statements – in rinsing water containing considerable volumes of carbolic acid (Carbol), sublimate and soap; the color of the water was a murky, brownish-red. The method proved successful in a 3% dissolved mixture of borate and human blood, as well as in blood-soaked garden soil, after desiccation for three months.

As these reports show, I was able in each case to diagnose human blood as well as hog and ram blood accurately.

500 At the start of my investigations it already seemed to me of considerable forensic interest to answer the question: from which animal species a blood sample originates in cases when no human blood is at issue. To determine whether or not the blood originates from man in any given case will be of decisive significance; it is nevertheless obvious that, whenever the human blood reaction is negative, the reliable identification of the animal species from which the blood stems could, under certain circumstances, provide important cues for the further progress of the criminal investigation. I refer in this context to poaching, for example. The diagnosis is also occasionally important when the statements of a defendant on the origin of blood stains found associated with him/her are to be investigated from the viewpoint of truthfulness. Not infrequently, murderers pour animal blood over traces of human blood, so as to conceal the same. In some of these cases, besides identification of the human blood, the determination of the animal blood species could be significant.

I am therefore making every effort to prepare such specific sera, useful for solving the above problem. These studies are extensive. So far, I can report the following results:

1. The serum of a rabbit, pretreated with hog blood, yields a precipitate in hog blood solution only; the precipitation is somewhat weaker in a wild boar blood solution, while all other specific blood types used as controls remain clear. Blood solutions from the following animals served as controls for all additional experiments:

Cattle, horse, donkey, ram, goat, hog, chicken, bat, pigeon, duck, goose, owl, crow, sparrow, rabbit, guinea pig, rat, mouse, hedgehog, dog, fox, cat, stag—Man.

2. The serum of a rabbit pretreated with horse blood yields a precipitate in a horse blood solution, and a slightly weaker precipitate forms in donkey blood solution. The other blood species remain clear. The serum of a rabbit pretreated with donkey blood shows reversed behavior.

3. The serum of a fox blood-rabbit yields a precipitate in the fox blood solution, and a weaker precipitate forms in dog blood; all other solutions remain clear. (Blood solutions from the wolf and jackal were not available; their behavior is presumably similar to the reaction in the dog blood solution).

4. The serum of a hedgehog-rabbit forms a precipitate in the hedgehog blood solution only. The controls are clear. (Animal species closely related to the hedgehog could not be investigated so far).

5. The serum of a cat blood-rabbit yields a precipitate in cat blood solution only. The controls remain clear. (Blood from other predators related to the cat was not available).

6. The serum of a ram blood-rabbit forms a precipitate in a ram blood solution; its precipitate is near-identical in the goat blood solution, and weaker in the cattle blood solution.

7. The serum of a cattle blood-rabbit forms a strong precipitate in the cattle blood solution; the precipitate is weaker in goat and ram blood.

The aforementioned facts reveal that it is possible to demonstrate the relationship between various animal species *ad oculos* in the test tube, a fact that was determined earlier concerning Man and monkey as well. This biologically significant finding should be taken into consideration in the forensic diagnosis of a specific animal blood type. However, definite results are obtainable with my reaction on the various aspects of inter-species relationships only when the serum is of the highest possible quality. For example: while

determining the relationship between the ram, goat, and cattle, the serum from a rabbit pretreated with ram blood immediately forms a strong precipitate in the ram blood solution; the precipitate is slightly less strong in goat blood and still weaker in the cattle blood solution. It becomes clearly evident, that cattle are less closely related to sheep than to the goat. When the strength of the serum is not high, no clouding whatsoever is obtainable in the cattle blood solution.

Excellent, high quality serum is the precondition required for any forensic application of my method. When the effectiveness of the serum is reduced, fateful errors could occur in the course of blood evaluation. I therefore require a serum for forensic use which, when added to a pale yellowish blood solution to the ratio of 1:40, will almost immediately, or at least within 1 minute, cause definite clouding. The clouding should not be delayed for one, much less for several, hours. Using such high-quality serum, I was able to produce a precipitate almost immediately even in very old blood, desiccated for twelve years. In my lecture delivered before the Scientific Association at Greifswald on June 5th of the current year, I demonstrated my reaction in human blood which had been dry for six years. While I was still adding serum to the 12 control test tubes, definite clouding was already evident in the first test tube containing human blood. The clouding was so obvious that it was visible from the highest seat rows of the large auditorium. The result of the reaction, of course, also depends on the concentration of the blood solution.

When only small blood samples are available, the fluid frequently shows hardly any color at all. In such cases the formation of foam during the shaking of a small test tube indicates that sufficient blood albumin has been dissolved.

It is occasionally difficult to obtain such high quality serum. The results depend not merely on the volume of blood used for pretreatment; I found that the condition of each rabbit is very important as well. Some rabbits yield an excellent serum after a few injections; others yield a completely useless serum after a much longer treatment period. I even found that despite continued treatment, the serum failed to improve; instead, it showed pronounced deterioration.

Ziemke's report indicates how much depends on the quality of the serum. He investigated the same blood solution with two different serum types. The reaction was positive in one case, and negative in the other. As a matter of course, such failures must be entirely excluded when using my method for forensic purposes. In my opinion, it is therefore imperative to assign the manufacture and control of the serum to an institute. In that case, experts would be able to obtain a tested, high quality serum at any time. It is certainly undesirable to let each medical examiner himself prepare the serum to be used for the diagnosis of human blood needed for a given case. The preparation requires prolonged practice and experience.

Larger volumes of the serum should be stored at a central location; it is necessary, therefore, to manufacture it in larger quantities. The use of larger animals than rabbits for obtaining serum would be desirable. My tests in this respect with a small lab failed completely. The animal yielded no trace of any precipitate, despite the fact that it had received, within five weeks, injections of approximately two liters of human blood and exudate fluid. Therefore, rabbits will have to be used for the time being; when they are large and vigorous, the serum yield will be approximately 50 cc.

I proceed as follows to obtain larger serum volumes: the serum of the rabbit is tested serveral times in the course of the treatment by taking blood samples of approximately 8 cc from the ear vein. When the serum proves to be usable, i.e. when it yields an almost immediate precipitate in the tested blood solution, the rib cage of the rabbit is opened in deep chloroform narcosis and a heart section is performed. The blood flowing into the sterile chest cavity is collected with a sterile pipette, collected into cylindric test tubes several cm wide and left to coagulate, while the test tubes remain in an oblique position. After separation of the serum, centrifugation is performed to obtain a completely clear serum. Since this does not always succeed with small, manually operated centrifuges I pass the serum through a Berkfeld filter. No obstacles whatsoever were encountered with this procedure. The serum obtained was absolutely clear and entirely sterile as well. For preservation I used either 0.5% carbolic acid (Carbol) or, more recently, chloroform as well. This mixture proved to be highly efficient so far.

With the above procedure, a larger quantity of serum can always be kept ready for mailing.

Other researchers determined that the serum from rabbits pretreated with human blood forms a precipitate in urine containing albumin. I used a similar method, therefore, to 501
investigate other human albumins. I found that the serum from a rabbit pretreated with human blood also causes clouding in human semen and in the purulent sputum (of tuberculosis patients). These are facts which deserve to be taken into consideration in the practice of medical examiners. Therefore my reaction is specific for human albumin.

Notes

1. *Disch. Med. Wochenschr.*, 1900, No. 46; Greifswalder medizinischer Verein am 1 December 1900, Referat *Muench. Med. Wochenschr.*, 1901, No. 8; *Disch. Med. Wochenschr.*, 1901, No. 6 (February 7); *Disch. Med. Wochenschr.*, 1901, No. 17; *Arch. Kriminalanthropol. Kriminalistik*, 1901, May; Verhandl. Naturwissenschftl. Vereins at Greifswald, Sitzung on June 5, 1901
2. *Berl. Klin. Wochenschr.*, 1901, No. 7 (February 18)
3. *Disch. Med. Wochenschr.*, 1901, No. 9
4. *Disch. Med. Wochenschr.*, 1901, No. 11
5. *Muench. Med. Wochenschr.*, 1901, No. 14
6. Societé de Médecine Légale, Referat *Disch. Med. Wochenschr.*, 1901, No. 26
7. *Disch. Med. Wochenschr.*, 1901, No. 26

Concerning My New Forensic Method to Identify Human Blood*

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317 Judges and experts have for a long time been most deeply concerned with the all important problem of distinguishing human blood from other blood types. Until now, though, a sure answer to this question has been impossible. One was able to diagnose fairly accurately human blood in the case of relatively fresh blood with the aid of blood-corpuscle measurement. In the case of dried blood, on the other hand, where the formed elements have been destroyed, even with the blood crystal test, the diagnosis was so unreliable that one could say it was impossible. Since in forensic practice one is almost exclusively concerned with such dried blood, one must be equipped with a practical, forensic method to determine also the origins of blood in this condition.

I was recently successful in discovering such a reliable method, which I will briefly describe in the following article. Concerning the details I refer the reader to my thorough studies which appeared in the *Deutsche Medizinsche Wochenschrift*.[1]

Busying myself with the biological differentiation of protein bodies of different birds' eggs, I established that the blood serum of rabbits, which were injected in the hollow of the stomach continuously with the whites of hens' eggs for a rather long time, produced a precipitation when added to a dilute solution of hen's egg protein. The same serum failed to produce any precipitation in other protein solutions which are not derived from eggs. In the course of my studies it was of great scientific interest to establish whether the protein substances of hen's eggs and hen's blood could be distinguished from one another with the help of this reaction. Following this, I injected rabbits in the stomach cavity with increasing doses of defibrinated hen's blood and discovered that the serum of animals pretreated in this way, produced no precipitation in a solution of hen's egg protein—at least at the serum's present titer. In the hen's blood solution, on the other hand, in which the blood corpuscles were dissolved by water, that is laked, precipitation appeared. The same serum produced no precipitation in the blood solutions of other animal species so that I had to assume the specificity of this reaction.

I now repeated this experiment *mutatis mutandis* with cattle blood. The serum of these animals pretreated with cattle blood produced precipitation only when added to a solution of cattle blood, never in the blood solutions of other animal species, brought in to act as a control. I prepared now to pretreat rabbits with human blood. At six-day intervals I injected into the stomach cavities of these animals approximately 10 cc of defibrinated human blood. After five of these injections, the animals produced an effective serum, which the following experiment demonstrates.

First I prepared solutions of a large number of blood types with ordinary tap water. To do this, I added water until the solutions were uniformly colored a weak red (dilution 1:100). In order to eliminate the remains of dissolved red-blood corpuscles which disturb the reaction, I either let the solution sit in a test tube or I filtered out the particles. I took approximately two cc from the clear solution I had extracted, placed it in small test tubes of approximately six mm in thickness and mixed it with the same amount of saline solution, double physiological strength (1.6%). It is very important to use in the experiments a blood solution in physiological saline solution, since normal serum, when mixed with tap water, frequently gives rise to turbid disturbances which can impair recognition of the specific turbid reactions. In physiological saline solution such disturbances do not occur when serum is added. These blood solutions, absolutely clear and colored reddish, were produced in this way from the following animals: cattle, horses, donkeys, pigs, mutton, deer, goats, dogs, foxes, cats, stags, female red deer, hares, guinea pigs, rats, mice, rabbits, chickens, geese, turkeys, pigeons—humans.

With a capillary tube removed from an injection needle, I now put 10 12 drops of the serum from the rabbits pretreated with human blood into each of my glass tubes. Relatively quickly a clear, especially striking turbidity made an 319
appearance in indirect sunlight only in the solution of human blood. All the other test tubes remained clear.

After observing longer, one noticed how the turbidity became increasingly intense and how finally a strong flocculated sediment formed.

I need scarcely mention that normal rabbit serum produces no turbid reaction in all these blood solutions.

I am now in a position (with the help of this reaction) to distinguish with certainty human blood from all the other blood types.

*Translation of: "Ueber meine neue forensische Methode zum Nachweis von Menschenblut."

in *Archiv für Kriminal-Anthropologie und Kriminalistik* 6: 317 320 (1901).

The reaction is very fine so that extremely small amounts of blood are enough to determine from which species the blood comes. In order to be certain in every case concerning the type of blood it is necessary that one pretreat rabbits with a great variety of blood types so that their serum can be utilized for diagnosis in suitable cases, provided that the specificity of this reaction occurs also *mutatis mutandis* with other blood types, which according to my experiments is most probable. I am presently occupied with clarifying this point.

That my reaction enables one to identify washed (laked) human blood points the way to the forensic use of the reaction. The deciding stroke was my observation that blood which had dried for a long time and then been dissolved in a physiological saline solution also produced a fine reaction.[2]

I have further busied myself with some important practical questions. I was able to establish that the reaction could discriminate human blood in foul-smelling blood samples which had been left three months in the laboratory to de-
320 compose. When it is a matter of decayed blood, one must naturally make the solution, diluted with saline solution, absolutely clear. To do this I use the Berkefeld Kieselguhr filter which I combine with a suction device which is easily attached to any water pipe. Since such a filter holds back all bacteria and sundry other corpuscular elements, one obtains a beautifully clear and sterile liquid with which one can then set up the reaction. I was also able to diagnose human blood stains without difficulty when the stains had been frozen in snow at $-10°$ for more than fourteen days.

It was equally possible from an assortment of soapy-water samples containing different types of blood to determine immediately the sample which contained human blood.

These experiments which I have briefly outlined here have already been confirmed in many other quarters, chiefly through the study of Wassermann and Schütze,[3] which appeared shortly after my publication.

These experiments also answered the question which I had raised, whether the reactions went so far as to differentiate very closely-related individual subjects such as man and ape. They showed that the serum of a rabbit pretreated with human blood produced a cloudy disturbance in a solution of ape's blood, though the disturbance was faint. This fact, though of great interest to natural science, should be of no importance whatsoever for our forensic practice.

Thus I am convinced that my method has been shown to be most useful for judges and experts.

I would be most thankful to these men if they would send me blood-stained *corpora delicti* to test in doubtful cases.

Notes and References

1. *D. Med. Wochenschr.*, 1900, No. 46; 1901, No. 6 and No. 17
2. *Note made during correction:* A short while ago Professor Beumer of the local institute for forensic medicine and the local First States Attorney, Mr. Hübschmann, handed over to me for testing several samples of blood dried on various objects, blood both from humans and from other mammals without any indication of origin. In every case I was able to diagnose with absolute certainty the blood type. One case involved dried pigs blood from the year 1889, another human blood dried in sand, which came from a murder committed in 1896. The other blood samples (human, pig, etc.) were from the years 1897, 1898, and 1900.
3. *Berl. Klin. Wochenschr.*, 1901, No. 7

Concerning the Development of the Biological Method of Protein Differentiation in the Service of Legal Medicine with Special Consideration of Our Own Research Results, (Personal Recollections)*

Paul Uhlenhuth

Freiburg i. Br.

/309 Professor Ponsold has requested that I report in a coherent fashion on the results of my efforts in the area of *biological protein differentiation* and especially mark out the method as well as point out the considerations and lines of thought, which led me to the discovery of forensic blood differentiation. I followed the request at first only hesitantly, because I believed that I had already presented this in its essentials in my first works. But, as I must assert after a review, there exists much between the lines, which one cannot express in an objective presentation of the research results, such as my many reports, but which might be of historical interest in understanding the development of the biological method of protein differentiation in the service of forensic medicine.

What made my decision especially easy, however, is the happy memory of those young years which like friendly stars are intelligible in the darkness of this time. During those years, I, as a young researcher at the beginning of my scientific career with light enthusiasm but also through hard work and difficult struggles, was able to join in the conquest of new territory, labor which was above all of critical importance for the law and for legal medicine in investigating the truth.

Even if in what follows, I try to represent this part of my life's work together with its practical results almost *in statu nascendi* in the spirit of Ponsold's historical viewpoint, permit me to express my personal experience. I would also heartily desire such an opportunity for our younger generation of researchers.

It was at the turn of the century when, as a young military doctor and assistant of our great master Robert Koch, I had
/310 the good fortune to be able to work and educate myself in his laboratory at the Institute for Infectious Diseases in Berlin, the so-called "triangle" on the Charité. At that time Koch stood at the height of his fame. His classical works on anthrax, wound infections, and on tuberculosis and cholera had made his name famous throughout the world, while his research on tropical diseases enticed him to undertake enthusiastically expeditions to distant corners of the world. At that time I came to know Friedrich Löffler at the Institute: he was the oldest student of Robert Koch. As his first and foremost assistant in the Imperial Ministry of Health, he had discovered in mucus the microorganisms of erysipelas, diphtheria, and trichinosis and had thereby already gained world renown. He was Full Professor of Hygiene in Greifswald. As the leader of the *Commission to Study Hoof-and-Mouth Disease*, which was set up by the Prussian Ministry of Culture and which carried out its work at the Institute for Infectious Diseases, he had made, together with Frosch, the significant discovery that the causative agent of this devastating animal disease was a microscopic filterable virus. When Frosch dropped out of the Comisssion in 1898, Löffler chose me as his successor. The facilities available in the Institute were not sufficient, particularly the stalls for the large animal subjects to be used in experiments. These animals were being housed in a make-shift manner at the city railway barn. As a result of these conditions the Commission was transferred to the Hygienic Institute in Greifswald, and I resettled there in 1899 as Löffler's co-worker. Although it was not easy for me to leave Berlin and the famous research laboratory with its disease section, nevertheless I surely viewed it as a lucky turn of fortune that I had the opportunity to continue this useful and important work in the more favorable country surroundings of Greifswald, a lovely little university town. It was also fortunate that I could continue this work, which had already led in Berlin to important results, with a man like Löffler to whom I was bound by a friendly devotion. Our further experiments concerning the nature and the control of this disease (hoof-and-mouth disease), especially concerning the active and passive immunization, were carried out with great difficulty in the Hygienic Institute and in a rented farm house close to the city. There in the country I took part in them until I was called to be director of the bacteriological section of the Imperial Office of Health in 1906. These experiments led, above all, to the discovery of a highly effective remedial and prophylactic serum which achieved great importance in the fight

*Translation of: "Über die Entwicklung des biologischen Eiweissdifferenzierungsverfahrens im Dienste der gerichtlichen Medizin unter besonderer Berücksichtigung eigener Forschungsergebnisse (Persönliche Erinnerungen)".

in *Deutsche Zeitschrift für die gesamte Gerichtliche Medizin* 39: 309 348 (1949).

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CONTINUED

2 OF 3

311 against this devastating disease.

The serum research, the youngest child of our bacteriological science, still stood at that time at the beginning of its development, but it already had celebrated illustrious triumphs. In 1890, Von Behring had made the important discovery that in the blood serum of animals, pretreated with diphtheria-toxin, specific substances appeared which were capable of neutralizing the toxin used in the injection while it was in the test tube and also when it was in the animal's body. This discovery has clearly shown itself to be extremely beneficial in the fight against this murderous disease of our children. It was the take-off point for all of immunology, a study to which I dedicated at that time a great part of my life's work. In 1894, Richard Pfeiffer was able to prove that in the blood serum of animals which were inoculated with cholera and typhus bacilli, specific immune bodies (antibodies) then appeared, which influenced the bacteria in a certain way, in that they broke up the bacteria when they were injected into the abdominal cavity of a guinea-pig (Pfeiffer's phenomenon). Two years later (1896) Gruber and Durham were able to detect more specific substances in the above-mentioned serum, namely substances which agglutinated the cholera and typhus bacteria in their culture suspensions (agglutinins). This reaction, well known as the Gruber-Widal reaction, has achieved great practical importance diagnostically. As a young assistant doctor in Oldenberg, I was one of the first who was able to affirm in practice the worth of this method in 1897. It was my very first experiment which I published.

These findings suggested that similar reactions would also appear in extracts produced from bacterial bodies as they had in the cultures themselves. It was already established that one could immunize with sterile filtrates from typhus and cholera bacilli cultures and thus obtain a serum with the same agglutinating properties as that which had been produced by inoculating with the pure cultures themselves.

Following this path farther, Rudolf Kraus then produced in 1897 the evidence that immune serum produces in filtrates of the bacteria cultures in question specific precipitates, and indeed these precipitates only appeared when an immune serum was brought together with the filtrate of the matching bacterial culture. Because of the demonstrable specificity, one had to assume that an equally important diagnostic significance would of necessity result from this precipitation reaction, as in the case of agglutination and the Pfeiffer phenomenon. That was, moreover, repeatedly the case with
312 glanders (Wladimiroff), anthrax (Ascoli), etc. Bordet then made in 1899 the important observation that, after inoculation with defibrinated blood, substances formed in the serum of animals which were pretreated in this fashion, substances which broke down the blood corpuscles (hemolysins) and coagulated them (hemagglutinins). Specific agglutinating and lytic antisera can also be produced from pretreatment with other animal cells i.e. ciliated epithelium, white blood cells, kidney cells, spermatozoa (Von Dungern, Metschnikoff, Moxter, Landsteiner, Lindemann, and others). Bordet found further that precipitins also formed in the blood serum of a rabbit after inoculation with cow's milk. Moreover, the reaction of milk serum was specific, so that one could differentiate the protein bodies of cow, goat, and human milk from one another (Fish, Ehrlich, Wassermann).

All these experiments concerning specific antibodies which gave us important suggestions in our research into immunity and serum, treatment of hoof-and-mouth disease, also powerfully inspired me to use every free moment to undertake my own experiments in this area. I can say with confidence that nothing in my career as a researcher has made a greater impression on me, and nothing so captivated me, as the *law of specificity* which governs the whole study of immunity. Indeed here, nature, our great teacher, reveals for us the splendid capacity of her smallest living organisms, the cells. Here she shows us that these cells are our greatest chemists and physiologists. We only give them the raw materials, and as playful as goblins, they produce from them the finest reactions, so sharp and certain in their reactions that the investigative soul stands still in pious reverence, as it would before a miracle.

With this impression I began my work in 1900 and I started *ab ovo* in the truest sense of the word, in that I set out in my work to determine whether specific precipitates developed in the serum of animals pretreated with egg albumin. I wanted to determine whether protein substances of different birds' eggs could be differentiated in this way. Thus, I inoculated rabbits in the stomach cavity with large doses of hen's egg-white solution and extracted in this fashion a serum that still produced turbidity, i.e. a precipitation, when added to a solution of hen's egg white diluted to 1: 100,000, while the chemical reactions in protein ceased at a dilution of 1:1000. The serum produced no reaction when added to solutions of various other kinds of protein (nutrose, somatose, Heyden nutrients such as peptone, casein, or horse, deer, mutton, or donkey serum). Only compounds of 313
egg albumin obtained from various sources reacted positively, a reaction that was also of practical interest (see below). The reaction was thus specific for egg albumin, as was demonstrated at almost the same time by Myers, working completely independently of us. Then, I tried to establish further whether it was possible with the help of this so unusually fine reaction to distinguish the albumin substances of various birds' eggs. The experiments, which I expanded to include eggs of chickens, doves, geese, ducks, turkeys, pheasants, sea gulls, and lapwings, led to positive results insofar as it was possible in this way to differentiate to a certain extent the albumin substances of the eggs, excepting the closely-related bird species. Pursuing further these biological attempts at differentiating albumin, I set up the task of proving whether it was possible to detect the differences between albumin bodies from a chicken's egg and those from chicken's blood, in other words, between two protein bodies from one and the same organism. Therein lay the key to the method which could distinguish different blood types, since this experiment demonstrated that *egg protein* could be distinguished without a doubt from *plasma protein* by means of the specific egg antiserum in that this antiserum produced precipitates only in the egg albumin, but *not* in the solution of hen's blood. At the same time, rabbits were inoculated with defibrinated hen's blood. The serum of animals, pretreated in this way, showed in a solution of hen's egg albumin no cloudiness after a rather long time or only a very weak effect, while in an equally diluted solution of laked hen's blood, the serum produced a strong precipitate.

Through this test it was proven that one was in fact able to differentiate egg albumin from the plasma protein of the hen. At the same time and through this test a fundamental fact was established; for the above-mentioned serum produced a precipitate only in a solution of hen's blood, while all the other blood solutions, from horse, ass, deer, ram, and pigeon blood, which had been introduced for comparison, remained completely clear. Moreover, normal rabbit serum produced no cloudiness in these blood solutions. This *interesting observation was the starting point for perfecting the biological method of differentiating the various types of blood.*

After I had reported on the results of these tests in my work, "A New Contribution to the Specific Test for Egg Albumin by Biological Means", which appeared in the November 14, 1900, edition of *Deutsche Medizinische Wochenschrift,* I presented these biological reactions of albumin and blood at the Greifswald Medical Association on December 1, 1900, at which time I was also able to demonstrate a corresponding specific reaction in donkey's blood. I took this
314 opportunity to make known that "I would be busy trying in an analogous way to decide the important forensic question concerning the distinguishing of human blood from that of animals". I will never forget that memorable session in which so many excellent men of the medical faculty, such as Löffler, Bier, Grawitz, Bonnet, Hugo Schulz, Beumer, Peiper, Schirmer, Moritz, Martin, and Krehl followed my presentations in suspense, and participated in the discussion in a lively fashion. I can still see in my mind how the famous physiologist, "the old man" Landois, a meritorious blood researcher, pushed back his glasses and, fascinated at seeing the reaction, shouted, "Blood is a very extraordinary juice".

I then proceeded to produce precipitating sera to test a great variety of blood types, a task I completed with considerable difficulty. Thus, I first achieved a high-grade serum to test cow's blood. My privy councillor, Löffler, set up a problem for me. From eighteen unlabeled blood solutions, which had been made from laked blood, and which Löffler had arbitrarily arranged in order, I had to select the tube containing cow's blood. The blood solutions were from the following animals: a cow, a horse, a donkey, a pig, a dog, a cat, a stag, a female deer, a hare, a guinea pig, a rat, a mouse, a rabbit, a hen, a goose, a turkey, and a pigeon. Human blood was also introduced into the test. After a few minutes I had solved the problem. The solution of cow's blood was the only one to display a typical cloudiness and a precipitin reaction, while all the other test tubes remained clear. My patience was put to a hard test, however, since I was at first unsuccessful in obtaining a serum which would precipitate human blood — a step which was surely the most important for forensic purposes. This was in itself not particularly noteworthy, since it turned out after further experiments that the individual characteristics of rabbits played a crucial role in the production of precipitating serums, so that approximately six pretreated rabbits sometimes yielded only one or two usable sera. Finally I had achieved a usable serum, which, when added to the same blood solutions mentioned above, only produced a precipitate in the solution of human blood, so that I was able to select the human blood without further ado. Of decisive importance, however, from a forensic standpoint was the fact that blood from humans, horses, cows, etc., which had dried for weeks on a great variety of materials, and had then been dissolved in a physiological NaCl-solution, could be differentiated immediately, even when it was a matter of very small blood stains.

Even when there could be no doubt after that, that the problem of forensic blood differentiation was solved in principle, still I could not at first decide whether to publish, since 315
I was most aware of the huge responsibility which was bound up with the publishing of a method, often critical in the administration of justice. I repeatedly checked my results using all imaginable controls. I let dry on a board (see below, p. 335) a number of smaller and larger stains of human blood and of blood from a great variety of animals and had Löffler and my trusted assistant, Schirmacher, who participated in these experiments with bright enthusiasm, hand over to me concealed samples, which they continually scraped off the board in order for me to determine their origin. Without exception I delivered the correct diagnosis, even though the extremely critical and careful Löffler in a joking manner repeatedly set traps for me. For a long time the manuscript lay completed. And at that point it was my young wife, who had followed my work with growing excitement and joy and who, full of apprehension as only women are, advised me not to wait any longer, especially since I had already published the fundamental tests on November 15, 1900 and had demonstrated them on December 1 at the Greifswald Medical Association.

When one day, I had identified by means of my own tests all the blood samples before me with mathematical certainty, in the evening I again sought out my privy councillor, Löffler, in his apartment. Loffler had assumed until then a cautious wait-and-see attitude. I read him my work. He was in agreement with it on every point. That same evening I brought my work to the post office. In about a week I received the proofs and shortly thereafter, on July 2, 1901, the article appeared with the title, "A Method To Distinguish the Various Blood Types, Especially For the Differential-diagnostic Test of Human Blood."[1] Fourteen days later Wassermann and Schütze reported in the *Berliner Klinische Wochenschrift* on similar results. Nevertheless, I was undoubtedly the first, and I had to thank my anxious wife for this honor.

If I have presented all this in such detail, I have done so because I wanted to show the younger generation how monstrously difficult it was for me to seize upon the right moment to make public a process involving such responsibility, a process on which, under certain circumstances, the fate of a man might rest. That means that together with the happy joy of the discoverer, the joy the young researcher knows, the joy with which one cannot get to the laboratory fast enough each morning, with that exultation, a calm and critical self-control must be maintained in order that one not be carried along to a hasty publication. This needs to be learned first.
316 When reminiscing, I reckon such times of tension and expectation, as I have often experienced in my later days, among the most beautiful moments of a life, filled with successes and also disappointments. On the other hand, the self-criticism and the period of testing and consideration ought never to last too long, lest the power to decide should suffer; otherwise, one is too late. For me that would have perhaps been fatal, since this discovery was the determining factor in the rest of my scientific career.

In this connection I must point out that, a short time after publication of my article, I received information of an observation which Tchistovitch had made in 1899 while studying immunization against the serum of eels, well-known as extremely poisonous. When he mixed this eel serum with an antitoxic serum, a cloudiness appeared after a few moments; if he used horse serum instead of the poisonous eel serum to pretreat his animals, he was able in the same way to establish analogous conditions immediately. Thereupon, Bordet confirmed this observation with the serum of a rabbit pretreated with defibrinated hen's blood. Accordingly, there can be no doubt that these creditable authors have established specific precipitins of plasma protein. What is of special interest, however, is that they have done this in a completely different context, through their experiments which were at that time totally unknown to us.

On the other hand, there should be no doubt that we first indicated the method to recognize and differentiate the different blood types in the course of our experiments concerning the biological differentiation of various birds' eggs, and especially through the difference which we established between the protein bodies of a chicken's egg and of chicken's blood, and, what is of chief importance, that we first worked out and recommended this method for forensic use. We were also the first to succeed in recognizing human blood, as such, in an old and dried state and to distinguish it from the blood of various animals, something previously impossible. These facts, which were not expressed clearly in the literature, or in my earlier works, deserve to be emphasized in the historical presentation of the development of biological blood differentiation.

II. In the greatest number of legal cases which were determined by the recognition and differentiation of human and animal blood, one had up to that point relied almost completely on the microscopical measurement of red blood corpuscles. However, in the case of dried blood, with which indeed forensic practice deals almost exclusively, these measurements established in no way a certainty, but a best only a *probable diagnosis* because of the contraction of blood cells caused by the drying process. A judge, however, could not begin anything with that. The medical examiners, 317
who carried out such experiments throughout the years, know best how depressing this deficiency was for the legal experts. Now, as if by magic, a change entered on the scene.

Even though it appeared that the method which we worked out for blood differentiation was finally perfected from the legal standpoint, not only after my own conclusive experiments with old blood dried on various substances, but also after decayed and frozen blood as well as blood mixed with different chemicals, such as soap, revealed its origins with certainty, still I went on using every opportunity to form a judgment myself concerning the forensic meaning of the method in its practical application. Thus I tested a great number of blood-spotted articles which were placed at my disposal in Greifswald by the director of the Medico-legal Institute, Professor Beumer, and also by the first state prosecutor, Hübschmann. I especially concentrated on state's exhibits of expired criminal cases, which were handed over to me by the Justice Minister for testing. After testing the blood-stained *corpora delicti*, which had been given to me without any further information, my verdict was compared with the relevant official reports. In every case I was able to make the right diagnosis, whether it concerned human blood or that of any animal. After the efficiency and dependability of the method was proven in the laboratory in this way, it was a lucky coincidence that it was able to stand its crucial test in the sensational murder trial conducted by the prosecutor's office in Greifswald against the sex murderer Tessnow. *This process was the very first in which our method found practical application.* Besides the charge of a sex murder against him, Tessnow was suspected of having butchered sheep in a grizzly, sadistic manner. In fact, I was able to detect on the pieces of clothing which were handed over to me for examination both human and sheep blood, a result which was of crucial importance to illuminate the facts of the case and convict the murderer. On the strength of my conclusions, Tessnow submitted a sweeping confession and was condemned to death.[2] Since I was naturally the only one at first who was fully conversant with the method of forensic blood testing, I was brought in as an expert to 318
delivery judgments, and I was thereby able to travel to every section of Germany at the request of the courts so that I could personally present the results of my tests at the various proceedings. This activity brought me uncommonly great satisfaction and compensation since I could grasp first-hand what importance this method of protein differentiation, which was at first a purely scientific method, had won in the search for the truth in legal proceedings, particularly since it contributed not only to condemnations but also to the freeing of defendants.

Even though the results of my labors were recognized and confirmed through many tests, still there were raised here

and there, as was to be expected in any delicate biological reaction, certain objections, which were, however, grounded simply in the faulty handling of the method, especially on the part of experts who were inexperienced and uneducated in serology. In view of the serious decisions which rested on the blood test in a legal process, I considered it necessary that the test be worked over according to certain uniform viewpoints which until then had held true. Thus, together, with the thoroughly critical, totally conscientious, and unforgettable forensic physician, Professor Beumer, the director of the Institute for Legal Medicine in Greifswald, I completed in 1903 an article entitled "Practical Primer For Forensic Medicine regarding the Blood Test Using the Biological Method".[3] This article underwent still further elabo-
319 ration as a result of experiments carried out in the following years with my students, Weidanz, Steffenhagen, Seiffert and others.[4]

III. Without going into all the details, I consider it necessary once more to point out in this context the most important of the precepts which we worked out.

Since the biological method is not a specific blood test, but rather a specific protein reaction so that suppurative sputum, seminal protein (gonorrhea secretive), albumin-containing urine, ascites, and other exudates, and possibly milk and colostrum, can react with an antiserum to human blood, so it is the first task of the expert to identify blood as such with the help of well-known chemical and physical methods. Only then does one move on to the biological determination of the origin of the blood. Thus in our "primer" we thoroughly treat of the production of a completely perfect antiserum, which must be absolutely clear, not opalescent, sterile, species-specific, and of a high-potency, not a very easy task granted the individuality of rabbits which are almost exclusively the animals in question.

The antiserum must above all display a prompt effect, i.e., it must be of a high potency because I require that the reaction or specific turbidity develop before our eyes within a few minutes, and that it appear so fast and clearly that even for a layman any doubt about the commencement of the reaction is out of the question. An antiserum which has this effect must be of the following strength: In a solution of 1 cc of serum diluted 1:1,000 with 0.85% saline solution, 0.1 cc must produce an immediate reaction within one to two minutes when carefully layered in. In a dilution of 1:10,000 or 1:20,000 serum in saline, the turbidity should set in within three to five minutes in the bottom of the test tube, a ring-like turbidity, gradually increasing in strength. One can see the reaction best by holding up a piece of black cardboard.[5] By observing these criteria, one has the inestimable advantage that he himself can successfully test tiny blood spots since we have seen the reaction take place within a short time even in dilutions of 1:20,000. Of especial importance, moreover, is the species specificity. Besides the relationship reaction, which we will pursue in more detail
320 below, one sees from time to time "heterologous turbid reactions", even in unrelated protein solutions. Such reactions are especially common when one adds high-potency, specific sera to protein solutions which were insufficiently diluted, that is when one does not work quantitatively. In immunity reactions such quantitative work is absolutely necessary.

This "overlapping" can of course be so strong in individual sera that even in greater dilutions turbid reactions, i.e. precipitates, can form. Still the practiced investigator will not confuse these reactions with specific turbid reactions if he notices the swiftness and intensity with which the reaction takes place. Nevertheless, they can be the cause of errors for the inexperienced.

The species specificity test of antisera, known to be of high potency (titer 1:20,000), takes place with solutions of heterologous antigen solutions of 1:100, 1:200 and, if necessary, 1:1000. After very careful tests of a great number of antisera, carried out in the Imperial Office of Health by my students, Manteufel and Beger, eighty-seven percent were shown to be absolutely specific, i.e., they alone did not produce a trace of turbidity at concentrations of 1:100 and 1:200.[6] Sera which still cause heterologous reactions even at 1:200 are unusable for forensic practice and ought not to be distributed (For more details, see below, "State Testing of Precipitating Sera", p. 347).

Regarding the execution and the course of the biological reaction, moreover, we have given exact directions, chiefly concerning the handling of the material used in the experiment to produce the extract from blood-besmirched surfaces. As in all forensic blood tests, the most important fundamental rule is that all containers, test tubes, and instruments be meticulously clean and sterile, and that all liquids be absolutely clear. In our experience simple physiological (0.85%) saline solution is the best as a solvent, but it must be allowed to act upon the relevant, pulverized substratum for a sufficiently long period. In order to achieve clear solutions by this process, every agitation must be avoided as much as possible. The test fluid, which under certain conditions is still clearly filterable, must display a foam when shaken, as the sign that enough protein has gone into solution. Then, the fluid must be diluted to 1:1000, and, when tested with nitric acid, can be recognized [as suitable] by means of a light turbidity. Besides the test liquid, control solutions must be introduced, controls which are prepared in the same way as the test liquid, using a solution of the same blood-species whose identity is to be established by the reaction. Solutions prepared from heterologous blood species must also be prepared. the selection of heterologous blood-species is unimportant (one must select steer and pig blood 321
controls to identify horse blood). Animal-blood solutions which display related reactions (see below) are naturally to be avoided. Then, the clear extracts must be tested with litmus paper for their reaction. At a dilution of 1:1000 they ought to show a neutral reaction. Strong alkaline and acidic solutions are to be discarded, but in practice they rarely come into play in view of the high dilution of the test liquid. If, in exceptional cases, they react acidically (leather, tree bark, etc.), they can be neutralized with 0.1% soda solution

or magnesium oxide.

The test solution is then put in test tubes I and II, one cc of the solution produced according to prescription from the blood stains in question being placed in e ch tube. The same quantity of homologous solution, i.e., a blood solution corresponding to the antiserum,[7] goes into tube III, while one cc of heterologous blood solution is put into tube IV and one into tube V (for example pig and cow blood). In tube VI is physiological saline such as that which was used to produce the test liquid. As a further control for certain cases we include still another tube, no. VII, filled with an extract from a blood-free piece of the substratum in question (see below).

0.1 cc of the antiserum which has been pretested (and with the prescribed titer 1:20,000) is then added with a graduated pipette (one cc with graduated marks) to each of the tubes filled with their respective solutions, with the exception of tube II. To this tube 0.1 cc normal rabbit serum is added. In adding the serum one must be careful that the serum runs down the wall of the test tube and is not dropped directly on the liquid. When the serum is added, it usually sinks to the bottom because of its greater specific gravity. The layering must be done very carefully or otherwise, the developing reaction will not appear clearly as a ring formation.

The following are valid criteria for judging the reaction. Tubes III, IV V and VI provide an indicator of the fitness of the serum used. In tube III obvious turbidity must appear at the bottom of the tube within a minute (the value of the antiserum). Tubes IV and V (specificity of the serum) and likewise tube VI (clarity of the serum) must not show a reaction, i.e. turbidity, within twenty munutes. Tube II must provide the evidence, i.e. the lack of any precipitation, that normal rabbit serum does not in itself bring about any turbidity. Only when the reaction in the six control tubes has run its course in the manner described above, and only when
322 tube I, in a positive reaction, shows a turbidity, i.e. a precipitation, of the same kind as in tube III, can the test be considered certain. Turbidity which sometimes develops after the twenty-minute period cannot be considered a positive reaction. In order to execute the test in the manner presented here, the test tubes *must not be shaken*. The reaction must be done at room temperature and not in the incubator. It is useful to repeat the experiment several times and it should be carefully observed and followed *in statu nascendi*. If the experiment is undertaken according to this prescription, all so-called heterologous turbidities, i.e., unspecific reactions and other "interference factors", can be excluded.

Here I must draw attention to an "interference factor" which merits close observation. Early we determined that strong extracts of tree bark and leather gave a "pseudo-reaction" as a result of their acidic content (tannic acid), i.e., that by the addition of antiserum as well as any other serum an obvious, often cloud-like turbidity or precipitation can result (Uhlenhuth and Dürck, Graham-Smith). This was not the case with other substrata which we examined, such as wood, glass, fabric material, iron, paper, stone, coal, cork, straw, sand, earth, etc. With the prescribed dilution of the test fluid to 1:1000, however, the turbidity resulting from the acid no longer occurs. In any case such a false reaction would reveal itself straightaway in tube II. For certainty, we have also required tube VII as a control on the substratum so that every possibility of error is ruled out. Where the failure to observe our suggested rules and controls can lead is demonstrated by a report from the state chemical laboratory in Lagos, recently published by Heindl. Here the careless testing of a stain on a waterproof raincoat, a stain suspected of being human blood, simulated a positive reaction, in that the extract from the raincoat alone gave a positive reaction with any serum whatsoever, a reaction which was later determined to have resulted from the rain-proofing substance (Fritz). If the expert had adhered to our rules, such a dangerous error would have been impossible, since tubes II and VII would have immediately revealed the interfering factor, as was the case in our earlier test with tree bark, leather, and so on. I refer in addition to the research of Fritz, Bessemann, and Baert, which was stimulated by this case of Heindl's, and also to my own treatise on that problem.[8]

Schoenherr was the first to research thoroughly this ques- 323
tion in my laboratory. In eighty-one extracts of various materials (tree bark, oak,[9] leather, rubber, plastic, roofing felt) he was able to establish twenty-eight pseudo-reactions (acidic reactions), and indeed these took place whenever he tested the extracts at the ratio of 1:10 (one part substratum and ten parts physiological saline solution). After diluting to 1:500 the pseudo-reactions disappeared so that, in fact, they do not come into play, if our rules are observed.

Concerning the details I refer to the work presently in print and appearing in the *Archiv für Kriminologie* as well as to the article of Fischer in the same journal.

Thus, the biological method, as we had worked it out, achieved such perfection that it satisfied all conceivable demands regarding its trustworthiness and dependability. It goes without saying that this is true only in the hands of an experienced expert. The best evidence of that is the fact that over the years our prescriptions were not altered in their essentials.

I would, however, not want to miss this opportunity to refer to the *capillary method* which was outlined by my friend Hauser and modified in my laboratory by Carnwath. Here I cannot go into the execution. For this method only the smallest quantities of the blood solution, i.e., the fragments of a droplet, are sufficient for the test. As a result, there would in practice rarely be a case in which the testing of a blood spot would meet with insurmountable difficulties, provided that the solubility of the blood was not diminished too much through aging or other causes. This method naturally required special practice and experience. Together with my students Weidanz and Angeloff, I have used the capillary method successfully to identify the provenance of blood in leeches and in blood-sucking insects (bed-bugs, fleas, lice, mosquitos, gnats, flies). By means of this test we were still

able to identify human blood in bed-bugs after fourteen days. The results were the same with human, cow, and goat blood in fleas, sheep ticks, and dog ticks.

In a selection of *Anopheles* mosquitoes, the transmitters of malaria, we were able to determine the presence of pig and cow blood, but not of human blood, which we had expected to find. The mosquitoes, we later discovered, had been caught in pig and cattle barns. So it was possible in a simple manner to determine the blood suppliers of individual carriers, a fact which can be of great importance in epidemiological research.

/324 The capillary method has come into vogue in many places for forensic practice, in place of the test-tube or Uhlenhuth-tube method (see also Merkel).[10] We ourselves have used it with advantage because the ring formation, which arises when layering the test liquid with the antiserum, appears in an especially striking fashion in the capillary tubes.

IV. To illustrate the forensic significance of the biological method, it is sufficient to refer to the opinions of court physicians which have played a determining role in illuminating the facts in countless cases of murder, bodily injury, moral transgression, theft of household animals, poaching, etc. I have brought together a collection of my own opinions, the especially important ones, and published them with Weidanz in my book, referred to above.

From the abundance of opinions which I have rendered during the years I want here to select only a few strking examples in order to illustrate the practical importance of the method.

1. A butcher, accused of a triple robbery-murder, alleged that the blood stains found on his shirt sleeves were due to his having butchered a cow. With the aid of the precipitin reaction I was able to establish with certainty that the stains were human blood stains. On the grounds of overwhelming circumstantial evidence, including this finding which was an important consideration, the accused was condemned to death. Shortly before his execution he made a comprehensive confession.

2. A man, on whose clothing were found blood stains, was arrested under heavy suspicion of murder. He asserted his innocence, however, stating that the blood came from a wound his horse had suffered. His story was not believed until I was able to prove the truth of his testimony by means of the precipitin reaction. The man was thereupon released from prison.

3. A man was accused of having stolen and butchered a pig, and of having concealed the body in a sack. He maintained that the blood stains on the sack came from a female dog which had given birth. I, however, was able to establish that it was a matter of pig's blood and thereby cleared up any doubt about guilt.

4. The following case is also interesting: a man who wanted to cheat on his pension was discovered one morning in his blood-stained bed. He maintained that he had suffered a violent hemorrhage. Since the medical examination gave
/325 no evidence of this, I brought into play the precipitin reaction in order to determine the origin of the blood. The test indicated the presence of cow's blood. When the man was directly confronted with the result, he admitted that he had poured out a bottle of cow's blood, which he had fetched for himself from the slaughter house, with deceitful intention.

In view of the great responsibility which such a forensic test brings with it, the conditions surrounding it are similar to the bacteriological determination of diseases which endanger the public, such as cholera. Considering the far-reaching consequences of such a diagnosis the imperial administration has issued exact instructions which are to be strictly followed, and, if these are not observed, the diagnosis of cholera is not recognized as valid. Further, experts are only admitted who have obtained proof of special training. If these demands are present in public health, they ought also to be necessary here, where the determining of human blood often decides a life-or-death issue in a murder trial. There even is a special training program for the carrying out of the Wassermann reaction for syphilis, a program which was worked out in the imperial health office and required by regulation for all official examinations.[11] Accordingly in 1903, I demanded that central offices be instituted where experts could be instructed in carrying out the forensic blood test. I felt that university institutes of legal medicine were the best suited for the job. From the central offices the experts can also acquire high-potency sera, which has been tested by the state.[12] It is a matter here of a serum reaction which brings about extremely fine biological processes; to observe and judge these processes requires a special course of studies. If these methods of testing are unfamiliar even to the c urt physicians, how much more are they strange to the court chemists, who are often called upon to carry out such experiments.

At this point it was a welcome turn of events that the official departments took a stand regarding this matter, so important for the administration of justice, on the recommendation of the Scientific Deputation for Medical Affairs. This recommendation ran as follows:

> The practical uses regarding the serum method of blood testing are already so widely disseminated in Germany as well as abroad, the results of the research so unanimous in their essentials, that no doubt can any longer be raised that this new biological method enables one in the majority of cases to determine with great certainty the origins of fresh and dry blood and to distinguish human blood from the blood of different animals. Though this excellent method naturally should 326
> not drive out the old, tested methods of blood identification, but rather should supplement and complete them, we vigorously urge that it be used in judicial practice.

On the basis of this recommendation the Prussian Justice minister issued on September 8, 1903 a disposition dealing with this question wherein the biological method was introduced into legal practice.[13] The Hygienic Institute of the

University of Greifswald, the Institute of Infectious Diseases in Berlin, the Institute of State Pharmaceutics in Berlin, and the Institute for Experimental Therapy in Frankfurt am Main were all named as institutes which were straight-away invited to undertake forensic blood research. Similar dispositions were released in Austria, Bavaria, Württenberg, Baden and also abroad in almost every nation. In order to have ready at all times a satisfactory serum, the Hygienic Institute in Griefswald was intrusted in Spring 1904 by the Prussian Ministry of Education with the production of high-potency serum, where I controlled the process myself. Later the bacteriological department of the Ministry of the Interior was also named. I was transferred there as director in 1906. The production and regulation of sera was all the more necessary as I was able to establish that sera produced by court physicians themselves, or obtained from other sources, fell totally short in many ways of meeting the prescriptions we outlined above. Above all were found among these strongly opalescent sera, which were not of sufficient potency to eliminate errors, i.e. false results. Unfortunately, as the result of adverse circumstances, it has in recent years not been possible for these bureaus to carry out the production of the necessary antisera so that the court physicians were no longer able to obtain satisfactory, officially approved antisera. Now that the governmental testing of sera,[14] testing we had formerly worked out in its details, and had held to be strictly necessary, has been introduced at the Institute for Experimental Therapy in Frankfurt am Main, and now that private serum laboratories have undertaken the production of the kind of antisera practical experience requires, one hopes that the constant difficulties in this respect have been removed. I also consider it strictly necessary that more attention be given the instruction of court experts in this matter
327 and that the blood tests be limited to state forensic medical institutes and possibly to hygienic-serological institutes which ought to be designated by name to the courts and police officials. In these institutes such experiments can continually be carried out by experienced experts. Since, in consideration of circumstances, the old official regulations are now forgotten and since considerable abuses now exist, abuses which my questionnaires to the forensic medical institutes brought to light, the question of forensic blood testing ought to be uniformly established and regulated. As the result of these regulations, chemists, criminal experts, official doctors, and health offices should not, in general, carry out these tests.

V. Of forensic importance, and also of interest to the natural sciences, are the results published in my first writings concerning the differentiation of albumin from various birds' eggs (see above). I established that the bonds of relationship among the animals achieve visible expression in the biological reaction. Thus, I hit upon the closely-allied idea of recommending the use of the precipitin reaction for the study of the relationships among the animals. By this means the blood relationships between horses and donkeys, among sheep, goats, and cattle, among dogs, foxes, wolves, and jackals, between pigs and boars, hares and rabbits, chickens and pigeons are demonstrated before us *ad oculos*. With certain gradual and temporal differences, the biological reaction runs approximately parallel to the degree of blood relationship and, in general, agrees with the animal taxonomy. In the case of reptiles and amphibians (v. Dungern) and of fish (Neresheimer, Dunbar, Kodama, and others) similar relationships exist. Nuttall confirmed these experiments and expanded them greatly, testing 900 different blood species with thirty different antisera and 16,000 reactions.[15] Of very special interest are the tests concerning the blood relationship between men and apes. The identification of these blood species was first brought forward by Wassermann and myself, and was confirmed and thoroughly studied by Nuttall. It was shown that a human antiserum produced almost as strong a precipitation in the plasma protein of the human-like apes (chimpanzees, gorillas, orangutans)—when analyzed quantitatively—as did in human blood. This serum reacted somewhat more weakly with the blood of baboons and long-tailed apes. Weaker still was the reaction in the case of new-world monkeys, the *Cebidae* and *Hapalidae*.
328 The blood of the *lemurs* (Halbaffen) reacted either only very weakly to serum of very high potency, or it did not react at all (Nuttall). That the serum of a rabbit pretreated with human blood calls forth precipitation not only in human blood but also in ape blood, and in addition produces such precipitates in no other types of blood, is forceful evidence for the blood relationship between man and the ape family. Moreover, considering the differences in the precipitates from the biological reaction, one must accept that different grades of relationship, some closer, some more distant, exist between man and the various types of apes, especially that the anthropomorphic apes (chimpanzees) stand closest to man and that, in general, the monkeys of the old world are more closely related to man than are those of the new world. Clearly, this evidence for the blood relationship between man and the ape family is worthy of being placed along side of all the other evidence which follows from comparative anatomy and from the history of evolution. Indeed this might be the most striking and startling, since one can demonstrate it in a flash in the test tube *ad oculos*. Thus this biological reaction is a solid prop for the theory of evolution as it was founded and developed by Darwin, Lamarck, and Häckel.

These experiments have been confirmed by many researchers (Hansen, Bruck, W. A. Schmidt, Yamanuouchi, and so forth). The serological studies of Mollison and von Krogh are especially impressive and interesting from the standpoint of animal taxonomy. By carefully measuring the quantity of precipitation, and by using transverse reactions, these studies came to the conclusion "that the phylogenetic relationship among the forms of life can be grasped more clearly and surely through the tested precipitin reaction than it can with the aid of morphological similarities."[16]

"If protein substances of related species are common, and if these substances are found no where else in the animal or

plant world, this fact must lay claim to greater importance than any morphological feature. The protein which a creature has inherited from its ancestors is to a certain extent a passport wherein the infinitely complex marks of its forefathers have been entered." It proves definitely, "that both species have a common stretch in their phylogenetic development which they completed before their present
329 differentiation. A distinction between protein relationship and phylogenetic relationship does not exist" (v. Krogh). If Ehrhardt is of a different view, his judgment at best must have reference to older experiments in which unsuitable methods were used.[17] Thus, our precipitin reaction as a method for biologically distinguishing protein must be considered at least equal to all the morphological research, and both of these methods will be most useful when they control each other's results.[18] Our precipitin reaction has thereby achieved great meaning for anthropology and zoology.

This rich biological method of observation has not yet been properly evaluated. For example, the apparent variation in susceptibility to infections and tumors (cancer) in supposedly closely related rodents led me to study thoroughly their blood relationships. To our surprise I was able to establish that, for example, between rats and mice there exists only a distant relationship so that it is easily possible to differentiate mouse and rat blood with an antiserum of not very great potency (see also Trommsdorf, Graetz, Steffenhagen, and Schönburg). This however, does not seem to take place with every antiserum in the same way (Otto and Cronheim). Rats and mice, of course, belong to two different families (*Epimys and Mus*). Recently we were also able to demonstrate that, by using an antiserum against field-mouse blood, one could distinguish field-mouse blood from that of a house mouse (white mouse), and that this was also possible in the reverse order, i.e. by using an antiserum against house-mouse blood.[19] Robert Koch already suspected such a difference between these blood types, when he conducted experiments concerning the varying susceptibility to the agents in mouse-septicemia and anthrax (1878). The field mouse displays a noticeable resistance against these agents compared to the house mouse. On the other hand, we observed that an antiserum against the blood of the house mouse reacted equally to the blood of a white mouse, a reaction which is perfectly understandable, because the white mouse is a pigmentless house mouse, and breeding is possible between them.

Although these relationship reactions are interesting, they are understandably the source of interference in forensic-medical practice. If, for example, the expert must confront the problem of distinguishing horse from donkey blood, or
330 sheep from goat blood, he comes up against unconquerable difficulties, since the precipitin reaction breaks down in these cases. In the attempt to distinguish related blood types Weichardt employed the so-called "saturation method." I cannot go into this method more closely here, since it has achieved no practical forensic importance, even in the hands of an expert.

A forensic opinion gave me the occasion to work thoroughly with the distinguishing of closely related blood types. In this case it was possible to go a step further. The court sent me a blood-stained walking stick with the request to establish the origin of these stains. The man, in whose residence the walking stick happened to be found during a house search, was under suspicion of having killed a deer or a smaller wild animal (a hare, a fox, or some other similar creature) and of having taken it away on the stick. The man, however, claimed that the stains were caused by goose blood; his mother had supposedly slaughtered some geese and hung them up. The walking stick stood below these geese and the blood ran down onto it. First, it was possible to establish that the serum of a rabbit pretreated with goose blood did not call forth a reaction in the solution of the blood-stained material scraped from the stick. Thus, goose blood was ruled out. Similarly by using a deer-blood antiserum, deer blood could definitely be ruled out. Now in order to determine whether it was hare blood, I attempted to produce a hare antiserum. Toward this goal I pretreated rabbits with hare's blood, although in view of the supposed close relationship of the hare with the rabbit, theoretical doubts against this procedure were raised. In order to obtain an effective antiserum to hare's blood in any case, three chickens were pretreated with hare's blood along with three rabbits. Since at that time it was closed season and therefore impossible to obtain fresh hare's blood, I used four-year old, dried hare's blood, which I dissolved in a physiological saline solution. To my great surprise all three rabbits produced usable antisera which precipitated with hare's blood. The three chickens also produced effective sera after four or five intramuscular injections with hare's blood.

The antisera obtained from the rabbits as well as that from the chickens reacted to hare's blood, but they displayed the following differences. Though the serum from the rabbits pretreated with hare's blood was added to a great variety of blood solutions, it produced a reaction only in hare's blood. The blood solutions of tame and wild rabbits remained completely clear. On the other hand, with the hare antiserum obtained from the chicken, there was no positive difference between hare and rabbit blood, since it produced precipitates both in hare and rabbit blood.

With the aid of the hare antiserum obtained from the
331 rabbits, I brought forth positive evidence that the blood found on the walking stick of the poacher was that of a hare. Through this "crosswise immunization," I was able in a similar way to differentiate positively chicken from pigeon blood. When I pretreated monkeys with human blood, I succeeded also in differentiating human from monkey blood by means of the human antiserum obtained from a monkey. The serum from this monkey, which has, since that time, accompanied me on life's journey as a stuffed specimen, called forth precipitation only in the human blood, not in the blood of apes. This interesting observation was confirmed by Landsteiner with human antiserum obtained from chimpanzees. Thus it is indeed possible in certain cases to produce

precipitins in the case of related animals such as chickens and pigeons, hares and rabbits, as well as humans and apes through reciprocal injections, and even to distinguish human from ape blood even when these antisera are, as a rule, not of high potency and are in general difficult to produce. On the other hand, it was not possible even with large doses of donkey's blood to produce from a horse specific precipitins against donkey blood. Even less was it possible to get such serum from sheep which had been pretreated with goat's blood because the plasma protein of these animals is too closely related. In this case, however, one must consider also that sheep are very poor producers of precipitins.

It is necessary that the expert take careful notice of the relationship reaction and, since he scarcely has at his disposal human antiserum obtained from apes, he should do as I have always done. He should add the note, "if ape blood has been ruled out by the judicial examination." Luckily this has practically no importance in our region. If need be, however, the decision can be reached with serum obtained from apes, as we have said. The same is true for the hare and the rabbit, the chicken and the pigeon, and so forth.

In practice, however, such an explanatory note is especially important when, for example, the case involves sheep blood (see above the Tessnow case), which by our method cannot at all be distinguished in practice from the blood of goats, deer, or cattle. The same is true of distinguishing horse from donkey blood. In these cases the diagnosis must be presented in conjunction with the judicial inquiry *per exclusionem*. In any case this must always be expressed in the opinion, since it has, in fact, happened that the accused has exposed the expert before the court.

We see, on the one hand, that the "crosswise immunization" means a certain progress in the differentiating of various blood types, but, on the other hand, the unsuccessful attempts at distinguishing horse from donkey blood, mutton from goat blood, show us that the precipitin reaction, otherwise so capable, finds its limit here. Where an equivalency of plasma protein can be biologically established, there is the possibility of crossing the animals, as is the case with the horse and the donkey. Mutton and goats are also indistinguishable biologically. Whether cross-breeding is possible here is still a disputed question. On the other hand, where a distinction of plasma protein is biologically demonstrable, cross-breeding seems to be ruled out. This fact is suited to quash any fantastic notions of breeding. Thus, these experiments can also be of practical use to the animal breeder.

It would be of particular interst from an anthropological standpoint if it were possible to distinguish the blood of different human races from one another. As is well known, C. Bruck, with the help of an antiserum against members of the white race, is said to have succeeded in distinguishing whites from members of the Mongolian and Malaysian races by means of weakly precipitating sera with the use of complement binding (see below). From the gradations in titer he derived the relationship of the different races to each other. According to our opinion, however, the differences in the titer were too small to permit sure conclusions. Moreover, Bruck's experiments could not be confirmed (Linossier and Lemoine, Marshall, Teague, Fitzgerald, and others). The experiments of Sutherland and Suk as well as those of Fischer and Raquet have not shown progress. Whether a "crosswise immunization" with the blood of whites and blacks would lead anywhere is unlikely, according to what we have said, since certainly among the individual human races mixing occurs to a wide extent (Europeans with Blacks, Indians with Eskimos). Nevertheless, such experiments should be attempted. Using antiserum to human blood obtained from the Robert-Koch Institute, we ourselves had the opportunity at our leisure to analyze quantitaively fresh blood serum from different races, from Englishmen, Armenians, Russians, Indians, Negroes, Arabs, and Mongols. In these experiments one must, of course, notice that the protein content of the blood sera can be subject to certain variations.

It was not possible to observe a distinction among blood samples of different races. The reaction proceeded very uniformly in a dilution of 1:1000 to 1:20,000. The reaction proceeded in the same manner in the blood of an ape which had been added to serve as a control.

We atempted also to distinguish the different canine 333/
breeds[20]/ which leave nothing to be desired regarding the variety of their outward appearance. Our experiment failed to distinguish with an antiserum to the blood of a pedigree German shepherd, quantitative differences in the blood of nineteen other supposedly pedigree dogs, a result which agreed with the well-known cross-breeding possibilities among the various canine breeds.[21] Although it would be very interesting from an anthropological standpoint we came no further with the present biological methods. The experiments, which we began with the Abderhalden protective-enzyme (Abwehrferment) reaction, could possibly reach the goal, but their execution is extremely difficult. Unfortunately, they had to be discontinued for other reasons.

VI. The idea came to me to use my method of distinguishing the different blood types to test whether it was possible to use this method in distinguishing the meat of different animals. From the start the prospects were good, because in a good cut of meat there is a large quantity of blood still present.

By numerous experiments I established that in a great variety of pig organs (spleen, liver, heart, muscle) dried for a year, a positive reaction occurred and, thereby, that the origin of these organs could still be ascertained exactly. This was my starting point in working out a method of distinguishing the different kinds of meat, which was of fundamental importance for meat inspection.

Through countless experiments we were able to demonstrate that the serum of a rabbit, pretreated with pig blood, produced precipitation only in an extract of pork, that from a rabbit pretreated with cat's blood only in an extract of cat's meat. Further, specific sera were produced for the identification of mutton and horse flesh, but at the same time the

possible relationship reactions between horse and donkey meat as well as that among sheep, goat, and cattle meat had to be pointed out. The importance of this method in testing chopped meat for the admixture of horse, dog and cat meat was accordingly self-evident. Moreover, I was able to establish the important fact for meat inspection that the specific identity is also successful in smoked products (pickled meat, pie). Thus it was possible to ascertain with certainty the origin of year-old smoked horse meat and ham. Similarly, we
/334 succeeded in determining the origin of horse sausage and sundry other German sausages, if the reacting protein bodies were not destroyed by cooking, as is the case with liverwurst.

The method of meat testing which I, together with Weidanz, Wedemann, and Borghmann, worked out in its smallest details for practical application, was confirmed and fully recognized by the work of Jess, Piorkowski, Nötel, Miessner and Herbst, v. Riegler, Groening, Ruppin, W. A. Schmidt, Schütze, Fiehe, and others.[22] Since it was impossible by using the current chemical and physical methods to identify with certainty horse meat, not to speak of the meat of any other animal, especially in sausage or other meat mixtures, the biological method for the practical inspection of meat was undertandably of extraordinary importance. For the inspection of foreign meat, the precipitin test to identify horse meat, carried out according to our instruction, has been required by law. Our instructions are found in the appendix "a" to the explicative guidelines "D" which went into effect 1 April 1908, regarding meat inspection[23] and the method is recommended officially for the inspection of domestic meat (see the relevant dispositions of Prussia, Würtemburg, Bavaria, and so forth as well as relevant opinions.[24] In the framework of meat-inspection laws, fresh, frozen, dried, smoked, pickled, cooked, and decaying meat can be subjected to testing by using the biological method. In all these cases the biological reaction reveals the origins of the meat, provided the protein bodies are not completely destroyed by cooking. Despite many efforts, the production of usable antisera for cooked-meat protein has not been successful. Regarding all the details of the technique and methodology I refer the reader to the works cited.

It should be mentioned that under the ban against prepared horse meat is included the introduction of horse intestines and dried horse blood. In these cases, too, the biological method has been used to advantage, as it has in the case of
335 fish meat (Uhlenhuth, Weidanz, Borchmann). Moreover, if it is possible to extract from either animal or human bones enough soluble, reactive protein, one can determine their origins, something of importance for forensic medicine (Beumer, Schütze, Steffenhagen, and Clough).[25] In forensic cases, however, involving bones which have been burned, bleached, or carbonized, or which have been in water for a long time, this is no longer possible (perhaps, however, by means of the anaphylactic reaction, p. 343).

VII. Though as a rule forensic practice deals with fresh material with which it is easy to produce the specific identification by precipitation, the investigation of relatively old blood stains may still, under certain circumstances, contribute to solving a crime and thus be of importance. In my first efforts I was already able to demonstrate that a positive biological reaction occurred even with blood stains that had dried for weeks or months, indeed for three, five, and eleven years. It was successful, too, with dried organs 1.5 years old. These results were confirmed by others (Biondi, Ziemke, Graham-Smith). Even in the case of mummified organs thirty to forty years old, and of those sixty to seventy years old, I was still able to determine their origin with certainty, while in the cases of Egyptian mummies, a thousand years old, and of a horse muscle a hundred years old as well as with mummies from the lead-lined cellar of the Bremen cathedral (100 to 450 years old) and with the head skin of an Inca skull, the precipitin reaction no longer provided a positive result. The supposed positive results of von Hansemann in the case of 3000–5000 year-old mummies were due to mistakes in the experiments (pseudo-reactions, see above), as I was able to establish. Nevertheless, I would like to take this opportunity to mention that I was indeed able to establish the derivation of individual Egyptian and Peruvian mummies as well as of the one-hundred year old horse muscle, mentioned above, through the anaphylactic reaction (see below p. 343).[26-27]

As I mentioned above, in the Winter of 1900–1901, I had smeared a board (see above p. 315) with different blood types (human, horse, cattle, pig, etc.) on which I carried out my first experiments with dried blood. On the occasion of the celebration of the thirtieth anniversary of forensic blood testing, arranged in my honor by the Greifswald Medical Association, my student, Zimmermann, confirmed that these blood stains produced a prompt, specific reaction.[28] Zimmermann was also able to provide a positive reaction in 336/
most cases with the old judicial exhibits which I had tested successfully at that time. Some of these exhibits had been stored for twenty-five to thirty years in laboratory test tubes. Only in a few cases had the protein lost its solubility and, therefore, its reactive capacity, despite leaching for several days. This is especially true where the blood samples are very small and are solidly embedded through absorption in the tissue fibers. Also, blood stains on a smooth piece of note paper from the year 1900, which I had diagnosed as pig blood, had become insoluble.

From these experiments it also emerges that the testing of relatively old blood stains can still be successful in the subsequent solving of crime. It is not possible to give a time period after which one ought not to expect the biological reaction to be successful. The failure of the precipitin reaction seems to be due primarily to a loss in solubility rather than to a loss in specificity. Such a loss in solubility is dependent on the workings of many outside influences. It is important that the blood dry as quickly as possible, since the protein is only damaged a little by the drying and in such a state can remain intact for many years, since, most importantly, it has been removed from the spoiling process. I therefore recommended at the time that fresh blood, which is found at the scene of a crime, and which is to be handed

over to the experts for examination, be soaked up with a pure piece of blotting paper and allowed to dry. In a petri dish Zimmermann was able to test blood of humans and of a great variety of animal bloods, which I had dried and stored in its substratum in test tubes—thirteen to thirty years old. In almost every instance the result was positive, even when occasionally several days were necessary to produce the solutions. As I mentioned above, I myself was able to produce from a rabbit a practical, usable antiserum to hare's blood even with dried hare's blood, four years old, by dissolving it in a saline solution.[29] Indeed I succeeded in obtaining especially high-potency, specific antisera (titer 1:80,000) with similarly preserved human and canine blood and with egg albumin, all thirty years old. All of these facts might also be of forensic importance for the distinguishing of blood types which are difficult to procure (for example, wild animals in the off-season, etc.).

VIII. In addition, the biological process of protein differentiation according to our experiments has also achieved considerable forensic importance in the control of food products and in the identification of adulterations. Thus, with regard to establishing the presence of egg yolk in dough
337 products and egg-yolk margarine we can succeed in distinguishing egg yolk from egg white with a specific antiserum to yolk.[30] (See also Otto-Lenghi, Emmerich and others). Adulterations of caviar with less-valuable fish roes could be demonstrated in my laboratory with certainty through the biological method (Kodama, Händel, Schern) since the sturgeon caviar can surely be distinguished from other fish roes by means of a specific serum. Combinations, i.e. adulterations, of nutrient preparations of protein for commercial purposes can be discovered through the biological method as we mentioned earlier. So we were able to prove that hematogin (Hommel) and marketable hemoglobin contained cattle meat. I call to mind the sensational legal process concerning a raw meat liquid extract "Puro" which was supposed to consist of fluid pressed from fresh ox meat, but which contained only dog protein. We were able to establish this by means of the precipitin reaction as did von Gruber and Horiuchi. Also, to identify bee honey (bee protein), i.e. to distinguish it from artificial honey, we advantageously called into service the biological process (see also Langer, Riegler, Galli-Valerio, Thöni and others). The same was true in identifying the provenance of milk products and cheese (Sion and Laptes). The biological method has also been called successfully into service to determine experimentally the origin of fat tissues (butter, bone marrow, margarine) insofar as soluble protein can still be extracted. The same is true for testing plant proteins (wheat, corn, rice, legumes, hemp, poppies, squash, almonds, mushrooms—champignons and others, yeast, etc.) as well as for inspection of animal-feed adulteration, for example, with Ricinussamen (Miessner) as the actual cases have demonstrated.

IX. In other areas such as physiology and clinical medicine, the precipitin reaction has also been shown to, be extremely valuable for the study of nutrition from a physiological standpoint (reabsorption relationships of foreign protein, the mechanics of albuminuria). Regarding this see Uhlenhuth, Citron, Ascoli, Hamburger, Moro, etc. One has also employed the precipitin reaction with more or less success to identify *Echinococcus* and *Taenia* tapeworm infections and for diagnosing cancer. In this respect, I refer the reader to our handbook article (see above). Attempts to feign sickness have been uncovered at the bedside, as was the case with a patient who simulated albuminuria. After chicken protein was identified in the urine instead of human protein, the patient confessed that he had put his breakfast eggs in 338/ his urine sample (Wegner). A man who wanted to obtain an annuity by trickery was discovered one morning in his blood-stained bed. He pretended he had suffered a violent hemorrhage. Because the medical examination produced no clue concerning this affair, the blood stains were handed over to me for examination. I determined that it was cattle blood, When he was confronted with this statement, he confessed to having emptied with deceitful intent a flask of cattle blood which he had gotten from the slaughter house.

Concerning a similar case, Merkel reports that he was able to show that a woman, who was under a doctor's care for a year because of an alleged gastric ulcer and had been collecting an annuity, had secretly sprinkled cattle blood into her spittoon.

I would like to allude briefly to my research concerning organ specificity, which took its start from my first experiments, mentioned earlier, concerning the distinguishing of the protein substances in a hen's egg and in hen's blood and which consequently bear a close relationship to the biological differentiation of plasma protein.

I determined that the crystalline lens of the eye is the only animal protein body now known that does not produce a precipitin reaction with a blood antiserum.[31] On the other hand, an antiserum produced by injecting a rabbit with lens protein gave a reaction only in lens protein, but not in the proper blood solutions, or in solutions of other organs. Thus, plasma protein and lens protein—two protein bodies of the same organism—could be distinguished with certainty. These experiments led further to the scientifically interesting conclusion that the crystalline lenses of mammals, birds, amphibians, and, in some lesser respects, of fishes, possess a biologically identical protein. For example, rabbits, which were pretreated with cattle-lens protein, produce a serum that causes an identical precipitation in lens protein of a human, a pig, a dog, a frog, etc., so that here, the law of species specificity of the biological method appears to have broken down. The lens, therefore, must be viewed as though it were a foreign protein body in the animal organism. Perhaps the explanation for this lies in the fact that the lens is purely an epithelial organ which is completely without plasma protein. The organ specificity of the lens has become the subject of far-reaching studies on the ophthalmologens (Römer, Crusius, v. Szily, Doerr, Kraus, Okamoto, Shibata, Uhlenhuth, Händel). Sachs has termed this reaction a lipoid-antibody reaction, and has demonstrated a similar

organ specificity with the brain.

/339 Without going further into the other studies on organ specificity, which have achieved practical importance in differentiating the protein bodies of milk and birds' eggs (white and yolk), I would like to refer briefly to the special case of hemoglobin protein which is of forensic interest. A. Klein and H. Pfeiffer were able to prove that the precipitins which form after injection with erythrocyte extracts (hemoglobin) of various animals are specific, i.e. they produce precipitation only in erythrocyte extracts of the same animal family as the animal which was used for their production. In the corresponding blood sera, on the other hand, precipitation does not occur with such an antiserum. Klein, therefore, believed that one could dispense with making a chemical identification by using such an antiserum. Moreover, the hemoglobin antisera cross react with related animals (horse-donkey, human-ape) just as the antisera against plasma protein. We ourselves were able to confirm that the serum- and erythro-precipitins, if not completely specific, were rather strongly specific. At least, we could not produce a clear reaction in dissolved blood with serum precipitins, when the serum used for immunizing contained no hemoglobin. Despite many attempts, we have not been sucessful in producing high-potency hemoglobin antisera (Uhlenhuth and Weidanz), a fact observed in other quarters. Moreover, there is no need of such sera, which has scarcely been tried in practice. For the rest I refer you to the relevant works (Leers, Hektoen and Schulhoff, Heidelberger and Landsteiner, Hijaschi and others).

Regarding the specificity of serum precipitins and erythro-(hemoglobin) precipitins, it is important to observe the forensically significant point of Mezger, Jesser and Volkmann.[32] The extract of blood encrustations, dried on wood, produced no or only weak precipitin reactions with the usual specific antiserum to plasma protein. On the other hand, an extract from a piece of wood under the blood crust, where the serum, having been pressed out by coagulation, soaked in, produced a clear reaction. It seems important to me in this connection to point out this observation.

Here I think it necessary to make some observations regarding the biological differentiation of sexual protein which Dunbar and I carried out. Dunbar was able to establish in the case of plants, and of animals as well, that the male and female sexual cells react against one another serobiologically and react to other tissue components of the same
/340 organism as if they were foreign (see also Graetz). He was able to demonstrate this especially with the sperm and roes of fishes. I myself with my coworkers Händel, Kodama, and Schern was able to produce proof that fish-roe protein can be sharply distinguished from fish meat of the same animal. It was also possible to show that the eggs of sturgeons can be distinguished from other fish roes (carp, roach, fresh-water carp, tench, salmon, herring, trout). The identification of caviar adulteration, mentioned previously, rests on this observation.

In a similar way we were able to sharply distinguish biologically frog-egg protein from frog-meat protein, while frog-spawn antiserum precipitated, if only weakly, extracts of tadpole protein of the same frog, but not the meat extract of the sexually adult frog (Uhlenhuth, Wurm, Hsia.[33] In these experiments one was able to make the significant assertion that the viscous egg envelope of batrachians, which, according to our experiments consists of mucin, and is most likely made up of admixtures of true protein, possesses the qualities of antigens, so that it is also possible to produce precipitins of an apparently specific character to mucins. Here lie conditions similar to those in the building of antibodies which I first demonstrated, antibodies against almost pure carbohydrate gum arabic.[34] Numerous experiments concerning carbohydrate antibodies in bacteria conform to this conclusion (Avery, Heidelberger, etc.). Recently I made an interesting observation while busying myself with the biology of the potato beetle and the methods of fighting this insect. In the case of frogs, different stages in development with respect to their protein bodies can be differentiated by the precipitin reaction. My work showed that similar conditions pertain in the different stages of development in the potato beetle. Above all, I succeeded with a precipitating antiserum against the eggs of the potato beetle in identifying egg albumin in the sexually mature, egg-carrying beetles by means of the precipitin reaction, while this reaction failed to take place with male beetles.[35] One ought to expand such tests to include the developing phases of other insects (for example, butterflies, caterpillars).

I would also like to remark that I attempted earlier to establish differerences in the blood of sexually mature men and women by using high-potency antisera against human sperm protein. These attempts turned up completely nega- 341/ tive results, while by chance I was able to observe that high-potency antisera to hen's egg white produced a strong precipitation in the blood protein of sexually mature hens as well as in the blood of a rooster.

X. My exposition concerning the biological differentiation of protein would be incomplete if I did not at least briefly refer to the two methods which, from a purely scientific standpoint, are of great interest since one is in a position to detect the least traces of protein by using them. These are the complement binding reaction and the anaphylactic process.

Complement binding (Bordet, Gengou), which has achieved great practical importance in diagnosing infectious diseases such as syphilis (Wassermann), glanders, and others, was recommended by Neisser and Sachs as a control and supplement to the precipitin method, which it parallels, as a rule, in distinguishing human from animal blood. In cases where the precipitin reaction is only indicated in very great dilutions, the positive result of this reaction can be documented in a certain fashion by the absence of hemolysis, while the appearance of hemolysis indicates a negative result. Complement binding has rendered us exceptional service in scientific laboratory experiments where we have been dealing with pure protein solutions, and I myself have used

it with success in such experiments.

With regard to the utility of the method in forensic practice, it is extremely complicated and difficult to carry out, compared to the simple precipitin process. It is also extremely sensitive. This extreme sensitivity is its principal disadvantage in its practical application, since it can still be positive when $\frac{1}{100,000}$ or $\frac{1}{1,000,000}$ cc of blood protein are present. Even human sweat gives a positive reaction under certain conditions, a reaction that can be most portentous in testing a sweat-soaked, blood-stained shirt. With the precipitin method such is not the case, even with high-potency antiserum. We were also able to determine that extracts from different substrata (sacks, foot wrappings, wool stockings) can contain misleading material, which could give rise to error. In the face of its great complexity, its exceeding sensitivity, and the many sources of mistakes inherent in the method, which cannot be overlooked, it cannot be recommended in any case for forensic practice even in the hands of an experienced expert. In practice we do not need a more exact reaction than the precipitin method, conducted ac-
342 cording to our directions. In every forensic case, I demand that the usual precipitin reaction be carried out according to the well-known prescriptions. If the reaction is positive, then a proper control is superfluous since no doubts can arise. If the precipitin reaction is negative, but the complement binding positive, then in practice, where indeed it is frequently a question of the life or of the death of a man, a judgement ought not to be given regarding the provenance of the blood, if that judgement is based solely on the positive outcome of the complement binding (Uhlenhuth and Löffler).[36]

The same point of view is valid also for using the method in meat inspection, where it can be called upon as a confirmation reaction to a positive result of the precipitin reaction.

Our judgement regarding the significance of the anaphylactic reaction is similar, a reaction well-known in living animal bodies. Its specificity, and the fact that here the smallest traces of protein are enough to induce typical anaphylactic effects in a guinea pig, suggest the thought that it has practical value in differentiating various sorts of protein. The comprehensive experiments, which I conducted with my friend and coworker Händel, led us to the conclusion that in all cases where the precipitin reaction can be used, the anaphylactic reaction can also be employed and that the result of the precipitin reaction alone can be viewed as decisive. The anaphylactic reaction is so sensitive that animals sensitized with urine—as with sweat—react positively with human serum so that the urines of different animals can be distinguished from one another.[37] This sensitivity, however, is a warning that one must be extremely cautious. Because of the circumstantiality, the considerable technical difficulties, as well as the difficulty in giving a judgement based on the hypersensitive reactions which appear differently in individual animals, this process is not sufficiently reliable for forensic practice and is, therefore, unsuited for it. Moreover, it is completely unnecessary. Nevertheless, the worth of the method should not be ignored for pure, scientific experiments where it can be used to expand and confirm the results produced with the precipitin method. This was true, for example, in my attempts at differentiating frog protein from fish meat, as well as my work in distinguishing frog eggs, tadpoles, and frog-meat protein (p. 340). Here the results agreed with the results of the precipitin method mentioned earlier. Where the precipitin reaction fails or for technical reasons cannot be conducted, one can 343
with advantage bring in the anaphylactic reaction for scientific problems. I have thoroughly studied these conditions together with Händel. It was evident that the anaphylactic method also failed in distinguishing related blood types. Indeed, because of its sensitivity, cross reactions occur more frequently than with the precipitin reaction so that one cannot distinguish rats from mice, as one can with the precipitin reaction (p. 329). On the other hand, we were successful with guinea pigs in establishing definite anaphylactic symptoms with extracts from several Egyptian mummies of the twenty-sixth dynasty (600 BC) and the twenty-first dynasty (950 BC), as well as with Coptic and Peruvian mummies from the cemetery at Ancon when we administered the follow-up injection of human blood (see p. 335). Also, in the case of fourteen year-old human blood which we set in the sun for a long time until it had completely decayed, the precipitin reaction failed, but the anaphylactic method still succeeded in making guinea pigs hypersensitive. Moreover, our further experiments with cooked horse meat, shell-fish meat, and cooked sausage (Uhlenhuth and Händel), as well as with cooked, charred, and decayed bones, produced a positive result where the precipitin reaction and the chemical protein reaction had failed (p. 335).[38] Here, however, a strong cross reaction occurred in later testing of the guinea pigs with heterologous protein. Hailer[39] and later Bürger[40] determined in my laboratory that strongly disintegrated protein (proteolytic products) lost specificity in the anaphylactic reaction. Hailer used for his experiments material he had obtained by thorough cooking with steam or with acid, or through peptic and tryptic digestion, as well as meat extract and nutritive preparation, Bürger amino acids, pure albumoses, protamines and acid albumin.

"The protein molecules, characterized by a species-specific construction of unspecific building blocks, collapse at dissolution into their building blocks. When these are injected, they are capable of producing a sensitizing stimulus in the organism inoculated. This sensitivity is, however, not specific, that is, typical anaphylactic symptoms make an appearance after a second treatment with heterologous protein. This nonspecificity of the resulting sensitivity even appeared when protein which was still coagulatable (species specific) was present in the solution used in pretreatment along with the proteolytic products" (Hailer). Finally, it is also an interesting fact, established by my students, that organs preserved for a long time in alcohol, can sensitize 344
guinea pigs, but only when finely ground organ material is introduced into the animal's body subcutaneously or intramuscularly (Dold and Aoki),[41] whereas they were unsuccessful with extract (Kodama).[42] Thus Klabe[43] was still able to produce positive, partially specific reactions with alcohol preparations 25, 38, 41 and 50–60 years old.

We also extended our experiments to include plant oils and fats, where precipitin reactions are also ruled out for technical reasons. Anaphylactic symptoms appeared at the follow-up test with the corresponding native plant protein in guinea pigs sensitized to raw linseed oil, colza oil, almond oil, and coconut butter, although these symptoms were not definite in all cases. Adulterations of animal feed with *Ricinus* seeds, field mustard, and corn-cockle can also be identified in this fashion (Schern).[44] We produced similar reactions to these with animal fats (butter, lard, beef-suet, neat's foot oil) by a second injection with the homologous serum, whereby the animals, sensitized to butter, reacted to the follow-up treatment with raw and cooked milk as well as to cattle serum. The symptoms were not always so convincing in these experiments that delivering a final judgement was possible in every case. Here great caution was demanded. I must at this point give up any closer discussion of all the other attempts at differentiating human and animal hair, skin, protein of organs (lenses), hemoglobin, and sexual proteins, and I refer the reader to the relevant works.[45]

I come now to the conclusion. I hope that in considering my personal experience and some of the results of research, I have succeeded in giving an overview of the growth and development of the biological differentiation of protein. I especially wanted to emphasize the path and the thought processes which led me to discover the method of recognizing and differentiating animal from human blood.

It should emerge from my explanation that the biological
345 process of protein differentiation has achieved fundamental importance not only for legal judgements and forensic medicine in all national states, but has also contributed to the study of animal taxonomy, of evolution, and of descent by means of its conspicuous identification of blood relationships. It has also furthered research on epidemological relationships in infectious diseases carried by blood-sucking insects. Moreover, it has proven itself indispensible for every-day meat inspection and control of food products (adulteration) and is prescribed by law. Finally it has rendered invaluable service in solving purely scientific questions in the areas of physiology, pathology, clinical medicine, anthropology, zoology, botany, and other branches of natural science.

In an effort to structure the precipitin reaction so that it is as reliable and as faultless as possible, I have worked out through technical directions and prescriptions to eliminate sources of error. By considering also the complement-binding and anaphylactic reactions, I pushed the precipitin method to the ultimate limits of its amazing capability. In my opinion one cannot contemplate any further sophistication with our present-day methods. Such sophistication would scarcely be necessary for forensic practice, since the precipitin method has shown that in the hands of an experienced expert, it has grown to meet all demands made of it. [The remainder of page 345, through page 348, consists of a lengthy Appendix, which discusses in detail the rules and regulations governing the state-controlled testing and quality control of antisera, and so forth. This appendix has been omitted from the translation.]

Notes

1. Uhlenhuth: *Dtsch. Med. Wochenschr.*, 1901, No. 7
2. For detailed information, see the book by Uhlenhuth and Weidanz, cited in footnote 4
3. Uhlenhuth and Beumer: *Z. Medizinalbeamte,* 1903, No. 5/6
4. Uhlenhuth: *Das biologische Verfahren zur Erkennung und Unterscheidung von Menschen- und Tierblut, sowie anderer Eiweisssubstanzen und seine Anwendung in der forensischen Praxis. Ausgewählte Sammlung von Arbeiten und Gutachten.* Jena: Gustav Fischer, 1905—Uhlenhuth and Weidanz: *Handbuch der Technik und Methodik der Immunitätsforschung von Kraus und Levaditi,* Bd. 2, p. 731, 1908—*Praktische Anleitung des biologischen Eiweissdifferenzierungsverfahrens mit besonderer Berücksichtigung der forensischen Blut- und Fleischuntersuchung, sowie der Gewinnung präzipitierender Sera.* Jena: Gustav Fischer, 1909; sowie entsprechende Handbuchartikel, Uhlenhuth und Steffenhagen: *Handbuch der pathogenen Mikroorganismen* von Kolle, Kraus, Uhlenhuth, 2nd ed., 1913, Uhlenhuth und Seiffert, 3rd ed., 1928—Uhlenhuth: *Über die biologische Eiweissdifferenzierung unter besonderer Berücksichtigung der forensischen Blut- und Fleischuntersuchung.* Leipzig: Akademische Verlagsgesellschaft, 1914
5. One should use uniformly thick, appropriately clean, small test tubes (precipitation test-tubes according to Uhlenhuth) with a flange, test-tubes which can be hung in a fitting rack.
6. Manteufel and Beger: *Z Immunitaetsforsch.* **33** (4/5), 1921
7. That is, a dilution of serum.
8. Uhlenhuth: *Arch. Kriminol.* **106** (5/6), 1940, and **109** (1/2) and **110** (3/4), 1942
9. Birch, beech and fir trees do not produce a false reaction.
10. Merkel: *Z. Aerztl. Fortbild.*, 1909, No. 19
11. Directions of the Federal Council for the Combatting of Cholera
12. Uhlenhuth and Beumer: *Z. Medizinalbeamte,* 1903, No. 5/6
13. *Veröffentlichung der Kaiserlichen Gesundheitsamtes, 1903,* vol. XXVII, 1, 42
14. *Works from the P. Ehrlich Institut and the Georg Speyer-Hause,* vol. 47, p. 28, Jena: Gustav Fischer, 1948
15. Nuttall: *Blood Immunity and Blood Relationship.* Cambridge: University Press, 1904
16. Mollison and v. Krogh: *Anthrop. Anz.*, 1937, no 3/4
17. Ehrhardt: *Die Verwandtschaftsbestimmugen mittels der Immunitätsreaktion in der Zoologie und ihr Wert für die phylogenetischen Untersuchungen,* Diss. Rostock, 1929
18. Mollison: Serodiagnostik als Methode der Tiersystematik. In *Abderhalden's Technik der biologischen Arbeitsmethoden,* Abt. IX, Teil 1, Heft 3, 1923
19. Uhlenhuth: *Z. Immunitaetsforsch.* **104** (2/3), 1943
20. Uhlenhuth: *Arch. Kriminol.*, in press
21. *Zuchtbuch für deutsche Schafterhunde,* vol. XXXI, No. 54984
22. Uhlenhuth, Weidanz and Wedemann: *Arb. Reichsgesdh.amt.* **28,** Heft 3
23. *Zbl. Dtsch. Reich* 1908, 60

24. Uhlenhuth and Weidanz: *Praktische Anleitung zur Ausführung des biologischen Eiweissdifferenzierungsverfahrens*, p. 161, Jena: Gustav Fischer, 1909.
Uhlenhuth: Die serologischen Untersuchungsmethoden von Fleisch- und Wurstwaren, Eiern, Fischen, etc. In *Handbuch der hygienischen Untersuchungsmethoden of Gottschlich*, p. 815, Jena: Gustav Fischer, 1927
25. Steffenhagen and Clough: *Klin. Wochenschr.*, 1910, No. 46
26. Uhlenhuth and Haendel: *Z. Immunitaetsforsch.*, 1910, 4, no. 6
27. Uhlenhuth: Festschrift für Schwalbe. *Z. Morph. u. Anthrop.*, 1914, 18
28. Zimmermann: *Dtsch. Med. Wochenschr.*, 1931, No. 6
29. Uhlenhuth: *Dtsch. Med. Wochenschr.*, 1931, No. 42
30. Uhlenhuth: *Dtsch. Med. Wochenschr.*, 1903, No. 5
31. Uhlenhuth: *Festschrift R. Koch* (60th Birthday, December 11, 1903). Jena: Gustav Fischer
32. Mezger, Jesser and Volkmann: *Dtsch. Z. Gesamte Gerichtl. Med.* **21**, No. 1
33. Uhlenhuth-Wurm: *Z. Immunitaetsforsch.* **96**, No. 2 (1939)—Hsiü: *Z. Immunitaetsforsch.* **98**, No. 5 (1940)
34. Uhlenhuth: *Dtsch. Med. Wochenschr.*, 1905, No. 14—Uhlenhuth-Remy: *Z. Immunitaetsforsch.* **79** (1933); **82** (1934); **85** (1935); **88** (1936); **92** (1938)
35. Uhlenhuth: *Kartoffelkäferforschung und bekämpfung*. Aulendorf: Edttio Cantor, 1948
36. Uhlenhuth and Löffler: *Klin. Jb.* **19** (1908)
37. Uhlenhuth and Händel: *Z. Immunitaetsforsch.* **4**, No. 4 (1910)—Rhein: *Z Immunitaetsforsch.* **19**, No 3. (1913)—Scheidin: Diss., Strassburg, 1914
38. Uhlenhuth-Haendel: *Z. Immunitaetsforsch.* **4**, No. 6 (1910)—Steffenhagen-Clough: *Klin. Wochenschr.*, 1910, No. 46
39. Hailer: *Abr. ksl. Gesdh.amt.*, Berl., **47** (1914)
40. Bürger: *Z. Immunitaetsforsch.* **33**, No. 2 (1914)
41. Dold and Aoki: *Z. Hyg.* **75** (1913)
42. Kodama: *Z. Hyg.* **74** (1914)
43. Klabe: *Arch. Tierhk.* **44**, No. 3/4 (1918)
44. Schern: *Arch. Tierhk.* **36** (1910)
45. Uhlenhuth and Händel: *Z. Immunitaetsforsch.* **4**, No. 6 (1910)—Uhlenhuth: Über die biologische Eiweissdifferenzierung unter besonderer Berücksichtigung der forensischen Blut- und Fleischuntersuchungen. *Nahrungsmittelchemie in Vorträgen*. Ed. by W. Kerp. Leipzig: Akademische Verlagsgesellschaft, 1914—Clough: *Arb. ksl. Gesdh.amt.*, Berl., **31**, No. 2 (1911)

Concerning a New Forensic Method to Differentiate Human from Animal Blood*

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/187 /188 To differentiate between human and animal blood is a problem which frequently confronts the forensic expert. If a case involves relatively fresh material, so that the morphological constituents, especially the red blood cells are still intact and a microscopical identification can be achieved with the help of a suitable solvent, then the problem is easily solved. It is then possible to diagnose human blood by measuring the size of the formed elements. But if, as usually happens, the case involves material which has dried for a considerable time on foreign bodies, on cloth, tools, walls, dishes, etc., so that the blood corpuscles, either through natural or artificial processes, have completely or to a great degree been altered in their form or completely destroyed, then it is extremely difficult to diagnose with any certainty whether such stains are of human blood or not. Indeed, in many cases the forensic experts admit that it is impossible, even with the use of blood crystal formation, to produce the desired identification. A process which would enable one, even in cases where the material was old and dried out, to determine in an unequivocal and easily executed manner whether that material came from human blood or not, must be viewed as a major step forward for forensic medicine. We have recently been busy working out such a method on which we wish to report here.

This new process grows out of Bordet's experiments on hemolysins and precipitins. Bordet demonstrated, for the first time in a systematic manner, that when red blood corpuscles of an alien animal species are introduced into the serum of an animal pretreated with those same blood corpuscles, specific substances appear which act upon the blood of the first sort in a certain fashion, and, indeed, some of these substances agglomerate these certain blood corpuscles (agglutinins) and some bring about their dissolution (hemolysins). Bordet was able to show further that these substances were specific, i.e. they acted only upon the blood which had been used for the injection. For example, a rabbit, pretreated with injections of guinea-pig blood, shows in its serum an increased presence of agglutinins and hemolysins only with regard to guinea-pig blood and not to any other type of blood. Bordet pointed out further that the reactive capacity of the animal organism to the introduction of foreign animal substances goes even further, and he showed that after the injection of certain animal fluids, new reaction products make their appearance in the serum.

Thus, he showed that, after the subcutaneous application of cow's milk to rabbits, the serum of these animals, when mixed with cow's milk, precipitated its casein (lactoserum). Tsistovitsch[2] and Bordet[3] were able to demonstrate further that also, when foreign blood serum is introduced, substances appear in the blood in the case of many animals, substances which precipitate the protein bodies of the serum used for injection. Nolf[4] repeated and confirmed these experiments. He was able to verify that the precipitated protein bodies in the blood serum were the globulins. In connection with these experiments, the animals were injected with still other types of animal protein, and the appearance of such reaction products was observed in many of them. Thus, after Myers[5] had introduced peptone, serum globulin, and crystalline albumin, and Uhlenhuth[6] egg albumin from hens' eggs and those of other birds, both saw substances appear in the serum of the animals pretreated with these proteins, substances which precipitated the corresponding protein types.

Our own experiments in this field began when we tested whether the substances which formed in the serum after the injection of animal fluids were of a strongly specific nature, i.e., whether they acted only on the fluid containing the protein which had been used for the injection. To this end, we first examined serum extracted after injections with milk. Just as C. Fisch[7] was able to do independently of us, we could demonstrate that substances appeared in the serum after injection which precipitated only the casein of cow's milk, but not that of goat or human milk, and so forth.[8] Accordingly, one of us recommended at the previous Congress for Internal Medicine[9] application of this new method to the special differentiation of different protein substances. From there we transferred our energies to working out a specific, forensic method, based on these principles, for differentiating human blood from other sorts of blood. We tried this first by using the agglutinins and hemolysins. We

*Translation of: "Ueber eine neue forensische Methode zur Unterscheidung von Menschen- und Thierblut."

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pretreated rabbits with defibrinated human blood, and then observed whether it was possible to make a diagnosis with the aid of the agglutinins and hemolysins, which formed in the serum of the pretreated animals. It soon turned out, however, that this process was not useful in practice, since the effects of the agglutinins and hemolysins were apparent when there was still a large number of preserved, red blood cells present in the human blood to be tested, in other words, when the blood was relatively fresh. Accordingly, we decided to use for the specific method, not the hemolysins and agglutinins which appear after injections of defibrinated blood, but rather the precipitins, which appear after injections of cell-free serum, whose specificity we had already demonstrated with lactoserum. Since, as we observed above, the precipitins[10] acted on the dissolved protein bodies of the blood, the globulins, and not on the structural elements as do the hemolysins and the agglutinins, whenever this first method is employed for diagnosis, the test object could be much older, and so altered that no morphological elements were present. The previous experimental methods were not capable of identifying older material. We proceeded in this manner: We undertook to treat rabbits with five to six subcutaneous injections each of 10 cc of cell-free, human blood serum at approximately two-day intervals. Approximately six days after the last injection, the animals were bled to death. They had done well under this treatment. The blood was placed on ice to separate out the serum. If one now adds ½ cc of this rabbit serum to a solution of human serum, diluted with physiological saline solution, or adds it to a dilute, laked solution of human blood produced with distilled
/189 water, an intense, cloudy precipitation appears almost immediately at room temperature, and even more intensely in an incubator at a temperature of 37°. A further question now was whether this precipitation was strongly specific, i.e., whether it occurred only when mixed with solutions containing human blood. To test this, we added serum from rabbits, which had been treated with human serum in the manner presented above, to the laked blood of all the animals which we encounter in daily life, as far as these were available to us, to blood from mammals, birds, fishes, altogether twenty-three different animals.[11] We then discovered that, in fact, the serum of rabbits, injected with human serum, acted in a extremely specific manner, i.e., it produced precipitation in no other blood type outside of the human, with one exception, represented by ape blood. In the laked blood of this animal we also obtained a precipitation after adding the serum of the pretreated rabbit, although only after a rather long time and to a lesser degree. This result is also of general scientific interest, in that it shows us that, in fact, the protein bodies of the ape are very close in their constitution to those of humans. For the exclusively practical goals of forensic medicine which we have pursued, this circumstance ought to cause no serious concern, since under the conditions in our land, blood stains of ape blood will scarcely come into question. Our results up to that point were still not sufficient to recommend the method at that time in practice. We had to see first whether the effect of our serum was still clearly visible, when the material to be tested was not fresh, as it had been in previous experiments, but older and transformed by time. Above all, we needed to know the thing which was most important in practice, whether in the case of such old material the process was still specific, i.e., whether or not, somehow, in old and dried types of animal blood, precipitations are produced by the specific rabbit serum, where this does not happen with fresh, animal blood types. Accordingly, in the month of October in that year we set out blood stains from humans and from all other animal species mentioned, on linen cloth; some we put on tools, for example, on a knife. These stains we produced artificially. We let these objects lie without any special care. Then, in the following January, after approximately three months, we extracted with 5 to 6 cc of saline solution one or more blood stains, each about the size of a dime, from all twenty-four test samples. These stains had, in the course of time, been transformed, and had turned brown as a result of the formation of methemoglobin. We then obtained a dirty-brown, cloudy liquid which we rendered completely clear by filtering through a paper filter. The solution must, without exception, be absolutely clear, if the result of the reaction is to be certain. Especially in the case of stains some of which from bird or fish blood it is quite often necessary to filter the fluid several times in order to remove the turbidity in the wash solution, a condition which arises from the presence of concentrated curdles, i.e., fatty impurities. We now filled each test tube with four to five cc of this solution of extract of blood stains, added to each tube ½ cc of serum from a rabbit, pretreated with human blood, and put each sample in the incubator at 37°. After twenty minutes, and occasionally sooner, the test tube in which the solution, extracted from the stain of human blood was contained, displayed a definite cloudiness; all the others remained clear, with the exception of the tube with ape blood, which showed a faint, incipient turbidity. After another fifteen minutes a definite, flocculent precipitation had been deposited on the bottom of the tube containing human blood. At this point we need not especially emphasize that the addition of normal rabbit serum, serum from a rabbit not pretreated with human blood, naturally did not produce any turbidity at all in human blood, i.e., in the solution extracted from a stain of human blood. Thus, the method enabled us easily to reach a certain decision with these blood remains, even in the case of old, dried blood substances, as to whether we were dealing with human blood or not.

For the practical application of the method we recommend the following. One should inject rabbits[12] subcutaneously five to six times in the manner described above with 8 to 10 cc of human serum. Six days after the last injection, one bleeds the animals by opening the carotids, and then places the quantity of blood extracted in the icebox to separate out the serum.[13] The experiment then proceeds in the following manner. The material to be tested is extracted as completely as possible in six to eight cc, occasionally more, of physiological saline solution. This solution, when filtered

to complete clarity, is divided into two equal portions which are poured into two sterile test tubes. To one tube is added ½ cc of the serum of a rabbit pretreated with human serum; to the other is added, as a control, ½ cc of normal serum from the same animal species, in this case, from a rabbit which was not injected with human blood. Then, four to five cc of a blood solution, laked with distilled water, or of a blood stain extract of another animal species, for example from pig blood or sheep blood, is placed into a third tube to serve as a control. To this tube is then added 0.5 cc of serum from a rabbit pretreated with human serum. All three test samples are set at a temperature of approximately 37°. If an apparent turbidity and the formation of precipitation begins within ½ to 1 hour in the tube which contains the suspected material submitted to forensic testing, and to which was added the serum of the pretreated rabbit, while the two others remain unchanged in their completely clear state, then one can make the certain diagnosis that the substance in question comes from human blood, so long as the anamnestic reaction of ape blood can be ruled out in the case of the test substance.

/190 A few weeks ago, we demonstrated the method we have described here, a method which we have often carried out, to the Director of the local Royal Educational Institute for Government Pharmacology, Professor Strassmann. To what extent this method will meet the requirements of each case, which vary so greatly from one case to another, and how far the method can be perfected to meet these needs, are questions which lie far outside our area of research. Thus, in the most cooperative way, Professor Strassmann and Dr. Ziemke of the aforementioned Institute declared themselves ready to study and to develop the method further in regard to these stated goals.

References and Notes

1. Follows a demonstration given by the authors at the Physiological Society in Berlin on February 2, 1901
2. *Annales de l'Institut Pasteur*, 1899
3. Ibidem, 1899
4. Ibidem, 1900
5. *Centralbl. f. Bacteriol.*, 1900, vol. 28, No. 8-9
6. *Deutsche Med. Wochenschr.*, 1900, No. 46
7. Studies on Lactoserum and on Other Cell-Sera. *St. Louis Courier of Medic.*, February, 1900
8. Cf: *Deutsche Med. Wochenschr.*, No. 30, 1900, Vereinsbeilage, p. 178 and *Ztschr. f. Hyg.*, vol. 36, 1, 1901
9. Cf: A Wassermann, Verhandlungen des Congresses für innere Medicin, 1900
10. Note added during correction: The most recent number (No. 6) of the *Deutsche Medizinische Wochenschrift* contains an article by Uhlenhuth in which the author, proceeding from the same principle, succeeds in using the precipitins for the differential diagnosis of human and animal blood.
11. Blood from the following animals was used: donkey, goat, cow, ox, calf, sheep, pig, dog, cat, ape (a small Pavian), guinea pig, rabbit, house mouse, house rat, goose, duck, pigeon, sparrow, eel, pike and tench.
12. For some time we have been testing whether, after injections of human blood, the precipitating substances also appear in the serum of other animals, larger than rabbits, since this would naturally be more convenient in practice. Thus, we are presently treating a goat with injections of human serum.
13. The human serum necessary for injection is easily obtained in these quantities from any larger hospital, where bleeding cups are often applied for therapeutic purposes. It is even easier and more convenient to get it from maternity hospitals by pressing out the placentas. Moreover, we ought to test whether the same substances appear in the serum of pretreated animals after the injection of larger quantities of human pleural transudates, or abdominal transudates, containing the same protein substances as the human serum. The action of the serum producing the reaction is stronger the sooner it is used after being removed from the rabbit. We are convinced that serum which is kept on ice still produces a reaction in a certain and prompt fashion fourteen days after its extraction. Thus, it is possible, if necessary, to dispatch the serum from a central station.

A Process for the Forensic Identification of the Origin of Blood. (Fixation of Hemolytic Complement)*

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1388 The eminently important question for forensic medicine, determining the origin of blood stains, has been solved from an unexpected direction as a result of Uhlenhuth's experiments and those conducted independently by Wassermann and Schütze. The so-called biological method for forensic blood identification is known to rest on the capability of the serum from animals pretreated with certain types of blood to produce a specific precipitation in a diluted solution of that very blood type. Although the trustworthiness of this process has been thoroughly tested in theory and practice over the last few years, and we ourselves arranged many experiments in which it proved itself completely, still we cannot hide from ourselves that the technical difficulties in practice are often great and that there exists a lively desire for a control of the experiment in view of the grave importance of the results. Therefore, we would like today to publicize a method of forensic blood differentiation which we have tried to advance in close connection with the progress of serum research. We seem to have met with the best success.

Moreschi's beautiful work, conducted under R. Pfeiffer's direction, stimulated us to undertake our experiments.[1] Moreschi reported about "a type of anti-complement serum effect which forms as the result of the cooperation of two substances, the first present in the serum of the pretreated animal, the second in the serum of the animal species (or in that of a closely related species) whose serum was used for pretreatment." Without wishing at this point to delve further into the theoretical meaning of this very interesting observation, we wish only to mention that this involves the same phenomena which Gengou described a few years ago, and is connected with the presence of amboceptors which sensitize protein bodies in the blood of animals pretreated with serum protein, etc.[2] Of special importance for our goal was the fact that "the very smallest quantities ($\frac{1}{100,000}$ cc) of normal serum sufficed to produce the anti-complement effect." This fact encouraged us to apply the phenomenon described by Moreschi to the identification of the smallest quantities of human blood, which are necessarily the givens in forensic practice. Our experiments based on Moreschi have met our expectations.

The experiments, which convinced us of the usefulness of this method are the following. To carry out the experiment, for every 1 cc of 5% sheep-blood suspension 0.0015 cc of amboceptor (the serum of a rabbit, pretreated with ox blood, that reacts also with sheep blood) and 0.05 cc of fresh guinea-pig serum as complement are used. The sheep-blood corpuscles used in this system are completely dissolved by the combined action of amboceptor and complement. The serum of rabbits pretreated with human serum served as an antiserum.The addition of 1 cc of this antiserum does not influence the hemolysis. A disturbance, i.e. an inhibition of hemolysis, however, was to be expected after the above steps were followed, if a trace of normal human serum was present. On the other hand, the hemolysis would of necessity be promptly resumed, if other types of normal serum were present. The experiment confirmed the correctness of our assumption, having been carried out in the following manner: 0.1 cc antiserum and 0.05 cc complement and varying amounts of different normal sera (each brought to a volume of 1 cc in a saline solution) are mixed and are left standing at room temperature for one to two hours. Then one adds 1 cc of 5% sheep blood and 0.0015 cc amboceptor and allows the mixture to stand for one to two hours at 37°. The results of the experiment, one of many similar ones, are shown in the following table:

As the table shows, only human and ape sera effect a cessation of hemolysis; all other types of sera, which were introduced, proved to be ineffective. It should not cause amazement that the sera of humans and apes behave essentially in an analogous fashion, when we consider the close relationship between these animals, though as a rule the latter produces a clearly weaker reaction. If we do not consider the common effect of human and ape blood, we are dealing with a phenomenon which is specific for human serum as the experiments show, a phenomenon which is so extremely fine that it is easily capable of identifying $\frac{1}{10,000}$ cc, and almost always $\frac{1}{100,000}$ cc, and occasionally even $\frac{1}{1,000,000}$ cc of human serum. The extreme fineness of this method suits it especially for the forensic differentiation of blood, which process involves the identification of the smallest traces of blood. Moreover, we were able most easily to differentiate the blood of human provenance from among extracts of blood stains dried three months earlier on linen, blood stains

Amounts of Normal Serum cc	Hemolysis which was begun by the addition of serum of:							
	human	ape	rat	pig	goat	rabbit	ox	horse
0.01	0	0	COMPLETE	COMPLETE	COMPLETE	COMPLETE	COMPLETE	COMPLETE
0.001	0							
0.0001	0	MODERATELY COMPLETE						
0.00001	a trace							
0.000001	complete							
0	complete							

which came from sheep, chickens, rabbits, guinea pigs, humans, oxen, and horses, and these stains were in a dilution in which the precipitating serum was scarcely able to produce a reaction.

Whether this method is superior to the one outlined by Uhlenhuth and Wassermann, only more experiments and practical experience can show. In any event we can immediately recommend it as a control and as a supplement to the precipitation method, and we can assert that it is equally as accurate as that method.[3] Moreover, it has certain advantages. First, the failure of hemolysis is a more apparent criterion than precipitation formation which quite often is
1389 only indicated faintly.[4] A further advantage of this system is that there is no need of clarifying a large quantity of solution for the reaction. For the Wassermann-Uhlenhuth method, this clarifying process, which is sometimes quite difficult, is absolutely necessary. In addition, the extraction of antisera to use in our experiment is easier. It is well known that it is very time consuming to obtain a high-potency serum suitable for the Uhlenhuth method, since the animals display the greatest individual variations in their ability to build precipitins. Thus, from a rather large assortment of pretreated rabbits, only a very few produce a usable serum. On the other hand, we have at our disposal examples of antisera which caused precipitation at their limit in a solution of human serum with a strength of 1:100 or 1:1000 but were capable of recognizing $\frac{1}{100,000}$ cc of human blood by using the method described. Finally the use of antisera is less restricted, in that the frequent presence of serum opalescence, which renders the observation of precipitation very difficult, is irrelevant for recognizing the hemolytic effect.

On the other hand, it might be possible that the results of the hemolytic method of identification could experience interference if unspecific, inhibiting substances are present in the objects submitted for testing. This obstacle, though unlikely must still be considered. It can be easily overcome by destroying the inhibiting effect of the human serum by cooking. In doubtful cases, a control for the test would be present in the form of a cooked solution. Regarding the technique for the process, execution of the experiment must at first be limited to those laboratories in which on-going results regarding the hemolytic effect have been collected. Presumably every hemolytic combination can be used as a reagent. It is only due to a circumstantial accident, their availability at the time, that we used the serum of a rabbit pretreated with ox blood, a serum reacting with sheep blood, as the amboceptor and guinea-pig serum as the complement. Further experiments should show whether still more appropriate combinations can be discovered.

At this point we want to say only a few words concerning the active mechanism which causes the reaction. We do not consider it essential, as Moreschi believes, that the binding of the complement and its fixation with the blood corpuscles, laden with the amboceptor, is caused by the precipitin produced by the common action of human serum and the antiserum. Rather we incline much more toward the interpretation already put forth by Gengou, that the complement fixation represents the effect of protein bodies of the blood which have been sensitized and dissolved by the specific amboceptors.

From this point of view the phenomenon which we have described can be explained without further ado. We are dealing then with the same principle which Ehrlich and Morgenroth first recognized, namely that the amboceptor, in and of itself, is incapable of binding the complement, that it must undergo an increase of its avidity by anchoring itself to the susceptible substrate so that it then is able to bind to the complement. Bordet and Gengou used this function of firmly anchored amboceptors to identify indirectly amboceptors of cellular elements in the serum. Gengou went an important step further when he transferred the effect of the antisera to the dissolved protein substances and demonstrated that one can be certain of the presence of amboceptors by means of the complement-binding function of protein solutions digested by specific antiserum. If one divides the antibodies of cells into agglutinins and amboceptors, one will be justified in differentiating protein antibodies into precipitins and amboceptors, so long as their identity has not been proven. Consequently we must temporarily base the complement-binding function of protein bodies of the blood, laden with specific amboceptors, on the mechanism of the process as described in Ehrlich and Morgenroth's interpretation. Their

*Translation of: "Ein Verfahren zum forensischen Nachweis der Herkunft des Blutes (Ablenkung hämolytischer Komplemente)."

in *Berliner Klinische Wochenschrift* 42 (44): 1388–1389 (1905).

important conclusion, based on principles derived by hard laboratory work, shows how the apparently impractical and theoretical study of immunity reactions has again produced results which, when applied in practice, have proven themselves of the greatest usefulness.

Strictly speaking, this method is naturally related just as little to the identity of the blood *qua* blood as is the Uhlenhuth method. It rather makes possible only the determination of its origin. It is a method to differentiate protein types of specifically varied provenances. Therefore, the identification of blood as such must be furnished separately whenever the method is employed.

We cherish the hope that the fixation method will prove itself in later tests and will constitute a welcome increase in our aids for forensic blood diagnosis.

Notes

1. C. Moreschi. Zur Lehre von den Antikomplementen. This journal, 1905, no. 37
2. Gengou. Sur les sensibilisatrices des sérums actifs contre les substances albuminoides. *Ann. Inst. Pasteur*, Paris, Vol. XVI, 1902
3. In forensic practice it is often important because of the small amount of material at hand that one unite both methods into one experiment. One first sets up the precipitin reaction. After noting the results the complement test is appended; the mixture remains standing for a while, and then the blood and amboceptor are added.
4. Moreover, in a court case it is often desirable to be able to display the evidence at the debate. This can be done most easily by centrifuging the undissolved blood corpuscles and by preserving the residue by adding a suitable preservative. The different color of the solution (red-colorless) will represent a marked difference even for the layman.

The Forensic Differentiation of Blood Using the Antihemolytic Effect. (Second Communication) *

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/67 In issue number forty-four of this journal we recommended for forensic practice a method to identify the origin of blood by means of the fixation of hemolytic complements, a method based on the experiments of Gengou and Moreschi.[1] Since then we have collected more facts concerning the suitability of the method and its technique. Proceeding
/68 from the fact that normal hemolysins, and those produced for immunity, display their effects according to the same mechanism, we have employed the hemolytic effects of normal serum to produce the reaction instead of the immune sera we used at first. Thereby the arrangement of the experiment can naturally be greatly simplified, in that in the case of normal hemolytic serum the two necessary reagents, the amboceptor and the complement, are ready to use in one liquid, while these reagents must be added separately when using artificially produced hemolysins. The hemolysin against sheep blood contained in normal rabbit serum has in our opinion shown itself to date to be the most appropriate, and this is for the following reasons. First, the rabbit is the customary laboratory animal, so that the extracting of serum should not create the least difficulty. Then, too, the hemolytic effect of different rabbit sera with respect to sheep blood is in general rather constant, so that one is not to a great degree dependent on the accidents of nature. Usually 0.25 to 0.15 cc represent the smallest doses which will lyse one cc of 5% sheep-blood cell suspension. Moreover, sheep blood is everywhere easy to obtain. If it should not be convenient to hire on a sheep at the testing center, entrusted with forensic blood differentiation, then the slaughter house can surely make the blood available. One can easily preserve this blood on ice for up to four days. Accordingly, the order of the experiment is as follows. First, in a pre-test, a completely lysing dose of rabbit serum must be established. In the experiment which is described below, this amounted to 0.25 cc. Now 0.25 cc of rabbit serum is mixed with the liquid to be tested for human blood and with the antiserum [2] (in our example 0.01 cc). The mixture is left to stand for one hour at 37°; then follows the addition of one cc of 5% sheep blood. Again the mixture stands for one hour at 37°. The reading can be taken after two hours. Failure of hemolysis indicates the presence of human blood. In a control experiment, set up in the same way, save that the solution to be tested for human blood is left out, the hemolysis must take place. A model experiment is shown in the following Table 1. Various amounts of human serum served as test objects.

Table 1

Amount of human serum cc	Amount of hemolysin (normal rabbit serum) cc	Amount of antiserum cc	hemolysis of 1cc of 5% sheep blood
1/1000	.25	.01	0
1/10,000	.25	.01	0
1/100,000	.25	.01	0
1/1,000,000	.25	.01	moderate
1/10,000,000	.25	.01	strong
1/100,000,000	.25	.01	complete
0	.25	.01	complete

As the table shows, the hemolysis is inhibited completely by the interference of $\frac{1}{100,000}$ cc of human serum, but even the presence of 0.000001–0.0000001 cc of human serum still reveals itself by clear alterations. Thus, the precision of the method leaves nothing to be desired. It seems to us that the small amount (0.01cc) of related antiserum is also noteworthy. There is at times an advantage in using smaller quantities for setting up the reaction, since in many cases a certain amount of antiserum appears to correspond to an optimum effect. In practice, it turns out that every antiserum to be used must, in any case, be tested regarding its effectiveness and then can be used in the test. We recommend 0.0001 cc as the amount of human serum to be identified. We consider it necessary for the acceptance of an antiserum for forensic purposes that it can identify at least this amount of human serum. Such a predetermination on the antiserum is exceedingly easy. It represents a reproduction of the above experiment, except that here the amount of human serum remains constant while the amount of antiserum varies. In Table 2 we present the predetermination of conditions for the antiserum used in Table 1.

*Translation of: "Die forensische Blutdifferenzierung durch antihämolytische Wirkung. II Mitteilung."

in *Berliner Klinische Wochenschrift* 43 (3): 67–69 (1906).

Table 2

Amount of human serum cc	Amounts of hemolysin cc	Amount of antiserum cc	hemolysis of 1cc of 5% sheep blood
0.0001	0.25	0.15	a little
0.0001	0.25	0.1	a trace
0.0001	0.25	0.05	a trace
0.0001	0.25	0.025	0
0.0001	0.25	0.015	0
0.0001	0.25	0.01	0
0.0001	0.25	0.005	0
0.0001	0.25	0.0025	0
0.0001	0.25	0.0015	a little
0.0001	0.25	0.001	moderate
0.0001	0.25	0.0005	strong
0.0001	0.25	0	complete

The increase in the hemolytic effect when an excess of antiserum is used, an increase apparent from the table, is due to the hemolytic amboceptors of sheep blood which are still present in unactivated antiserum. The antiserum is extracted from a rabbit and must, therefore, also contain the normal amboceptors of such blood. Thus, an excess of hemolytic amboceptors results and, as we can report according to our relevant experiments, such an excess appears always to frustrate the demonstration of the Gengou-Moreschi phenomenon of the anti-complement effect.[3] One could easily remove the normal amboceptors of the antiserum causing the interference by absorption with sheep blood, but such measures seem superfluous for carrying out the reaction, since, as the table already shows, lesser amounts of antiserum are perfectly suited for our method. This is, moreover, a lucky circumstance, since it protects against too hastily wasting this valuable material. In order to provide an approximate basis for further experiments we would like to mention that according to our experiences with usable antisera, 0.02 cc represents as a rule the optimum quantity.[4]

69 We would like to recommend in any case the use of normal hemolysins as the best for practice. The technique is thereby made extremely easy, and any interference from any sort of disturbing antibodies is ruled out. It would, of course, be desirable to replace sheep blood with that of a smaller animal, although obtaining sheep blood is, in our opinion, not a serious difficulty. It must be left to further experiments to demonstrate whether other hemolytic combinations of blood and serum, extracted from laboratory animals, can be recommended.[5]

Concerning the relationship of our method to the tested Uhlenhuth-Wassermann reaction we can in essence only recommend what we have already presented when we described our first experiments dealing with hemolysins produced through immunization. Just as the biological precipitin method to identify the origin of blood represents the application of the important principle of protein differentiation discovered by Wassermann, so our process, strictly speaking, is based only on the identificaiton of protein. Regarding accuracy, our method is at least as reliable as the method using precipitation. Indeed, we do not want to neglect to mention that we have had a positive result of the fixation reaction, even when no preciptation formation could be detected. In any case the strength of the precipitation and that of the fixation capability do not stand in direct proportion.[6] Therein we can see the fundamental reason for the supposition, already expressed in our first work, that our method possibly involves a different class of protein antibodies which act as amboceptors in Gengou's sense.[7]

Be that it may, it seems to us that the fixation process ought to be included with the Wassermann-Uhlenhuth method in forensic practice. We are convinced that the forensic expert will declare it a welcome change to be able to reach his decision, one so full of responsibility, by basing it on two methods, which mutually control and supplement one another. We ourselves advantageously employed this combined test in two forensic cases which were handed over recently to the institute, and we considered it of special value to be able to base the identification of human blood on a positive result in both experiments.

The first case involved a small tree leaf on which were found a few blood stains. An extract of these was produced in the least possible quantity of saline solution. 1 cc of the solution served to set up the precipitation reaction when 0.1 cc of antiserum was used. A weak but clear turbidity ensued, and finally a precipitation formation. The remaining 0.2 cc of the extract was increased ten times in volume (2 cc) with physiological saline solution. This solution, diluted to one-tenth of its strength, was still usable in the fixation reaction. Increasing amounts of this solution were each mixed with 0.25 cc of rabbit serum (as a hemolysin) and 0.02 cc of the same antiserum which was used for the precipitin reaction. These mixtures are left to stand for one hour at 37°. Then, sheep blood is added. The same experiment is simultaneously repeated, but the antiserum is not added, in order to determine whether the solution to be tested has an anti-hemolytic effect in and of itself. Table 3 shows the result.

Table 3

Amounts of the 1 10 diluted extract solution cc	1 cc of 5% sheep blood, 0.25 cc of rabbit serum	
	a) 0.02 cc antiserum	b) 0.2 cc saline solution
0.5	0	complete
0.25	0	complete
0.15	strong	complete
0	complete	complete

As the table shows, 0.025 cc of the original solution still causes a complete inhibition of hemolysis. The fixation method was thus able to identify human blood in a fortieth of the amount used for the precipitin method.

The second case which we examined involved a few small blood stains found on a wooden hammer. This experiment was set up exactly in the same fashion as indicated for the first case. While in 1 cc of the extract obtained, the addition of the antiserum resulted only in a very weak, but nonetheless apparent reaction, 0.2 cc of the same solution still produced a total inhibition of hemolysis in the fixation

experiment.[8]

Our method has proven itself not only in a laboratory test, but also under the serious conditions of real practice. Thus, we think we can recommend it as most advantageous to include in the forensic blood test next to the officially recognized reaction of Uhlenhuth and Wassermann the method of complement fixation of normal hemolysins which we have presented.

Notes

1. M. Neisser and H. Sachs. Ein Verfahren zum forensischen Nachweis der Herkunft des Blutes. *Berlin Klin. Wochenschr.*, 1905, no. 44
2. As in the Uhlenhuth-Wassermann reaction, the antiserum is rabbit serum which comes from rabbits pretreated with human serum.
3. The cause of this phenomenon certainly lies in the fact that the need for complement to produce hemolysis becomes less when the amount of amboceptor increases so that traces of free complement are still available to produce the effect.
4. The cooperation of the normal amboceptor contained in the antiserum suggested to us to utilize simultaneously the normal hemolysins to sheep blood contained in the antiserum as hemolytic reagents. In actuality this is easily possible, and accordingly, the arrangement of the experiment follows these specifications. First, one determines the dose of antiserum which will completely lyse sheep blood. Then one allows a mixture of this amount of antiserum and of the solution which is being tested for human blood to stand for one hour at 37°. Then, sheep blood is added. One must weigh another consideration against the advantage of this simplified technique for daily practice. Since the hemolytic complements of sera become ineffective rather rapidly, the hemolysins contained in antiserum will lose their effectiveness under normal preservation conditions. But, when we conserved the antisera in a frozen state at −12°, the hemolysins were preserved. At least, when we tested five-month old antisera for its hemolytic action in sheep blood, we found that the sera possessed the normal hemolytic effect.
5. We have until now been able to fulfill this need only by calling upon amboceptors obtained in the immunization process. In this respect the combination—guinea-pig blood, specific amboceptor obtained from a rabbit, and normal rabbit serum as complement—has proven itself useful in our view. The advantages of this combination lie in the fact that all of the animal sera to be used in the experiment comes from rabbits. There is only the guinea pig as a second blood donor. The use of this second method is always convenient, whereas obtaining sheep blood could be impractical. Moreover, when a case involves the identification of the blood from one of three related animal species, sheep, goats, or oxen, then sheep blood must be avoided as a reagent in the interest of clean experimental conditions.
6. Recently A. Klein also reported relevant observations (*Weiner Klinische Wochenschrift*, 1905, No. 48).
7. Wasserman and Bruck (*Med. Klinik*, 1905, no. 55) support this view in a very interesting article which appeared while this study was at the press. In ingeniously arranged experiments they use the fixation process to differentiate bacterial extracts and to demonstrate that even old bacterial extracts produce the complement binding function when the corresponding immune serum is added, although these old bacteria, as opposed to freshly obtained extracts, cannot be precipitated.
8. Note added during correction: In the meantime we have also had the opportunity to participate in a forensic blood test in which the bloodstains in question were not of human blood, but of pig blood. The use of the fixation process gave the same diagnosis (pig blood positive, human blood negative).

Prof. Dr. Franz Josef Holzer 1903 1974
Courtesy Verlag Franz Deuticke and National Library of Medicine

Prof. Dr. Leone Lattes 1887 1954
Courtesy Prof. A. Fornari, Prof. S. Perugini and *Haematologica*

Prof. Dr. Vittorio Siracusa 1896
Courtesy Prof. Dr. A. Fiori

Section 4. Blood Grouping

Leone Lattes (1887–1954) was one of the best known medico-legal and general serologists in the first half of this century. He became involved in blood grouping in its early years, and even his earliest papers demonstrate a grasp of the subject that was not widespread at the time. His book *L'individualita del Sangue nella Biologia, nella Clinica e nella Medicina Legale* (1923) became a classic, editions of it being issued in German, French, and in 1932, in English. The Lattes papers indicate the early techniques, in which the agglutinin in the stain was sought for determination of the ABO group. Tests for isoagglutinin in blood stains are still referred to as "Lattes" tests. The 1927 paper recounts a number of his cases. This paper was written in German (he wrote papers in German and French, as well as in Italian). Lattes' obituary appeared in *Haematologica* 38 (11) in 1954. Siracusa's paper introduced the elution procedure for detecting agglutinogens in dried blood, and it discusses the so-called absorption-inhibition method as well. The two were used side by side in these studies.

Franz Josef Holzer (1903–1974) was a well-known medico-legal blood grouping specialist who studied in this country for a time with Landsteiner. His 1931 paper introduced an inhibition procedure for grouping bloodstains which was used for many years. In 1937, he discussed the secretor characteristic as a marker in forensic investigations. The 1953 paper reviewed the current status of blood grouping, especially in its medico-legal applications. Dr. Holzer spent much of his career at the University in Innsbruck.

On the Practical Application of the Test for Agglutination for the Specific and Individual Diagnosis of Human Blood*

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/310 The importance of the natural hetero-agglutinins for the specific diagnosis of blood, according to the method proposed by Marx and by Ernrooth, has been very restricted following the further works appearing on this subject. Without citing here works whose results are contained in the treatises, (see the chapter *Agglutination und Hämolyse* of Landsteiner in the *Handbuch der Biochemie* of Oppenheimer, and *Die forensische Blutuntersuchungen* of Leers), one can say that from all the research, it appears that the test for the hetero-agglutinins can be considered only a preliminary one to the precipitin test.

The limited significance of this test, on which many hopes were founded because of its simplicity, depends on two rules of reason. Contrary to the assertions of Marx and Ernrooth, the test can turn out negative, even when it is surely a matter of heterologous blood, either because it is a case of blood from very young individuals in which agglutinins do not exist (see Dungern, Halban, Landsteiner, Baecchi) or because the stains became dried, sometimes even for a brief period (Uhlenhuth, Martin) or finally it is because the stains were altered by some chemical or physical agent (Carrara, De Dominicis). Therefore, all authors up to now unanimously think that the unsuccessful agglutination of the human globules, in the presence of extracts of bloodstains, is not a valid reason for excluding its being heterologous blood.

/311 On the other hand, not even the positive outcome of the agglutination is valuable in demonstrating that it is a matter of heterologous blood, since the reaction can occur through the intervention of the isoagglutinins that one so frequently finds in the human blood. Therefore, the test of Marx-Ernrooth does not have any precise significance, in a practical way whether the results are negative or positive and it would be absolutely necessary to reject it.

However, the authors have proposed criteria, according to which it would be possible to distinguish isoagglutination from heteroagglutination. Thus they have argued the heteroagglutination is more rapid and more intense than isoagglutination, that the isoagglutinins are much more labile and likely to disappear in a very short time, that the heteroagglutinins, unlike the isoagglutinins, lose their efficacy in the presence of a serum of the same type as those of cells that are used in the test and that heteroagglutination is regularly accompanied by hemolysis. The existence of these differential characteristics was, however, not at all confirmed. From the works of Martin, Uhlenhuth, Landsteiner and Leiner, Moss, Baecchi, etc., it is clear that they have no objective foundation and that very often, isoagglutinins exhibit behavior perfectly identical to that of the heteroagglutinins.

The only real difference that exists between the two types of agglutinin is that the heteroagglutination occurs regularly whenever the individual supplying the human cells used in the test is of inconsistent type for isoagglutination, making the selection irregular and variable according to the cells used, until human cells are found that are refractory.

Baecchi, who was recently occupied with the value of the Marx-Ernrooth test for the specific diagnosis of blood, did not hesitate to propose applying it to the practice of forensic medicine. He proposed, namely, to follow the agglutination test with the extract of a stain, not only with one variety of human erythrocytes, but with several. In that case, if all these globules became uniformly or almost uniformly agglutinated, it would be possible, with great probability, to believe that the blood under consideration is of heterologous origin, while when there are conspicuous differences in the agglutinability of the cells, it would be possible to conclude that the stain is of human blood.

It would be necessary, nonetheless, to carry out the test on 312
a great variety of globules, since, doing it as this author does, on only four types, makes uniformity of behavior possible even for isoagglutination.

Baecchi, however, apart from his perfectly theoretical justification in recommending this test, leaves us uncertain about its practical applicability, and particularly, as to the number of the types of cells it would be necessary to test before arriving at a certain diagnosis. On the other hand, the multiplicity of tests serves to rob this technique of its greatest advantage, that is to say, of its simplicity and rapidity.

In order to eliminate the inductive uncertainties in the test of the heteroagglutinins by the possible presence of iso-

*Translation of: "Sull'applicazione pratica della prova di agglutinazione per la diagnosi specifica ed individuale di sangue umano."

in *Archivio di Antropologia Criminale Psichiatria e Medicina Legale* **34** (4 ser. **5**): 310-325 (1913).

agglutinins, it seems to me appropriate to confront the problem in its fundamentals, seeking to annul the action of the isoagglutinins, rather than to insist on the alleged differences of these two series of bodies. It is not possible at present to destroy the isoagglutinins in such a way as to preserve the possible heteroagglutinins, but the action of the latter can be demonstrated by recourse to a simple artifice. It is sufficient for this purpose to adopt for the reaction, not any already arbitrarily chosen human cells, but those that have been demonstrated to be refractory to isoagglutination.

All authors who have occupied themselves with this issue have noted that certain human cells are agglutinated by certain human bloods and others are not. Marx and Ernrooth also observed that certain varieties are not agglutinated by any human blood serum. The irregularity of the behavior of isoagglutination is then discussed. In reality, isoagglutination is not at all irregular, but it is characterized by certain rules, which serve to clarify the differences in behavior of single sera or single cells, and permit a choice to be made among them, when it is necessary to choose cells appropriately reactive for this diagnostic test.

From the works of Landsteiner, Langer, v. Dungern and Hirschfeld, Jansky, Moss, little known in the forensic camp, the results clearly show that isoagglutination is dependent upon the presence of certain specific groupings on the red cells, susceptible to reaction with corresponding agglutinins present in other serum, and thus manifesting the phenomenon of agglutination.

According to the above-mentioned works, the specific groupings of the human erythrocytes able to react with the
313 isoagglutinins are only two, denoted by the letters A and B; they can be present separately or coexist, or both can be lacking. Landsteiner expressed the rule that a normal blood never contains the agglutinins able to agglutinate its own erythrocytes (and, in reality, one finds autoagglutination only in pathological circumstances), and it contains instead agglutinins able to react with the groupings that it does not have. Thus, in conclusion, human bloods can be divided into four groups: 1) groups with cells containing the grouping A, with serum containing the agglutinin called β, able to agglutinate B cells; 2) groups with cells with structure B and agglutinin α; 3) groups with the cells having A & B structures and lacking isoagglutinins; 4) groups with non-isoagglutinable cells, that is to say, lacking A and B, and serum containing the two agglutinins α and β.

This division issues clearly from the above-mentioned works, but it was expressed in this form only in the works of v. Dungern and Hirschfeld.

And very notable is the fact that the percentages of the single types, quoted, independently of one another, by v. Dungern and Hirschfeld in Heidelberg, and by Moss in Philadelphia, were just about identical.

Moss, in 1600 tests carried out on the sera and cells of one hundred individuals, did not find any exceptions to the grouping scheme indicated here.

Among the four groups listed above it is the fourth that matters to the problem occupying our particular interest.

The red cells that belong to it would have no affinity for the isoagglutinins. Therefore they would have to constitute an excellent reagent for the test of Marx-Ernrooth, for the direct interpretation of just what is necessary to eliminate the action of the isoagglutinins.

In order to find these cells easily I followed the pathway indicated by Landsteiner's rule. According to this, the bloods containing cells with no isoagglutinable groups are those whose serum contains the two agglutinins α and β.

These bloods, according to the figures of v. Dungern and Hirschfeld and of Moss, represent about 40% of all the bloods, and are, therefore, easily encountered by examining a certain number of sera and cells chosen arbitrarily.

The technique used certainly is of great importance for 314
the proper appreciation of agglutination, as I was able to convince myself in research done for other reasons. Moss tested for agglutination in small tubes, and perhaps this technique should be judged the most appropriate, were it not for the necessity of taking blood from a vein. The microscopical examination in hanging drops often gives uncertain results with agglutination, given the possibility that the cells can collect upon one another by the simple action of gravity.

It does not seem appropriate to me to introduce an agglutination test on too minute a scale, since too many positive reactions would be required, often masking the reality of the situation. In my opinion the "traces" cannot be accepted as indicative of a positive reaction. According to Baecchi, "The formation of small and few groups of corpuscles are observed in the most peripheral parts of the drop, given simply stacking of these same corpuscles at their borders." On the other hand, I would, like Baecchi, call such reactions negative. These facts correspond quite well to the data obtained in tubes by considering as positive agglutination (by analogy to bacterial agglutination), only cases in which the cells are really agglutinated, that is to say, reunited in an irregular accumulations of many globules, with superposition, and not merely their collecting upon one another. It is undeniable that sometimes one has small, clear diamond shapes according to this criterion, since often not all of the corpuscles become agglutinated. In my records I assigned three degrees of agglutination: the first—positive—in which the formation of the accumulations is evident, but there remain free corpuscles; the second—strong—in which the majority of the corpuscles are agglutinated; and the third—total—in which all or almost all the corpuscles are within the same mass.

The tests were done in hanging drops with red cells washed twice and suspended at about 5% in physiological saline solution. I allowed one loop of these cells to react with two loops of serum, diluted by half. In order to obtain the necessary serum and cells easily, I aspirated seven to eight drops of blood from the ball of the finger in pipettes constructed especially for the purpose, and containing many glass beads. Shaking these pipettes defibrinated the blood, and the globules were then separated from the serum by centrifugation. Only the cells being necessary, I collected a

RED CELLS

SERA		O				B				A								AB
		L.L.	G.E.	L.J.	MAZ	R.V.	RIC.	R.R.	R.P.	C.V.	G.C.	P.L.	ET.	ALF.	M.C.	R.S.	Z.G.	C.C.	C.M.	L.C.
$\alpha\beta$	L.L.						N	+	+	+	+	+	+	+	+	+	+	+	+	N
	G.E.						N	+		+	+						+	+		+
	L.J.						N	+	+	+	+	+	+	+	+	+	+	+	+		
α	RIC.										+	+	+	+	+	+	+	+	+	+
	R.R.										+	+	∴	+	+	+	+	N		+
	O.E.										+	+	+	+	+	+	+	N		+
	R.P.										+	+	N	N	N	+	+	+	+	+
β	P.L.						+	+	+	+										+
	ET.						N	+	+	+										+
	ALF.						N	+	+	+										+
	M.C.						N	+	+	+										+
	R.S.						+	+	N	+										+
0	G.B.																			-
	L.C.																			-

N = unexpected negative (see text) [Designated in the original text by heavy type minus signs]

315 drop of blood directly in 1 cc of physiological saline solution. The observation of the reactions were made after ½ to 1 hour of constant temperature incubation.

In order to identify the non-agglutinable cells I allowed the cells and the sera of a certain number of normal individuals (twenty-four in all) to react so as to distribute them in the various groups on the basis of their behavior in the agglutination test.

316 I report in a table (see above) the results of these tests indicating for every serum and cell the category to which the results belong, omitting from the table only a few of the tests conducted, which gave patterns with cells and sera that were too incomplete.

From this table the division of the bloods, examined in the way indicated above, is very clear. There are two classes of bloods in which the sera reciprocally agglutinate each other's cells and these have, respectively, the cells A and the cells B.

These two classes do not occur with the same frequency, and according to the nomenclature of v. Dungern and Hirschfeld, the one that is encountered most frequently can be designated A, and the less frequent one B.

We have then a group with A cells and agglutinin β able to agglutinate the B cells (blood Aβ), another with B cells and α agglutinin (blood Bα). Another still, with cells lacking A and B namely the non-isoagglutinable, and with serum containing α and β agglutinins (blood O$\alpha\beta$). A last class, representable by ABo, has a serum not provided with isoagglutinins, and its cells are agglutinated by sera from the other three classes.

The twenty-four bloods that I examined were divided in the following way: 6 O$\alpha\beta$; 5 Bα, 11 Aβ, 2 ABo.

In the table are reported in heavy type some reactions which do not correspond as one would have thought to the above-mentioned division. [Heavy type is replaced in the translation by the letter N.] Certain cells containing the groups A or B were not agglutinated by sera containing the corresponding agglutinins $\alpha\beta$.

I found only negative exceptions, that is to say, in certain cases the agglutination did not occur where it could be expected; but I never observed abnormal, positive agglutinations in opposition to the division of the bloods into four groups. It is not impossible that in the cases in which agglutination was absent, it would have occurred eventually, but was so weak as to be unappreciable with the technique used. Still, even v. Dungern and Hirschfeld have noted that not all the homologous bloods behaved identically. Thus, certain agglutinins α did not react with all the A cells but only with a part of them. By absorbing, moreover, a serum α with A cells (namely, by treating it with an excess of A cells and by separating it by centrifugation), there often remain agglutinins for the other A cells. So that the biological configurations A, B, α, β, cannot be considered as unitary entities, but as corresponding to series of substances that can assemble according to certain relationships. 317

V. Dungern and Hirschfeld admit, therefore, that all the bloods of one and the same group are not identical, but that a further subdivision exists among them; and even with the use of animal sera "absorbed" with human cells of single groups (experimental conditions rendered much more complex by a partial superposition of the groups) there are differences that can more properly be called individual, and theoretically permit the diagnosis of the single individuals.

The possibility that certain agglutinations are unexpectedly negative does not have great importance for the choice of the globules to be used in the Marx-Ernrooth test. Since, for this, one must use non-isoagglutinable globules, only the eventuality of exceptional positive isoagglutinations would invalidate the results of the test, causing one to be able to confuse isoagglutination with heteroagglutination. Instead, the O cells presented perfectly uniform and identical behavior with respect to the isoagglutinins, not having been agglutinated by any of the sera tested. It is, therefore, possible to identify human cells in the way described above, which are both theoretically and experimentally refractory to isoagglutination.

They are, therefore, very well suited to serve as reactive cells for the reaction of Marx-Ernrooth. The positive value of the reaction under these experimental conditions is not being able to attribute agglutination to isoagglutinins. The results of all the previous work show that the hetero-agglutinins act uniformly on all the human cells, and furthermore, this characteristic has been considered by Baecchi as the one which can differentiate them from the iso-agglutinins. I tried a simple control; since the experiments of other authors were already decisive on the matter of the action exercised by the animal bloods on the non-isoagglutinable O cells.

The tests were carried out by treating the cells of LL and GE in the table with extracts of the bloodstains of the more common animals (rabbit, guinea pig, dog, chicken, etc.) and unanimously showed positive agglutination.

318 These results coincide with the analogous tests of v. Dungern and Hirschfeld. These authors have, nevertheless, observed that the serum of the anthropoids (chimpanzees) can, in some cases, fail to agglutinate the A or B cells. This analogy between the serum of the anthropoids and that of man, is not surprising nor does it in any way change the value of positive agglutination of the O globules, which often occurs even with blood of higher apes.

Negative results from the reaction carried out on these cells cannot, as is clear, have any decisive significance. In fact, as was mentioned above, it can happen that even heterologous bloodstains are inactive on human cells.

The reaction of Marx-Ernrooth carried out on non-isoagglutinable human cells reacquires value for its precious properties of rapidity and simplicity. It can be objected that the identification of the needed cells is rather complex and requires testing of a number of bloods. But even if one wanted to examine as many bloods as I myself did (that does not seem necessary), suffice it to say that this set of tests has been accomplished once and for all. It may be noted that, in the blood of adult individuals, the relative properties of agglutination are maintained unaltered for an indefinite time period, such that once an individual carrier of O cells is identified, one can employ these globules for the reaction at once, eventually carrying out a control test from time to time.

As long as the test was done in that way, it had notable simplicity and certainty, but it cannot, nevertheless, compete with the precipitin test. The latter is, in fact, of much more general applicability, and especially has the advantage of permitting the direct recognition of human blood, while agglutination cannot directly demonstrate neither the presence nor the absence of human blood, but only the presence of non-human blood. The agglutinin reaction, performed with known cells, can lend notable service, nonetheless, when confirmed by the precipitin reaction, and especially in the cases which it is appropriate to orient oneself to the nature of the bloodstain rapidly, before having set up the precipitating sera. Besides, the positive outcome of the agglutinin test can indicate directly, whenever the precipitin test with anti-human serum is by itself positive, the simultaneous 319/ presence of non-human blood in the human bloodstain.

To the agglutinin test is, therefore, attributed a value in itself, different from and better than the simple criterion of confirmation.

Isoagglutination has also been used, as is known, for the individual diagnosis of human blood. The authors who proposed this application,, namely Landsteiner-Richter and Biffi, based it on the fact that autoagglutination is a completely exceptional phenomenon in healthy individuals; therefore, when a stain shows positive agglutination with the cells of a particular individual, it is possible to exclude that bloodstain from having come from that same individual.

Everyone agrees that if, instead, agglutination does not occur, one must stop and declare that no response can be given.

Baecchi justifiably thinks that one must not stop at this point, because if a stain does not agglutinate certain corpuscles, that does not mean that it cannot very well agglutinate some others. Therefore, he thinks that comparing the behavior of the stain with that of the blood of the individual suspected of crime and to a series of erythrocytes of diverse origin, one can arrive at two orders of conclusions. First of all, if one finds a type of corpuscle to which the two bloods react entirely differently, it cannot be doubted that their differentiation is indisputably established. Since, according to this author, it can be supposed that all the bloods are capable of isoagglutination, it would always be possible, following this indirect method, to exclude a given stain as having come from a particular person, given that, with a sufficiently large series of corpuscles, it is practically impossible to find two stains that resemble one another. Therefore, it would always, or in most cases, be possible to decide on the non-correspondence of a given bloodstain with a given person. In the second place, it would be possible in favorable circumstances to arrive at a direct individual diagnosis of a stain, when the stains furnished by an individual demonstrate a behavior completely identical to that of the stains to be diagnosed, using a suitable series of corpuscles of various origin.

Well, now, these clear, new facts notably restrict the prac- 320/ tical value of these conclusions.

Above all, there exists in every series of bloods a percentage which is destitute of isoagglutinating capacity, and this is equal to about 10% according to the experiments of v. Dungern-Hirschfeld and Moss and also according to mine. In the series of normal individuals studied by Baecchi the percentage is the very same (7 in 63) if, for the above-mentioned reasons, the "traces" are considered negative. Baecchi has said that these bloods would have demonstrated agglutinating properties, if tried with a larger series of cells than he employed. This is, in reality, very improbable.

The two o sera of my table (GB and LC) were tried on twenty-two and twenty-three different types of human cells, respectively, and none of them ever agglutinated. One can,

therefore, affirm that bloods exist which lack isoagglutinins.

Therefore, when a stain is constituted by a blood of that kind, unless circumstances are very favorable for freshness and preservation, the diagnosis of exclusion of a particular individual will turn out completely uncertain, even if this blood were endowed with isoagglutinating properties, since these would be able to disappear in the stain.

But there is still another more important possibility, namely that the two bloods which are under consideration for identification or differentiation belong to the same group. Then they can resemble each other to such degree as to lead to more dangerous errors. The perfectly analogical behavior that can exist between two different bloods renders very problematical the assertion that one can always diagnose exclusion. Even in Baecchi's tests, one finds that there are groups in which the smallest quantitative differences of the agglutination of four varieties of corpuscles would not permit exact differentiation of the single individuals. But also, adopting a very extensive series of corpuscles, as in the tests I followed, we can observe the same behavior in different bloods belonging to the same group. The possible existence of exceptional reactions (those in which agglutination is absent), those which distinguish a blood from the others of the same group, allows one to suppose that, by multiplying the number of tests still further, it would be possible finally to find a cell that differentiates two similar bloods. But it does

321 not seem to me necessary to take account of such a theoretical possibility in practice. In the first place, these exceptional reactions can be verified equally with different bloods, as my table clearly shows. In the second place, the number of red cells that would have to be examined in order to lend certainty to finding a difference between two bloods of the same group in every case would have to be considerable, such as to remove from this procedure every element of practicability. In the third place, the imprecision of the number of cells on which the investigation would have to be based would not allow the results to have the degree of certainty indispensable for forensic judgments. If we think, in fact, of obtaining from a stain and from a suspect's blood identity of behavior with respect even to an extensive series of cells, we can conclude that the two bloods belong to the same group; but at the state of our knowledge we will not be able to assert that the two bloods belong to the same person, or even to different persons. We can always think that, multiplying again the number of cells tested, we might find some difference, and that, therefore, the bloods can be different. Thus one cannot with any argument establish that the absolute identity of behavior excludes the possibility of another blood of the same group. In my experiments I obtained at various times absolute identity, even in the exceptions, of the behavior of different bloods with respect to a non-negligibly large series of erythrocytes.

By this I do not intend to deny that there can exist and does exist an individuality in the human blood, but I believe only that it is not manifest through these characteristics so clearly that we can demonstrate it at present by means of isoagglutinins.

I think, therefore, that to speak now of direct individual diagnosis is premature. This does not exclude the possibility of searching thoroughly and going beyond the simple diagnosis of human blood, and it is actually possible to make a distinction directly between the different bloods, which has very notable forensic importance. In addition to the prudent negative diagnosis of a stain (not belonging to a given individual) of Landsteiner-Richter and Biffi, a positive, direct diagnosis seems to me perfectly warranted, not yet of the individual, but rather of the group. Having established that the stain is human (preferably by means of the precipitin test) one can find some value in the isoagglutinins, whose resistance to different agents was established in preceding 322 work, in order to circumscribe the number of individuals to whom the stain can belong, and assigning to it one of the four groups of which we spoke above. This can be attained by allowing the extract of the stain to react with A cells and with B cells, or better yet, having seen the possibility of exceptions, on several varieties of both A cells and B cells. If only the A cells become agglutinated, it is a matter of a Bα blood; if only B cells, of Aβ; if both, of O$\alpha\beta$. When neither A nor B become agglutinated, the diagnosis will have to remain inconclusive, since it could be a matter of ABo blood, but the destruction of the agglutinins which might have been present could also have occurred.

Only when blood is very fresh and manifestly unaltered, will one be able to presume that it should be assigned to this last group.

One must, in addition, establish the group to which the blood of the suspected individuals belong, and one can then make the timely comparisons easily and possibly draw conclusions from them.

I carried out some of these tests with recent bloodstains, setting up conditions under which I would not be subject to any preconceived notions or suggestions, and I obtained favorable results.

A colleague brought me a stain on blotting-paper, three days old, with a request to indicate to which of seven persons it belonged. The extracts of the stain were tested with A cells (GC) and with B cells (CV) which never showed exceptional reactions. The extract of the stain agglutinated the B cells and not the A ones. The sera and cells of the seven persons indicated were examined. One of these bloods belonged to the group O$\alpha\beta$, two to the group Bα, four to the group Aβ. Therefore I judged the stain as belonging to one of these four, which matches exactly.

This result is already notable, having succeeded in excluding three persons out of seven; but when the number of persons to be distinguished is smaller, the response can be completely individual.

I was given a stain on blotting-paper that was five days old, with an offer to state to which of three people it belonged. The extract of the stain agglutinated the A cells as much as the B cells. Of the three persons, the first has O$\alpha\beta$ blood, the second Aβ, the third Bα; I judged, therefore, that

the stain belongs to the first of these, the one which matches exactly.

323 One cannot give a definitive value to this laboratory research, the conditions being much more complex in practice. Nevertheless, in the expectation that other studies will establish the limits of applicability of this group diagnosis, the following points do not seem doubtful to me. First of all, although more modest, an individual direct diagnosis is more certain. On the other hand, the value of this research surpasses that of the simple reaction between the stain and the cells of the individual suspects, since, whenever that reaction gives negative results, it permits no other conclusions; but the investigation of the groups can be made to distinguish whether this negative result depends: 1) on similarity of blood group of the stain with that of the suspect; 2) on the fact that the stain does not contain isoagglutinins; or 3) on the fact that the suspected blood belongs to a non-isoagglutinable group, (0ab). This distinction, obviously, has considerable importance.

If one succeeds in establishing that the blood of the stain and that of the suspect belong to different groups, the response will be easy, since it certainly cannot be a matter of the same blood.

When, instead, it turns out that the stain and the suspected blood belong to the same group, this coincidence can have, as is clear, very great judicial importance.

An individual diagnosis of exclusion can be indicated within the limits of the same group (and only when it is not a matter of the group ABo lacking isoagglutinins) on the basis of different behavior with a variety of erythrocytes from the same agglutinable group; and as is stated above, and can be seen from the table, it will perhaps be possible in some favorable case to be done. But a direct and sure individual diagnosis of identity between the two bloods cannot, for now, be achieved, since whenever they have identical behavior with respect to a long series of erythrocytes of diverse origin, the possibility that they belong to different individuals cannot be exluded.

I conclude as follows:

1. Among the four groups into which the bloods can be divided on the basis of their capacity to participate in isoagglutination, there is one in which the red cells are refractory to isoagglutination.

2. The study of these cells is very timely as is thier use for the specific, negative diagnosis of human blood according to
324 Marx-Ernrooth, the interfering action of the isoagglutinins being eliminated in this way.

3. For the identification of the individual origin of a bloodstain, its assignment to one of the above indicated groups can be very useful.

4. It cannot always be considered possible to establish by means of agglutination that a stain does not belong to a given person. This negative diagnosis will almost always be impossible when the blood of the stain does not contain isoagglutinins, and it will be extremely difficult and uncertain when the stain and the blood of the individual suspect belong to the same group. The assignment of the stain and of the suspected blood to some one of the groups can illuminate the individual origin of the stain more than the absence of the simple reaction between the stain extract and the cells of the suspect blood.

5. The one positive, direct diagnosis obtainable by means of isoagglutination up to now is the group diagnosis. It is premature to speak of individual, direct diagnosis, since different bloods belonging to the same group can agglutinate in an identical way a lengthy series of test cells.

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Two Practical Cases of Individual Diagnosis of Human Blood*

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298 The results of the experiments that I have been conducting for many years on the individual diagnosis of human blood have an application in two practical cases which are, to my knowledge, the first of those kinds of diagnoses to actually be performed. The publication of these cases, demonstrating the resolution of one of the more important problems of judicial practice, will undoubtedly interest everyone involved in forensic medicine. Simultaneously, the technique used in this diagnosis, indicated in a general way a previous publication of mine† will show that it is necessary to submit to the exigencies of the particular case in point, and show some flexibility according to the circumstances.

The first case was not of a judicial nature, but a purely private matter.

The fifty-year old worker, R. G., appeared at the Institute for Forensic Medicine in order to explain a situation that had gravely disturbed the tranquility of his family for a good three months. He came for advice to a colleague, Dr. Bertola, who indicated to him that the Institute of Forensic Medicine was the only place where he might perhaps be able to clear up this affair. At R's first explanations, and having seen his interest in the thing and its relationship with the studies we had been pursuing, I accepted gladly the assignment.

R. told how on a Sunday, he had put on a dress shirt and
299 gone to a town near Torino where he stayed until late at a country inn of some friends. The next day he took it off in order to wear it again the next Sunday; he then noticed that there were blood stains on the shirt, but he ignored it. The next day his wife, A. G., a woman of high character, very jealous, who habitually accused her husband of disloyalty, asked him for an explanation of those spots, proving in her mind that he had once again been disloyal and that the stains surely demonstrated that he had had relations with other women during his stay outside Torino. (This had been further confirmed by a fortune-teller!) The husband claimed, on the other hand, to be innocent and had intended from the beginning on the contrary to keep the stained shirt in the hope of being able to demonstrate his true innocence with it.

The discussions surrounding this matter became continual and so harsh so as to render life impossible for every member of the family, so much so that even strangers in the neighborhood were implicated. R. was extremely desirous of clearing up the mystery of the stains so that peace could return to the household. While the wife explained the stains in the above-mentioned manner, he advanced the following hypotheses as to their origin:

(1) Being in the habit of going to buy meat from the butcher, he thought it possible that on the Sunday on which he wore the shirt he soiled his fingers with blood in choosing the meat, and then pulling out the shirt to urinate he soiled it.(??)

(2) On the Monday on which he left the shirt, he came to find his wife's friend, the one who helped her to make the beds. This friend was menstruating and remained for some seconds alone in the bedroom. He supposed that she was able to use the shirt to dry the menstrual blood.

(3) It was more probable that the suspicious wife, not having ever been able to prove his supposed adultery, had made the stains with real blood, seeking to provoke a confession from him.

These last two hypotheses aroused anger and incredible resentment.

The shirt, of the finest linen, shows in the first part, on the anterior area near the edge, an oval and irregular spot about 5 × 2 cm, rather dense; other similar ones, 2 × 2 cm., are near the edge; other thinner stains, with the appearance of having been rubbed, are located at a distance of 10 to 25 cms from the edge. Given the appearance of the stains and R's age, I suspected that that man could be afflicted with prostatic hypertrophy and that the blood came precisely from a hemorrhage of the urethra. He said that for a year and a half he had had to get up two or three times almost every night to urinate; he never had any retention of urine nor observed 300 any hemorrhaging; the outflow of urine was somewhat diminished. He often experienced sensations of fullness in the rectum.

By rectal exploration was noted a rather hard prostate gland, protruding noticibly in the rectum. On the sacrum

*Translation of: "Due casi pratici di diagnosi individuale di sangue umano."

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† See in this journal, 1915. In a previous communication (*Giorn. dell'Acc. di Med. di Torino*, 1916) I indicated further useful modifications of the technique, which will be reported in a forthcoming issue.

and the buttocks were found a large and itchy eczema. The wife many times observed blood stains on the sheets, but she always attributed them to the scratching of the eczematous skin. She never saw blood on the shirts.

Summing up all the possible origins of the blood stains, excluding, for obvious reasons, the hypotheses of the wife, the diagnosis had to be made among bovine blood, urethral blood of R. G., menstrual blood of her friend T. E., and blood of the wife A. G.

With a small trace of stain one performs the species diagnosis with high titer anti-human precipitating serum, according to the usual technique, with the following controls: human serum, human stain, ox blood, physiological saline solution. It showed very evidently that the stain is human blood; it immediately showed an opaque ring just as the one within the human controls.

This limited it, therefore, to the individual diagnosis of three people, and I was prepared to carry on with it.

As a result of my previous work, the first objective was to obtain an extract of about the same volume as the blood from which the spot was constituted. Since the spot was not crusted on, but impregnated the material, it was convenient to obtain the piece of dried blood by removing from the stained piece a similar, clean piece of the same surface. In order to do a similar comparison with the greatest possible exactness, after various tentative experiments, I went back to the following method. Having chosen the largest and most dense stain, I delimited the smallest area of linen that contained it, I counted the threads in the one direction and in the other with the help of a strong lens; the threads showed 180 in one direction and 65 in the other.

Then in the clean part of the shirt I cut 10 pieces of cloth with the same number of threads. To do this, I cut with the scissors somewhat larger pieces, then with a thin needle I separated the excess threads, and finally,with straight cutting scissors and with the help of the lens, I cut the edges of the protruding threads from the edges of the rectangular pieces of linen. Dried in a thermostat, the 10 pieces were weighed with maximum precision with the following results:

1. 0.0900 g	6. 0.894 g	
2. 0.0900 g	7 0.893 g	
3. 0.0898 g	8. 0.893 g	Average
4. 0.0898 g	9 0.0890 g	0.0895 g
5. 0.0898 g	10. 0.0885 g	

301 The stained material, cut in the same way, weighed 0.0944 grams. Therefore, deducting the average, the maximum and the minimum weights of a similar piece of unstained material from this number, the weight of the dried blood was, on the average, 0.0049 grams, but it could vary between 0.0059 and 0.0044. Considering that the residue of dry blood is 20%, in round numbers, of the total weight (Hammarsten), it was necessary to add 0.0176 grams of distilled water in order to renew the volume of blood (basically, that amount small enough to avoid hypotonicity). Then to increase the volume of the extract in a way that would have certainly diluted it by half, taking away, therefore, from the maximum weight of the blood, it was necessary to bring the extract to a volume of 0.0472 cc with physiological saline solution. As a practical matter, I rounded the figures, and in small closed weighing bottles made of emery I put the cut out stain, following its contours, adding to it 0.02 cc of distilled water and 0.03 of physiological saline solution, leaving it to steep for twelve hours in an ice box and squeezing it repeatedly with a glass rod. Then, squeezing the piece of cloth between two pieces of glass I could, with a small capillary pipette withdraw a dark brown extract, to be used for the tests; its dilution with respect to the blood could vary from 1:2 to 1:3, and therefore can be regarded as appropriate. This extract was collected in a small tube, closed with a rubber stopper.

According to the proposition of Landsteiner and Richter, they would test the agglutinating property of this extract with respect to the red cells of the three suspected persons, mixing two loops of extract in a hanging drop with one of a 5% suspension of washed cells. All three of the tests turned out negative. I went on then to the agglutination tests, according to the same technique, of running test erythrocytes with the extract and with the sera of the individual suspects, diluted 1:3 with physiological saline solution. Besides these, some other complementary tests were done, letting the cells and sera of the three individuals react with one another. These were distinguished by a simple mark inserted by my 302 colleague, Romanese. As test cells I used a pair of A cells and a pair of B cells, which I had already noted in my previous research. The results obtained were the following (23-25 February 1915) [as shown in Table I].

I did not keep track of quantitative differences in the 303 agglutination, since in the three month old stain the agglutinins could be weakened compared to the fresh sera.

The meaning of these designations was the following:

I. R. G. (husband)
II. T. E. (friend)
III. A. G. (wife)

The above tests show that the husband and the friend belong to group βA, the wife, instead, to group $\alpha\beta$0. And since the stain belongs to group βA, the results exclude the possibility that the blood could have come from the wife. Having arrived at this point, it was necessary to distinguish the blood of the husband from that of the friend, although the hypothesis that the blood came from the latter was somewhat improbable, and vigorously denied. At this point it would have been necessary to carry out tests with long series of B cells to see if the two bloods behaved differently with respect to any one of them. But as I explained elsewhere, the outcome of this difficult investigation is quite problematical, and the experiment would have been justified only if no other way could have been found. Since among the elements of the problem was the circumstance that the friend's blood could only have been menstrual blood, it was much simpler here to distinguish the source of the blood, and thus the investigation became a matter of determining whether the stain was from menstrual or common blood.

At this time, the diagnosis of menstrual blood based on the

Table I

1. Serum	Cells	Agglutination
I	Cab. (A)	–
I	Gra. (A)	–
I	Cav. (B)	+
I	Ro. (B)	+
I	II	–
I	III	–
L.L. ($\alpha\beta$)	I	+
Cav. (α)	I	+
2. Serum	**Cells**	**Agglutination**
II	Cab (A)	–
II	Gra. (A)	–
II	Cav. (B)	+
II	Ro. (B)	+
II	I	–
II	III	–
L.L. ($\alpha\beta$)	II	+
Cav. (α)	II	+
3. Serum	**Cells**	**Agglutination**
III	Cab. (A)	+
III	Gra. (A)	+
III	Cav. (B)	+
III	Ro. (B)	+
III	I	+
III	II	+
L.L. ($\alpha\beta$)	III	–
4. Stain extract	**Cells**	**Agglutination**
"	Cab. (A)	–
"	Gra. (A)	–
"	Cav. (B)	+
"	Ro. (B)	+
** "	I	–
"	II	–
"	III	–

** Test of Landsteiner and Richter

glycogenic reaction of the vaginal cells, according to Wiegmann, had become widespread. Numerous experimental tests done on ordinary blood, and on both fresh and old menstrual blood for the past seven years, had convinced me of the specificity, certainty and practicality of that reaction. I found the technique proposed by Brandino particularly suitable; a great number of vaginal cells could be observed even on small and very old stains. In no case did the reaction fail.

Having taken small pieces of the stained shirt in different places, I subjected them to discoloration in 2% hydrogen peroxide for twenty to thirty minutes. After then rinsing them in physiological solution, I treated them on a microscope slide with Lugol solution. At no point could I observe glycogenic cells. A diffused light violet color appeared on the cloth and small violet spots, which must have been due to 304 starch from the ironing. Besides direct observation of the material, scrapings of the bloodstain were also examined; but even in this way I could not observe the glycogenic cells. Given the consistency and the certainty of the reaction, one could conclude that the stain was not one of menstrual blood, nor could it have belonged to T.

The definitive conclusion of the above inquiries was, therefore, that the stain was of human blood and it was the blood of R. G.

This result restored the peace to family G.; not only were the components of this case indeed in concert with an appreciation of biological principles, but, from the response of a physician completely disinterested in the question, a new and plausible origin of blood was also brought to light, namely, the hypotrophic prostrate which, naturally, they had not thought of before. Having seen this sequence of events more than once, they observed blood stains on the sheets, being able to exclude eczema as their origin, and this served to confirm the reality of much that I had explained.

In another practical case I had occasion to apply the individual diagnosis. This time it was a case of great judicial importance. An individual was suspected of a most serious homicide. Besides the general capacity to commit a crime, the judicial circumstances seemed to be singularly stacked against him. Especially important among them was the presence of numerous blood stains on the overcoat that he wore. Now, he explained these stains as having come from a heavy nose bleed resulting from a blow he received to the nose. There were, however, strong reasons to believe that the blood of the stains could have belonged to the victim. Thus, circumstances were present in which only the individual diagnosis could be valuable, since it was a matter of human blood beyond question.

During the autopsy of the body of the victim, I withdrew some blood from the heart. From this I prepared serum, lightly rose-colored, a part of which I dried at low temperature, and a part I kept fresh in an ice box. I also attempted to keep the red cells for the agglutination test, but by the time I accomplished this, they had changed too much and thus could not be used. The time that had elapsed be- 305 tween the death and the autopsy was about forty-eight hours. After forty-eight hours I did the agglutination test. The stains were at least four days old, irrespective of the differing versions of their origins. The blood of the suspected individual was taken the same day as the test. The serum was diluted by half, the cells prepared in a 5% suspension.

Because I was examining the serum of the victim, I was motivated to make an observation which was important for the proceeding, and on which I initiated other investigations. Using the usual technique (two loops of serum and one loop of 5% cell suspension) with this serum, diluted by half, I consistently observed the phenomenon of true and proper rouleaux formation, roughly simulating an agglutination. And this was visible while using cells, so that from many tests there were no isoagglutination results. The dilution of the serum by half was not enough, therefore, as it is in the case of sera taken from the living, to eliminate pseudoagglutination. The pseudoagglutination from rouleaux, is, as shown by my previous work, the consequence of an excessive concentration of the serum employed. Well, the determination of the dry residue of serum taken from the cadaver showed that it had acquired a noteworthy concentration.

In fact, 7.5 cc of serum gave a dry residue (at 100°) of

1.1880 g. According to the tables of Hammarsten, the dry residue of serum corresponds to about 1/11 of the weight of water (= volume). However, in this serum it was a little less than twice this value. Since the serum was only lightly tinted rose, this increase of dry residue is, in all probability, attributable to a diffusion of water from outside of the vascular system, exactly the phenomenon I was to study. The fact remains that diluting the serum of the cadaver at 1:4 rather than at 1:2 inhibits pseudoagglutination, proper agglutination remaining unaltered.

Thus, the conclusion formulated in my previous work, namely, that in order to obtain certain results in the isoagglutination test, it is necessary to dilute the serum 1:2 (that becomes 1:3 in following the proportions of added cell suspensions used by me) is applicable only when the serum was taken from the living. In serum drawn from a cadaver a greater dilution will be necessary, according to the degree of concentration of this serum, to be determined on a case by
/306 case basis where it is possible. In general one will be able to avail oneself of the criterion that the dilution must be such as not to provoke pseudoagglutination by rouleaux in cells that do not show isoagglutination. In my case the serum of the cadaver was used in the dilution of 1:4.

The extract of the stains was made in the following way. The stains were crusted on smooth woolen cloth, so that they could be cut away with a pointed bistoury, almost without damaging the material. Most of the stains were collected in a weighing bottle and dried at 37°. The aggregate weight was 0.0656 g. Not all of this weight was blood, there still being present much woolen cloth. The stains were then treated with a quantity of solvent, presumed the minimal amount necessary according to the above stated criteria, namely, with 0.2 of distilled water and 0.2 of physiological saline solution. It was kept for six hours in an ice box, macerated and pressed repeatedly with a small glass rod. The blood readily dissolved, and there remained a mass of threads. These were gathered into a small ball that was, as much as possible, squeezed out with a wide pincers. After this, the threads were washed repeatedly with distilled water, dried out, and weighed. They weighed 0.020 grams. Therefore, the real weight of the dry blood from the stains was 0.0656 − 0.0200 = 0.0456, corresponding to approximately 0.18 cc of fresh blood. This blood would have had to be diluted to a volume of 0.36 cc. The extract already prepared with 0.4 cc of solvent was, therefore, a slightly greater dilution, but was still perfectly appropriate for the tests.

The investigation directed at the individual diagnosis was naturally preceded by the demonstration of the human origin of the stains by means of the precipitin test, in the manner indicated in the above-mentioned case.

The test of Landsteiner and Richter followed successively, that is to say, the agglutination of cells from the individual suspect by means of the stain extract, and turned out negative.

I went on, therefore, to the agglutination tests with test cells of known group, proceeding, as in the previous case, with a pair of A test cells and a pair of B test cells. I report now all the results obtained, neglecting, for the reasons mentioned above, the simple quantitative differences. [See in Table II]. 307/

Table II

Serum of the suspect, diluted 1:2, with red cells	Agglutination
Cab. (A)	+
Ros. (A)	+
Ro. (B)	+
Ov. (B)	+
of the suspect	−
Liquid serum of the victim, diluted 1:4, with red cells:	**Agglutination**
Cab. (A)	−
Ros. (A)	−
Ro. (B)	+
Ov. (B)	+
of the suspect	−
Dried serum of the victim, redissolved, with red cells:	**Agglutination**
Cab. (A)	−
Ro. (B)	+
of the suspect	−
Stain extract with red cells:	**Agglutination**
Cab. (A)	+
Ros. (A)	+
Ro. (B)	+
Ov. (B)	+
*** of the suspect	−

*** Test of Landsteiner and Richter

Besides these tests others were done as controls with my blood, which has non isoagglutinable cells and serum containing the two agglutinins α and β. The sera in the dilutions employed did not agglutinate my cells at all; besides, my serum, which strongly agglutinated the four examples of test cells used, was completely inactive with the cells of the individual suspect.

The blood of the suspect belonged, therefore, to group O $\alpha\beta$, that of the victim, to group Aβ.

The extract of the stain reacted like the blood of the individual suspect; and it was differentiated distinctly from that of the victim by its isoagglutinating power with the Ro. and Ov. cells. Therefore, one could conclude: (1) that the stains found on the cape did not belong to the victim; (2) that it was perfectly credible that they came from the suspect.

With this answer the positive, indirect diagnosis was 308/ achieved and the medico-legal inquiry was finished. Certainly, such a response could not, of course, be assumed to be a decisive element of proof, considering the novelty of the investigation. (Much time was needed before probative value was attributed to fingerprints in trials!) Therefore it is appropriate to take note that the course of the inquiries on the case had to abandon definitively the original theory, and to diminish the suspicion of that particular individual; from that came the greatest demonstrative value of the biological, medico-legal inquiry.

(In another assessment on blood stains which I did with Professor Carrara in a homicide case, the magistrate asked us if a stain on the lining of the jacket of the accused came from the blood of the person accused (as he maintained), or from that of the victim, already looked at by the other experts. We withdrew blood from the accused in order to attempt the test.

However the scantiness and the small size of the stain scarcely allowed the specific diagnosis, and would not give a sufficiently concentrated extract of high enough strength, so that all the tests gave negative results and the inquiry failed).

On the Technique of the Isoagglutination Test for the Individual Diagnosis of Blood*†

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400 The technique of the practical individual diagnosis of blood that I described in my recent work (1) is founded directly upon the results of experimental inquiry. Having thus demonstrated the essential importance of serum concentration for the certainty of the test, there followed the necessity of knowing the titer of the stain extract and, therefore, the weight of the dried blood to be dissolved. Now, inasmuch as this technique shall be retained as the most scientific and rigorous, and has furnished me with excellent results in two particularly favorable cases (2), it remains a fact that it cannot be considered applicable in most medico-legal circumstances.

A technique more suited to general use, and particularly for the investigation of small stains, in which the possibility of weighing the blood is quite problematical, would therefore be highly desirable.

In the recent experiments of De Domincis (3), having confirmed the practical interest in the isoagglutination test, has been proposed a technique, reminiscent of Landsteiner
401 and Richter (4), directed precisely at the investigation of common bloodstains.

He proposed placing a drop of cell suspension on the surface of the microscope slide, and to tease apart a pair of threads of the stained material, about 4 or 5 millimeters long, and then putting on the cover slip. Under these conditions agglutination can be observed which would otherwise have been very weak or absolutely negligible.

One observes the agglutination proper where the liquid in the preparation appears more distinctly hemoglobin-colored.

This technique undoubtedly has the value of great rapidity and simplicity, besides that of being applicable to very small stains. But, these advantages and the technique's adaptability do not suffice, and, to the contrary, they have an entirely subordinate value. The first indispensible requirement is that the test be able to demonstrate isoagglutination with certainty, consistency and, especially, specificity.

From my previous research of a general nature, which must be kept in mind when considering attempts at practical application, it is clear that the isoagglutination test cannot have any value at certain concentrations of agglutinating serum; more precisely, it must be kept within the broad limits of 1:2 to 1:25, and, practically, it can be said, within 1:3 and 1:10. At lower dilutions, true isoaggulatination can be simulated by non-specific pseudoagglutination; at greater dilutions, the agglutination is no longer appreciable.

Now, in the technique of De Dominicus, the concentration is not calculated, and the test is so devised that the same concentration is employed to meet the widest variations in conditions. And precisely, if during the unravelling of the thread material, the blood readily dissolves in the drop of cell suspension, the concentration will become more than 1:15 and the agglutination will not be visible, as with a blood that would not show it under other circumstances. If, instead, the blood diffuses slowly around the edge of the thread, it will be able to bring about a concentration sufficiently elevated to cause pseudoagglutination. De Dominicis recognizes that with concentrated blood solutions, as well as with the concentrated bloodstains, one can have agglutination, which is 402/ as much as saying pseudoagglutination.

In the test proposed by him, the concentration of the serum at every point in the procedure depends on too many uncontrollable factors: the unravelling, the rapidity of dissolution and diffusion of the blood, the movements of the preparation, and even the thickness of the stained thread upon which will depend the thickness of the compressed liquid layer between the microscope slide and the cover slip.

I wanted to introduce controls into the proposed technique, using serum and cells whose relative behavior in isoagglutination was perfectly known to me from hundreds of experiments, while adhering strictly to the specifications of De Dominicis. It is incontestable that in certain cases, blood and cells capable of giving an agglutination reaction do so in a distinctly observable way. On the other hand, the test very often shows negative results, probably because the blood diffuses into the drop during the unravelling process.

These negative results, obtained with blood-red cell combinations with which agglutination would have to be positive, invalidate the consistency of the test.

I ignore that which De Dominicis understands exactly as "indicative agglutination", since he qualifies it by saying that under other conditions "it would have to be considered very slight or as downright negligible". As I have already stated explicitly elsewhere, in order to be able to admit that agglutination has occurred, one must see true, irregular clumping of the cells; the simple groups formed by contact are not enough to demonstrate it. Even less is it so in the test of De Dominicis, since in it one can easily see that the cells might amass themselves around all of the artifacts of the preparation, air bubbles, threads of the material, heterogeneous granules, and the edges of the preparation, by the simple physical action of capillarity and without the intervention of agglutination. Such more or less irregular masses and incidental contact of cells with debris are observed also with blood-red cell combinations which would certainly be negative for agglutination.

Experimenting with the same combination of blood and red cells both agglutinable and non-agglutinable, respectively, it was possible at times to "guess" partly the difference, which included looking at the order of magnitude of the "general outlines", and recognizable only because the result had been previously known.

403/ But I maintain, in contrast to such a view, and in speaking not of blood and red cells of known type, but of a case to be resolved, that no one would have dared to risk a diagnosis of positive agglutination.

However, this would not be the dangerous aspect of the De Dominicis test, since the negative results at least have the value of not compromising anything, it being possible to admit that a previously agglutinable blood has lost this property. As I indicated in the work already cited, only positive agglutination has diagnostic value, never the negative.

Unfortunately, however, the test of De Dominicis can give rise to pseudoagglutination.

It happened often that in using either threads of material from a dense stain, or even a thinly crusted blood which I did many times, one could obtain all around the trace of blood a picture of sufficiently intense agglutination, even experimenting with a combination of blood and red cells in which isoagglutination could not possibly occur, and at other times with stains and red cells of the same person.

The excessively high serum concentration around the blood trace can, therefore, sometimes cause pseudoagglutination.

Besides, during the unravelling of the stain, proposed by De Dominicis, one can spread around in the cell suspension clumps from the blood that constituted the stain. These clumps are easily confused with a true and proper agglutination result, and in any event they impede a certain appreciation of the results of the test.

Therefore, the door remains open to grave errors of judgment.

On the whole, the test of De Dominicis is inconsistent; at certain times it yields a result that actually conforms to one obtained when applying the more rigorous scientific techniques. But, on the contrary, it can yield the two opposite defects: that of not revealing a true and proper agglutination or else that of simulating a completely non-existent one. Without a doubt, this happens principally because in certain cases, the concentration of serum remains so low, while in others, it becomes too high.

It being truly important in forensic medical practice to obtain a simple and reliable method for the individual diagnosis of common bloodstains, he sought to eliminate the two above-indicated causes of error and to reconduct the test 404/ under the conditions demonstrated to be necessary by scientific inquiry, by avoiding the preparation of a titrated extract of the blood, frequently impossible.

The difficulty to be resolved resided in the contrast between the necessity of having a strong enough serum concentration to see agglutination well, and the danger that pseudoagglutination might manifest itself as the result of an excessive concentration. Now it seems to me very difficult with a purely empirical approach to the dissolution of the blood to obtain, in a consistent way, that serum concentration optimum which the theoretical inquiry has shown to be around 1:2–1:3.

More easily attainable in fact is the intentional elimination of the pseudoagglutination, not *prior to* but *after* the reaction is terminated.

The tests on microscope slides and in small tubes demonstrated that once the cellular clumps, characteristic of true agglutination, were constituted, a very considerable dilution was not enough to make them disappear. On the other hand, with excessively high serum concentration, one can have a regular or irregular mass of cells (pseudoagglutination), but a small dilution suffices in this case because the cells immediately separate one from another.

I report some of the tests carried out, which I repeated many times with various dilutions, and always with the same result.

(1) 0.05 of α serum is mixed in a tube with 0.05 physiological saline solution and 0.1 of a 5% suspension of red cells of Group A. After 15 minutes, a few loops of the liquid are withdrawn, after shaking, and examined in a hanging drop. Virtually all the cells are agglutinated in a large clump. If 1 cc of physiological saline is added, the clump persists. If another 1 cc of physiological saline is added (a dilution of the serum at this point of about 1:44), the clump still persists.

(2) 0.05 of $\alpha\beta$ serum is mixed in a tube with 0.05 physiological saline and 0.05 of a 5% suspension of red cells of Group A. Examining the material in a hanging drop after 10 minutes: large clumps of cells. 0.1 of the liquid is withdrawn and mixed with 0.9 of physiological saline: the clump persists. If another 1 cc of physiological saline is added (dilution of about 1:60), the clump still persists.

(3) A small drop of $\alpha\beta$ blood is mixed in with $\alpha\beta$ serum, and the mixture agitated. Examination on a slide or in a hanging drop shows almost complete pseudoagglutination

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*Translation of: "Sulla tecnica della prova di isoagglutinazione per la diagnosi individuale del sangue."

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† A summary was communicated to the Acc. di Med. di Torino April 7, 1916.

(also identifiable were numerous, short little masses). 0.05 of physiological saline is added to 0.1 of this liquid. Upon examination, just about all the cells are free, there being some
405 limited pseudoagglutination. If 0.1 of physiological saline is added to 0.1 of this liquid, all the cells appear free.

The same results are obtained by carrying out these dilution experiments on slides or in hanging drops, just as well as in tubes.

From these tests it can be concluded that the clumps of true agglutination persist even at a dilution of the serum at which agglutination would not occur in any appreciable way. But the clumps from pseudoagglutination (masses) disappear as soon as the dilution is such that pseudoagglutination is no longer manifest (1:2).

On the basis of these facts, the previous dilution of the serum does not appear indispensible to the reliability of the isoagglutination test, at least for most practical purposes. But with reference to stains, those from which a titrated extract cannot be obtained, one can proceed in such a fashion that the maximum concentration of blood is possible. With the agglutination that eventually occurs, a successive appropriate dilution will permit the determination of whether it is a true or simulated agglutination.

Thus one can practically apply this method.

It is necessary, as done by Landsteiner-Richter and De Dominicis, to add a portion of stained material directly to the cell suspension.

Therefore a thin crust of blood is placed on the slide, or in dealing with material, a small square of a couple of millimeters on a side. This form is preferable to that of the thread, since with it, one can obtain a stronger concentration of serum, either all around, or also in the network of the material. If the material is thick, it can be advantageous to cut a small hole in the center of the fragment, where the cell suspension can sit enclosed, in a little cell, as it were.

It can be necessary instead to unravel the threads of the material, as De Dominicis proposes, because this unravelling very often brings about a rapid dissolution of the blood into the suspension. It is convenient to proceed in such a way as to make the bloody material as compact as possible, collected together at the same point, but without causing an excessive gap between the slide and the cover slip, with a corresponding large increase of the quantity of liquid interposed between them. One, therefore, adds a small drop of cell suspension and covers it instantly with a cover slip,
406 taking care not to displace the blood trace, then eventually fills up the preparation with other drops of cell suspension.

So far the hanging-drop method is without a doubt better for a scientific study of isoagglutination, for in this case one must simply evaluate a simple microscopic preparation. In these preparations the flow within the liquids is almost impeded, and because of that, the serum dissolves only in the zone of the liquid adjacent to the bloodstain, reaching there its maximum concentration. There is, nevertheless, often a problem, namely that the cells move only very little and struggle to reunite in order to agglutinate; it is appropriate for that reason to have some minimal lateral movements back and forth under the cover slip, in order to make the interaction of the cells easier but carefully avoiding, however, the blending of the liquid.

The preparation is then left to sit in a humid chamber, and it is examined two or three times, displacing it as little as possible, and finally after a half hour it is perfectly useless to prolong the observation further. Positive agglutination is usually visible in a few minutes: it is manifest by the clumping of the cells in the liquid zone immediately adjacent to the blood trace. Sometimes, instead, the cells unite on the glass plate, especially when one has stains on materials, in such a way that the agglutination is not easily recognizable in a direct way, but makes it necessary, therefore, to displace the preparation, as will be further indicated below.

Often (not always however!) in working with a blood-red cell combination that cannot give agglutination, one observes a clumping, even an intense one, because of pseudoagglutination; so that, although true agglutination is *usually* more intense than pseudoagglutination, one cannot make a diagnosis using this criterion.

In order to distinguish the two phenomena it is necessary to proceed with the dilutions. Since the serum is concentrated only in one small zone surrounding the blood trace, where, precisely as noted by De Dominicis, the hemoglobin color is observed, it is sufficient to mix it with the rest of the suspension contained in the preparation in order to obtain a considerable dilution of this zone. To accomplish this, the cover slip is removed, and the liquid material that is far from the edge of the fiber or the thin blood crust residue is blended using the edge of the cover slip, and the preparation then 407
covered again using another, larger cover slip (18 × 18 instead of 15 × 15). With this maneuver, which must be done in every case, a duplicate result is obtained. First of all, if the agglutination had taken the form of being spread out, or if the clumps were concealed in the network of the material, the blending of the liquid renders very evident, and thus verifies the agglutination, if it exists. In the second place, a homogeneous dilution of the serum of the stain is obtained throughout the preparation.

The dilution obtained in this way is certainly completely empirical; but it greatly surpasses that needed for the verification of pseudoagglutination. In order to get a rough idea of it, I left drops of blood (about 0.05 cc) to dry on a pane of glass for further testing. The round drop dries there, dividing itself into many sections. Withdrawal of a portion of a section is adequate. For the experiment, I withdrew a section of about 1/20 of the drop and divided it into halves, each one of these corresponding to 1/40 of 0.05 of blood or, in round numbers, to 0.001 0.002 cc of blood.

At least 0.02 cc of cell suspension was necessary for the preparation, and thus, if the blood was completely dissolved, its dilution would not have been less than 1:10. Usually, the dilution is even higher because the blood is not completely dissolved, and because the quantity of suspension is greater.

The mixing of the liquid makes the clumps of cells, previously limited to the vicinity of the blood trace, spread out into the entire preparation. If it is a question of true agglutination, it could easily be distinguished among the numerous isolated cells. If instead, it were pseudoagglutination, the clumps, also voluminous, completely break down and are no longer observed among the isolated cells.

Following this technique, it was always possible in many tests to successfully eliminate the cause of error due to pseudoagglutination, which is certainly the one to be feared most in this case. Failure to break the bloodstain into small bits causes the spreading out of the clumps from the same bloodstain in the preparation to become inevitable, and renders the judgment uncertain.

408 The test yields results nearly consistent with small blood crusts (of course the blood on which the systematic examination of the serum was done had previously demonstrated its power to agglutinate the cells which were used), at least under the conditions I tested, those being dried blood, unaltered, and no older than ten months. Agglutination is usually indicated by large clumps and does not lend itself to doubts.

The results with blood impregnated materials are less consistent; sometimes, specifically in experiments with isolated threads, an agglutination that would have been expected is not seen with certainty. Obviously, the "compactness" of the bloody substance being less under these conditions, a sufficient concentration of serum in the surrounding liquid for bringing about a certain agglutination is not reached. In this sense, the test presents the same problem as that of De Dominicis, though more rarely; and the greater consistency is attributable to having omitted the unravelling of the fabric, and also of having sought every device to augment the concentration of the serum.

As disagreeable as the possibility of these negative results are, they are not, as I already stated above, of great importance, because one does not in any case attribute probative value to negative agglutination.

I believe that these above-proposed modifications of the technique, based on the laws governing isoagglutination, and which permit the carrying out of the test in a simple and certain way on small blood traces (particularly in the form of small crusts) make a further contribution to the attempts at individual diagnoses.

Given the great practical importance of the problem, I hope that other investigators will want to take these methods under consideration, in order to affirm their value and improve them in the points where they might be wanting.

References

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3. Sulle indicazioni individuali del sangue. *Cesalpino* 11-437, 1915
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Practical Experience Concerning Blood Group Determination in Stains*[1]

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402 Italian legislation does not at present allow tests concerning paternity except in very special cases.

In Germany blood group determination has already been widely used for the very important medico-legal goals of recognizing, or better, of excluding paternity. While scientific experiments in this field have developed fruitfully in Italy under Mino, we have not had at our disposal practical experiences for the reason given above.

The forensic cases, which we have had to examine, concern, therefore, the individual identification of blood stains.

The number of cases which I have examined is, indeed, modest in comparison to the frequency with which the question should be submitted to testing. Without a doubt, this results from the fact that the judges inquiring into the cases, as well as the forensic physicians, are not sufficiently familiar with the possibility of making determinations or, at least, of obtaining useful, individual indications. Although I have only done about ten cases, in fact, no single expert, so far as I know, has at his disposal equal *practical experience.*

I, therefore, think it useful to present all of these together, even though some of them have already been individually presented.[2]

I hope that this representation of these real cases, taken from actual practice, almost all of which were favorably resolved, will convince the forensic physicians of the importance of this new examination method and of the necessity of introducing it into regular forensic medicine.

In addition, I would like to indicate some technical processes which broadens the possible applications of the method, and simplifies the process of carrying it out. In the
403 older cases, of course, I could not yet use the new processes, which were in need of further study.

I shall now enumerate the individual cases.

1. Dried blood stains, approximately three months old, on a shirt (not a court case).

It was necessary to determine whether the blood came (a) from a cow, or (b) from a human being; and if human, whether from a certain man, from his wife, or from a friend of these two (in this case menstrual blood was involved).

The Method Used: Production of a titrated blood extract by indirect weighing of the blood (average difference between a particle of blood and a series of several other particles of the same size). Test for isoagglutinins; microscopic method in the hanging drop.

Results: The precipitation caused by rabbit antiserum to humans showed that it was a question involving human blood.

The Landsteiner-Richter test (hereinafter abbreviated L.-R. V.), applied to the three persons, was negative.

The grouping proved that the stain contained the agglutinin β; it belonged, therefore, to Group II (Aβ).

The grouping of the three persons by means of serum and blood corpuscle testing demonstrated that the man belonged to Group II (Aβ), the wife to Group I (O$\alpha\beta$), and the friend to Group II (Aβ). The absence of menstrual glycogen cells eliminated the friend as a possible source.

Judgment: The blood stain came from the man.

2. Four day old, dried blood stains, on a piece of cloth. A court case (murder). It was necessary to determine whether the stains came from the suspected perpetrator of the crime (nose bleed) or from the victim.

The Method Used: Production of a titrated blood stain extract by weighing. Testing for isoagglutinins by means of the microscopic method in the hanging drop.

Results: L.-R. V. against the blood corpuscles—negative. The grouping showed that the stain contained both isoagglutinins α and β; it belonged, accordingly, to Group I (O$\alpha\beta$).

The grouping of the accused (by means of serum and blood corpuscles) showed that he belonged to the same group I (O$\alpha\beta$); the grouping of the victim (obtained by using the serum extracted during the coroner's examination) showed that he belonged to Group II (Aβ).

Judgment: The blood stain comes from the accused and not from the victim (accused was released).

3. Very thin blood stains, dried for about one month on linen cloth. Court case (murder). It was necessary to determine whether the stains came from the accused (previous abrasion).

Method Tried: Empirical extract preparation, in this case 404
of an extremely small quantity of blood, too small to weigh. The extract was ineffective against the blood corpuscles of the accused [who belonged to Group II (Aβ)], as well as against the test blood corpuscles A and B. No isoagglutinins could be demonstrated.

Result: Negative.

4. Fifteen to eighteen month old blood stains adhering to a silk cap. A court case (murder). It was necessary to determine whether the blood stain corresponded to the blood of the victim (preserved in a dry state).

Method Used: Direct microscopic method (cover-slide method). Control by means of elective agglutinin-absorption.

Result: In the stain, the isoagglutinin α was demonstrated (with weak reaction); the absorption reaction showed that the blood from the stain removed the agglutinin β from a serum $\alpha\beta$; it thus contained the agglutinogen B. It, therefore, belonged to Group III (Bα). The blood of the victim (well-preserved) contained strong agglutinin α and the agglutinogen B; it belonged, therefore, to the same group.

Judgment: The blood from the stain corresponded to the blood of the victim.

5. Small, scaly blood stains, more than three weeks old, found in a trouser pocket. Court case (murder). It was necessary to determine whether the blood from the stain came from the accused. The accused could offer no explanation regarding the origin of the stain.

Method Used: Direct cover-slide method.

Result: The L.-R. V. was negative. The grouping demonstrated the presence of agglutinin β in the stain; it belonged, therefore, to Group II (Aβ).

The grouping of the accused (with serum and blood corpuscles) showed that he belonged to the same Group II (Aβ).

Judgment: The blood from the stain corresponded to that of the accused. A microscopical examination of the sediment of the extract produced for serum preparation showed fragments of a crushed flea. The stain was thus caused by crushing the hemophagous insect (accused set free).

6. Numerous, thick blood stains on a shirt, two or five months old depending upon the proffered explanation. Court case (murder). It was necessary to determine whether the stains came from the accused (nose bleed). (The victim's blood was not available).

Methods Used: 1. Stain extracts titrated by weighing. 2. Cover-slide method with dried extract sediment. 3. Elective absorption.

Results: The use of extracts in the hanging drop resulted in uncertain results. The cover-slide method, undertaken with dried-extract sediment, showed with complete certainty
405 the presence of both isoagglutinins α and β. The L.-R. V. was negative. The absorption experiment, carried out with two successive portions of the stain, removed absolutely no agglutinin; there was thus no isoagglutinogen present. The blood from the stain belonged to Group I (O$\alpha\beta$).

The grouping of the accused (by means of serum and blood corpuscles) demonstrated that he belonged to the same Group I (O$\alpha\beta$).

Judgment: The blood from the stain corresponded to that of the accused.

7. Numerous blood stains, two and a half months old and dried on smooth stones. Court case (murder). It was necessary to determine whether the stains came from the accused. The accused had a small, festering wound on one finger which the judge interpreted as the result of a bite, but which the accused claimed was a small work injury. The wound, it was alleged, had bled and thus stained the stones along the path that the murderer used to get away from the scene of the killing.

Methods Used: Direct, cover-slide method (the case was especially suited for this method). Control by means of elective absorption.

Results: L.-R.V. negative.

The grouping demonstrated the presence of very active agglutinin β in the stain. By means of the absorption test it was shown that the blood from the stain removed the agglutinin α from a test serum $\alpha\beta$. Thus, agglutinogen A and agglutinin β were contained in the stain; it, therefore, belonged to Group II (Aβ). The blood of the accused was tested (serum and blood corpuscles), and it belonged to the same Group II (Aβ).

Judgment: The blood from the stain corresponded to that of the accused (As a result, an indictment was issued in this case against the accused).

8. The examination of this notable case was requested not by the officials, but by the ophthalmologists and psychiatrists who were to answer the important forensic question regarding simulating the effects of accidental injuries on the job.

A girl (with pronounced hysterical symptoms) was injured in the eye with iron clips which harmed only the conjunctiva outside the cornea. The chips were removed with a magnet, and the small wound was on the point of healing cleanly. In the eye clinic, however, it was noticed during the morning round that the bandage was plentifully soaked with pure blood, which had partially trickled down; what is more surprising, this took place more than one month after the accident. The ophthalmologists were unable to find any source of the profuse bleeding, either from the conjunctiva which had almost completely healed, from the eye lids, or, even less, from the completely sound cheeks. Moreover, no one was able to observe the bleeding. As a result, they consulted with the psychiatrists with the idea that it could be some mysterious, hysterical bleeding. The psychiatrists, however, were skeptical regarding the situation; they turned to me, asking 406
that I test the blood from the bandage and compare it with that of the girl's.

Methods Used: The bandages were examined a few hours after their removal; they were soaked with blood that had run to some extent, and in the deep layers the blood was so fresh that I was able to produce from it suspensions of well-preserved blood corpuscles. Thus, the agglutinogen content could be directly determined by measuring capability of the blood corpuscles to agglutinate. The agglutinin determination was carried out by means of the cover-slide method with extract sediment dried on the slide itself.

The grouping of the girl's blood with serum and blood

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*Translation of: "Praktische Erfahrungen über Blutgruppen-bestimmung in Flecken."

in: *Deutsche Zeitschrift fur die Gesamte Gerichtliche Medizin* 9: 402-410 (1927).

corpuscles showed that it belonged to Group II (Aβ).

Results: a) The first bandage, removed without any special attention, produced the following results.

	Agglutination
Bandage blood and girl's blood corpuscles (L.-R.V.)	strong
Girl's serum and bandage blood corpuscles	"
Test serum α and bandage blood corpuscles	"
Test serum β and bandage blood corpuscles	"
Bandage blood and test blood corpuscles A	"
Bandage blood and test blood corpuscles B	"

Of course, the sera were used in the dilution 1:3, and the observations were carried out at a temperature of 25°. The results of the reaction demonstrated the following: 1) that the bandage blood did not come from the girl; 2) that the blood was possibly from an animal since the reactions, all positive, belonged to the category of heteroagglutination. Although, from the outset, the situation did not indicate this, it was necessary to carry out a species diagnosis: namely, by means of sero-precipitation and the test of O blood corpuscle agglutination. The precipitation reaction, carried out under strict controls with anti-human serum from rabbits, produced a *negative result.*

Judgment: The blood which had soaked the bandage we were given did not come from the girl, but from an animal.

b) After I had communicated these results indicating deception to my colleagues, the girl was strictly isolated. Nonetheless, the blood-soaking occurred again six days later. I now examined the bandage which was sent to me with the same test sera and blood corpuscles we used in the first test; i.e., I repeated in parallel the tests on the first bandage (with unchanged results), though the blood-corpuscle suspension from the first bandage, having been preserved in the icebox, appeared in a somewhat altered state.

407 With the second bandage I obtained the following results:

	Agglutination
2nd bandage blood and girl's blood corpuscles	negative
2nd bandage blood and test blood corpuscles A	negative
2nd bandage blood and test blood corpuscles B	strongly positive
2nd bandage blood and test blood corpuscles O	negative
Test serum α and 2nd bandage blood corpuscles	positive
Test serum β and 2nd bandage blood corpuscles	negative
Girl's serum and 2nd bandage blood corpuscles	negative

The parallel precipitation tests conducted with the same antiserum from rabbits (at a dilution value of 1:1000) produced the following results: the failure of any disturbance to appear in the case of the first bandage; on the other hand, a strong precipitation ring in the case of the second, a ring which was still very clear, when one further diluted the serum by half (1:2000).

Judgement: The blood of the second bandage was certainly different from the blood of the first; it was human blood and surely belonged to Group II (Aβ), the same as that of the girl. (Further experiments showed that it was not a question of menstrual blood). I still have not found out what practical conclusions were drawn from my experiments. Concerning this case, Professor A. Sacerdote and I will bring out a publication.[3]

This case points up how the source of errors resulting from heteroagglutination possess not only a theoretical value, but they must in practice always be kept in mind, even when there is no suspicion. Even when I have not specially mentioned it, the precipitation reaction with anti-human serum was performed in every case as an indispensable test, and so too the agglutination test with O-blood corpuscles was carried out in almost every case. Only when the blood demonstrated a differential effect on the human blood corpuscles, usually the ones used in the test, and when, in addition, the amount of blood was too small (cases 1, 4, 5), did I sacrifice the test.[4]

Regarding the technical aspects, I consider it unnecessary to repeat everything here which I have dealt with separately in other works.[5]

No matter how great the preference of some authors, and especially of the great expert, Dr. Schiff, may be for the microscopical method, it remains completely barred from the area of forensic, individual diagnosis of blood stains for 408 obvious reasons.

In the context of the microscopical technique, the process which I worked out (called by Schiff "the Lattes cover-slide method"), merits the first place because it is so easy, as even Dr. Schiff admits; one adds the smallest traces of dried blood to a fitting suspension of blood corpuscles in a usual microscopical preparation; in carrying out the process, one protects against the threat of error: 1) by diluting through mixing of the preparation; 2) by the use of lecithin suspensions (spherical blood corpuscles); and 3) by keeping the temperature between 20° and 25°.

This process can be used straightaway if the stain is found in a crusted state (even if it is very small), as in my cases 4, 5, and 7. On the other hand, in the cases in which the blood has thoroughly soaked into some material, no good results are produced when a piece of the material is added directly to the preparation, especially on account of the excessive thickness of the layer of liquid which is contained between the slide and cover slip. The production of titrated extracts can sometimes lead to good results (cases 1 and 2); in other cases this method fails on account of its unavoidable inaccuracy. Moreover, it is complicated and time-consuming, despite the useful application of the torsion scale to weigh the blood and the dilution liquid. According to my latest experience, it is preferable to substitute the extraction procedure with the cover-slide method, suited for blood crusts, by using a special technique which I conceived and perfected for my case 6, and which functioned outstandingly in the many tests of case 8.

If no blood crusts are present, but only blood-soaked material or substratum, I produce for myself artificial crusts. This is done most easily in the following manner (provided the blood is not insoluble). The stains are first cut and are macerated for a few hours in the icebox with a very small amount of distilled water (so that there is not present an excessively low salt level); with a capillary tube, the extract is absorbed from the material between two pieces of glass;

Case	Type of Bloodstain	Age	Microscopical Method Used	Individuals Used for Comparison	Possibilities of Origin	Diagnosis of the Stain	Judgment Concerning the Individuality of the Blood
1.	Soaked into linen	3 months	extract; hanging drop	1. cow 2. man II (Aβ) 3. woman I (Oαβ) 4. woman II (Aβ) (menstrual blood)	1. Accidental staining 2. Uretheral bleeding 3. Willful staining	Human, not menstrual blood group II (Aβ)	Belongs to man II
2.	Soaked into scarf	4 days	extract; hanging drop	victim II (Aβ) accused I (Oαβ)	1. Nose bleed 2. Murder	Human blood I (Oαβ)	Belongs to accused
3.	Soaked into linen	1 month	extract; hanging drop	accused II(Aβ)	1. Previous bruise 2. Murder	Not feasible	Negative
4.	Dried on silk	15 18 months	1. cover-glass method 2. elect. absorpt.	accused III (Bα)	1. Previous head wound 2. Murder	Human blood III (Bα)	Agreement with the accused
5.	Dried on scarf	3 weeks	cover-glass method	accused II (Aβ)	1. From accused (?) 2. Pocket stains through bloody hand	Human blood II (Aβ)	Agreement with the accused
6.	Soaked into linen	2 or 5 months	1. extract; hanging drop 2. indirect cover-glass method 3. elect. absorp.	accused I (Oαβ)	1. Nose bleed 2. Murder	Human blood I (Oαβ)	Agreement with the accused
7.	Dried on stone	2 ½ months	1. cover-glass method 2. elect. absorpt.	accused II (Aβ)	Bleeding from a wound of the murder near the scene of crime	Human Blood II (Aβ)	Agreement with the accused
8a.	Bloody bandages	fresh	1. indirect cover-slide method	suspected girl II(Aβ)	1. Hysterical bleeding	Animal blood	Does not belong to suspect
8b.	Bloody bandages	fresh	2. blood cell agglutinability		2. Simulation of accident injury	Human blood II (Aβ)	Agreement with the suspect
9.	1. Dried on straw 2. Soaked into scarf 3. Soaked into wood	4 months	1. cover-glass method 2. indirect cover-glass method	accused II (Aβ)	See note on page 407 [footnote 4]	1 and 2 human blood II (Aβ) 3. not feasible	Agreement with the accused

using a fan, very small drops of the extract are then dried at low temperatures on a slide. This process is repeated so that four to six droplets are placed on the same spot. Thus, one obtains a series of slides on each of which is a thick bloody crust, about the size of a pinhead.

To this crust one adds the corresponding blood corpuscle suspension and covers it with the cover slide without mixing the crust and the suspension. The agglutination, when positive, manifests itself clearly on the edges of the encrustation.

410 I can warmly recommend this process since it has produced for me outstanding results. The possible applications of the cover slide method to a variety of blood stains ought to be disseminated to all forensic physicians. Of course, if the stain is not soluble or produces no isoagglutinins, one will have to employ the absorption tests to aid in the examination. The absorption tests are in any case to be evaluated as a control, so that one can determine the agglutinogen as well as the agglutinin, i.e., can obtain an integral representation of the blood group. The elective absorption, however, has a much narrower field of application than does the direct agglutination test, since it demands much greater amounts of blood.

From all my cases, one can see that in some of these (cases 4, 6, 7, 8b) it was possible to produce only one demonstration of agreement, though one very important for the forensic goal in the cases; in other cases, given the state of affairs before the court, it was possible to answer the forensic question most specifically, in that one could either offer an indi-

vidual diagnosis of exclusion (negative), as in cases 1 and 2; or one could utilize a secondary finding (cases 5 and 8). Only in a single case was it impossible to obtain a usable result.

I hope that these practical cases will awaken general interest and encourage the systematic use of individual blood stain diagnosis.

Notes and References

1. Delivered at the 15th Meeting of the German Society for Legal and Social Medicine in Düsseldorf, September, 1926.
2. *Arch. di antropol. crim. e med. leg.* **37**, 3, 1916: **44**, 1923; **45**, 1925; *Rass. internaz. di clin. e terap.* **72**, 192
3. In the *Arch. di Antrop. crim. e med. leg.* 1927
4. Note added during correction: At the request of Prof. Goroncy (Königsberg in Prussia), I recently dealt with another case of murder (I do not know the legal circumstances).
   Age of the stain: About 4 months
   Cover-slide method to identify agglutinins:

Dried blood of the criminal	Group II (Aβ)	(Pronounced reaction)
Small crusts on straw	Group II (Aβ)	(Pronounced reaction)
Bloodstained vest	Group II (Aβ)	(weak reaction)
Blood spotted wood chips	Not able to be determined	

5. See my contribution: "Methoden zur Bestimmung der Individualität des Blutes" in *Abderhalden's Handbuch der Biol. Arbeitsmethoden*, 1927

The Isoagglutinable Substance of Blood and its Demonstration for the Individual Diagnosis of Stains*†

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362 The procedure followed until now for attaining the individual identification of human bloodstains consisted of the demonstration of the isoagglutinating power of the stain under examination with fresh, appropriately selected cells. The isoagglutinins, contrary to what was asserted for years by various authors, resist harmful influences such as drying, putrefaction, moderate heating and exposure to air quite well; thus, in a good number of cases they can be identified easily enough in the stains, resulting in the direct assignment of the blood to a blood group (group I: O cells; serum a & b; group II: A cells; serum b; group III: B cells; serum a; group IV: AB cells; serum o).

Still, in other cases it is not possible to demonstrate the existence of any isoagglutinating power in a stain. This negative result can be due above all to the circumstances of the blood under consideration, which, even if still very fresh, can belong to the group distinguished by the absence in serum of both the human isoagglutinins. But even the existing iso-
363 agglutinins in fresh blood can be altered and destroyed by harmful influences (age of the stain, excessive heating, chemical influences, etc.) as is verified by stains which have become insoluble in water.

A negative result in an investigation on isoaglutinins in a stain is, therefore, without significance, and the method does not permit, in that case, any conclusion about individual origin.

Even in the first attempts at individual diagnosis consideration was given (Biffi) to utilizing for the diagnostic reaction not the isoagglutinins, but the cells contained in the stain. Biffi believed that it was possible to restore them to the original condition in order to test the specific isoagglutinability. But, given the delicacy of the reaction and the practical impossibility of reconstituting the dried out cells in their integrity, the idea could not be applied. It resulted further from other investigations that isoagglutination is a complex phenomenon, in which a specific element could be distinguished by the selective absorption of the agglutinins, and a non specific element and the reunion in clumps of red cells and the flocculation (Lattes)[1].

It would be sufficient therefore, for diagnostic purposes, that the first could be conveniently ascertained.

The selective absorption, already studied by Landsteiner and his collaborators, and then by several other authors, is a reversible process (according to the temperature) in such a way that the greater part of the agglutinin bound to the cells can be recovered in solution; it would be possible for this property to acquire practical value.

Professor Lattes has, for the record, advised me to carry out investigations directed at the eventual utilization of this phenomenon for diagnostic, medico-legal purposes; namely, to verify abstractability of a true and proper agglutinability from the capacity of the cells in a stain to selectively absorb isoagglutinins and yield them up again at a higher temperature, and this, even when the stain has undergone alterations so as to render the demonstration of the agglutinins impossible (this test because of its simplicity is always the method of choice when it can be done).

Encouraging experiments in this direction have already 364
been accomplished by Schütze[2] (in his experiments for other reasons) showing the agglutinin-absorbing capacity of the residue of distilled water extracts of stains, in other words, of the globular stroma.

As for the possibility, thus far not studied, that the agglutinable substance of blood, even though altered or denatured, is in a position to absorb the isoagglutinins, it prompted us to examine the analogous situation in bacterial agglutination. It turned out, in fact, from various investigations that germs whose typhus or proteus bacilli are able to absorb specific agglutinins to the very same extent after cooking as in the fresh state (Scheller, Friedberger-Pinczower, Kumagai, Dessau, Lange)[3], or else after treatment with dilute acid without showing the phenomenon of agglutination (Eisenberg and Volk[4], Wassermann[5]).

On the other hand, numerous investigations exist from which it emerges that bacteria treated with various chemical agents are modified little or not at all in their agglutinability. They could still be able to conserve the property of absorbing specific agglutinin.

*Translation of: "La sostanza isoagglutinabile del sangue e la sua dimostrazione per la diagnosi individuale delle macchie".

in *Archivio di Antropologia Criminale Psichiatria e Medicina Legale* 43 (4 ser. 14): 362-384 (1923).

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† The results of this investigation were communicated to the R. Accademia Peloritana of Messina in May, 1922. See the Atti of the Academy, vol. 30, 1922.

Thus, cholera vibrios, killed with chloroform vapor, can still be agglutinated (Bordet)[6].

Bacteria subjected to the action of various bacteriocidal substances conserve agglutinability (Van de Velde)[7].

Typhus bacilli treated with formaldahyde can be substituted for living ones in the reaction of Widal (Widal and
365 Sicard)[8]. They conserve the sensitivity to the agglutinins for several months (Nicolle)[9].

Thus pure carbolic acid, the colloidal silver and sublimate (Righ)[10] does not modify the intensity of the agglutination of living bacteria.

Silver nitrate (1%), sublimate (1%), potassium dichromate (1/2 N), osmium tetroxide (1%), acetone (50%), ether, chloroform, formalin (10%), and hydrogen peroxide (25%) do not exercise any influence on typhus bacilli (Busacca)[11].

Other substances would increase the affinity of the bacteria for the agglutinin: weak solutions of osmium tetroxide (Imai).

Others, on the other hand, would destroy it: sodium hydroxide (Porges)[12], 95° alcohol (Busacca)[11] or they would limit it: pure acetone and acids (Busacca)[11], alkali (Dreyer and Blake)[13]. According to Busacca, the behavior of the typhus bacteria to the alkali, is different from that of the second substance employed: there was no change with ammonia, a notable reduction with sodium hydroxide.

I carried out various experiments both with blood altered by physical or chemical agents, and with blood in which isoagglutinins could not be demonstrated for other reasons
366 (nature of the group, old age).

I. Blood Treated with Physical Agents

In this series of investigations I tested the capacity for absorption of isoagglutinin either of blood simply dried, from five to twenty days before, or of blood rendered insoluble by heating at 100° for 5–10 min., or also of red cells washed and baked. I did not think it necessary to repeat the absorption experiments on fresh cells, the results of which I am by now very certain.

The samples of blood, belonging to a determined group, carefully pulverized (in small tubes by means of crushing with a small glass rod), or else the cooked cells were left for several hours at ordinary temperature in close contact with an appropriate quantity (sometimes little, at other times an excess) of fresh serum providing the two isoagglutinins (serum a &b); after that the material was centrifuged and subjected to two types of investigations:

1. The residual isoagglutinating properties of the serum, separated by centrifugation, was tested.

The preparations were made in hanging drops and observed after about 15 mins, shaking the microscope slide frequently; the serum was used in a 1:3 dilution in order to avoid every possibility of pseudoagglutination that would confuse the results.

2. The sediment was suspended in an excess of cold physiological saline (preferabaly at 0°) and washed two or three times; following incubation in a small quantity of new physiological saline for 15 min at 45°–50° (a temperature which from experience purposely created the most favorable opportunity for the extraction of the agglutinins fixed to the cells) and rapidly centrifuged with a water mantle at 45°.

I then tested the isoagglutinating properties of the supernatant fluid (containing the agglutinins yielded up in the heat from the sediment) on fresh A and B cells.

It would be superfluous to report all the experiments done, inasmuch as they yielded concordant results, although quantitatively somewhat different. I maintain that a reason for these differences, of however small importance, must be sought in the fact that the absorption power of a stain varies 367
by reason of the fineness of its pulverization, the degree of which cannot easily be assessed in a single test.

I will report some of the more significant experiments.

1. Dried blood

0.05 cc of blood with group A cells (S. V.) dried for three weeks, is pulverized and suspended in 0.1 cc of serum a & b (L.L.) freshly diluted by half. After half a day the material is centrifuged and separated and the serum is strongly colored by hemoglobin:

- Absorbed serum + fresh A cells (S.V.) = no agglutination
- Absorbed serum + fresh B cells (C.C.) = total agglutination

The sediment was washed at 0° and extracted for 15 min at 45° in 0.03 cc physiological saline solution and centrifuged while hot:

- Extract from the sediment + fresh A cells (S.V.) = intense agglutination
- Extract from the sediment + fresh B cells (C.C.) = negative agglutination.

Other experiments with B blood gave concordant results. In addition to suspending the dry blood in excess of serum (1 cc), the successive test with the extract from the sediment gave still very rapid and intense specific agglutination.

(The sera ab (L.L. and S.G.) used in these and in the succeeding experiments agglutinate the A cells as intensely as the B cells).

2. Heated blood

0.025 cc of blood with type A cells (S.V.) dried and pulverized, was added to 0.1 cc of physiological saline solution in a small tube that was stoppered and then immersed 5 min in a boiling water bath. The tube was cooled and 0.1 cc fresh serum a + b (L.L.) is added and mixed up thoroughly. After half a day at ordinary temperatures it is centrifuged:

- Absorbed serum + fresh A cells (S.V.) = negative agglutination
- Absorbed serum + fresh A cells (S.E.) = negative agglutination
- Absorbed serum + fresh B cells (C.C.) = total agglutination

The sediment, washed at 0°, is extracted for 15 min at 45° with 0.1 cc of physiological saline solution and centrifuged

while hot:

- Extract from the sediment + A cells (S.V.) = moderately positive agglutination
- 368 Extract from the sediment + A′ cells (S.E.) = positive agglutination
- Extract from the sediment + B cells (C.C.) = negative agglutination

In other experiments the absorption is not complete, at least with certain cells, with a first portion of stain, but only with a second.

0.05 cc of A′ blood (S.E.) dried and suspended in physiological saline solution was baked at 100° for 5 min, then treated with 0.1 cc of fresh serum ab (L.L.):

- Absorbed serum + fresh A′ cells (S.E.) = weakly positive agglutination
- Absorbed serum + fresh A cells (S.V.) = positive agglutination
- Absorbed serum + fresh B cells (C.C.) = strongly positive agglutination

The serum is absorbed further with 0.025 cc of blood A′ (S.E.) dried and baked, as above, in physiological saline solution:

- Absorbed serum + fresh A′ cells (S.E.) = negative agglutination
- Absorbed serum + fresh A cells (S.V.) = negative agglutination
- Absorbed serum + fresh B cells (C.C.) = strongly positive agglutination

In all the experiments in which the stain and the absorbing serum were mixed in proportions similar to those indicated, the absorption of the agglutinins appeared complete and wholly specific, if not in the first test then at least in the second. The subsequent test of the agglutinin extraction on the other hand, often gave less clear and sometimes even negative results, clearly because of the scarce quantity of agglutinins recovered, due either to the small quantity of serum employed (precisely with the intention of obtaining the complete absorption of one of the two agglutinins contained in it), or to the procedure of washing the first sediment from the extraction, or perhaps to the temperature during extraction, which in some experiments surpassed the indicated 45°–50°.

Such negative results were obtained, for example, in the following experiment:

0.05 cc of pulverized B blood (C.C.) was suspended in 0.1 cc of physiological saline solution and baked 5 min at 100°. 0.1 cc of serum ab (L.L.) was then added, and after
369 incubation for several hours the mixture was centrifuged:

- Absorbed serum + fresh A cells (S.V.) = total agglutination
- Absorbed serum + fresh A′ cells (S.E.) = total agglutination
- Absorbed serum + fresh B cells (C.C.) = negative agglutination

The sediment is washed twice with physiological saline solution at 0°, and afterwards extracted with 0.1 cc of it for 15 min at 45°–50°, then centrifuged while hot:

- Extract from sediment + fresh A cells (S.V.) = negative agglutination
- Extract from sediment + fresh B cells (C.C.) = negative agglutination

(only 3–4 cells appear doubtfully associated with one another).

In order to better investigate the possibility of recovering the absorbed agglutinins even from baked stains, I conducted other experiments using greater quantities of serum, so that the agglutinable substance would be maximally exposed to agglutinin. Thus in the following experiment, the proportions of serum were such that absorption did not turn out to be complete.

To 0.05 cc of B blood suspended in physiological saline solution and baked above 100°, was added up to 0.3 cc of fresh serum ab (L.L) After twenty-four hours, the material was centrifuged:

- Absorbed serum + fresh A cells (S.E. and S.V.) = total agglutination
- Absorbed serum + fresh B cells (C.C.) = slow, weak agglutination

The sediment was washed twice with physiological saline solution at 0° and extracted with 0.05 cc of it at 45°–50° for 15 min:

- Extract of the sediment + fresh A cells (S.E. and S.V.) = negative agglutination
- Extract of the sediment + fresh B cells (C.C.) = evident agglutination (persisting to a 1:3 dilution of the extract).

Very clear results have been obtained using red cells, rather than dried blood, baked at 100°.

0.2 cc of A blood (S.V.) is suspended in physiological solution and washed twice; the liquid is removed as much as possible and the cellular sediment sprinkled with boiling physiological saline solution, then the small tube is immersed into a boiling water bath, where it is left about 10 min.

A shiny, homogeneous suspension is obtained, in which, however, the microscopical examination shows, moreover, the pressence of small amorphous lumps and of a few recognizable cells. This suspension is centrifuged and the sediment resuspended in 0.1 cc of fresh ab serum (L.L.).

After an hour it is centrifuged and prepared in the usual 370
way:

- Absorbed serum + fresh A cells (S.V.) = negative agglutination
- Absorbed serum + fresh A' cells (S.E.) = negative agglutination
- Absorbed serum + fresh B cells (C.C.) = intense agglutination

The sediment was washed and extracted at 45° in the usual way with 0.1 cc of physiological saline solution:

- Extract of the sediment + fresh A cells (S.V.) = strong agglutination
- Extract of the sediment + fresh A′ cells (S.E.) = strong agglutination

Extract of the sediment + fresh B cells (C.C.) = negative agglutination

II. Blood Treated with Chemical Agents

Having withdrawn blood of a determined group, I allowed the washed red blood cells to be in contact with various chemical agents for a time, not less than twenty-four hours, and at an ordinary temperature of 22°. Then I removed by washing or neutralization the substance employed and I proceeded to the tests of absorption and of extraction of agglutinins from the cellular sediment, using the same technique employed in the preceding experiments.

Substances employed: Hydrochloric acid, acetic acid, sodium hydroxide, ammonia, mercuric chloride, silver nitrate, potassium permanganate, potassium dichromate, osmium tetroxide, formaldehyde, ethyl alcohol, ethyl ether, chloroform, acetone.

1. Hydrochloric acid

0.05 cc of blood with A cells (S.V.) are twice washed; the sediment is added to 2 cc of HCl solution at 3.7% (1N). Macroscopical homogeneous suspension: a clear solution is reobtained by centrifugation. After twenty-four hours in HCl, the cells, washed three times, appear shrivelled under a microscope and present the features of discrete agglutination. The addition of agglutinating serum a and b in hanging drops did not modify this appearance.

The absorption test on such cells, twenty-four hours in HCl, washed three times and kept for forty-eight hours at 0° with 0.1 cc of fresh serum ab (S.G.), and centrifuged, gave the following results:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

371 Absorbed serum + fresh B cells (P.G.) = strong agglutination

Another 0.2 cc fresh serum ab (S.G.) is added. After twenty-four hours at 0° it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = weak agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

The sediment is washed three times with physiological saline solution at 0°, resuspended in 0.02 cc of physiological saline solution, and extracted for 15 min at 45°, then centrifuged while hot:

Extract form the sediment + fresh A cells (S.V.) = rapid, almost total agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

2. Acetic Acid

(a) 0.05 cc of blood with A cells (S.V.) is twice washed; to the sediment is cautiously added dropwise 2 cc of glacial acetic acid (rapid addition provokes complete, instantaneous hemolysis) and one gets partial hemolysis with the formation of small reddish-brown membranes and of a few white masses (cellular stroma).

After twenty-four hours the material is washed repeatedly until the odor of the acetic acid is no longer present, and reduced to minute fragments by crushing in the same small tube with a small glass rod. The sediment used in the single tests was about ½ or ⅓ of the initial cellular sediment. To it is added 0.1 cc of fresh serum ab (S.G.). After twenty-four hours at 0° it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = strong aggutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

The decanted serum is absorbed again for twenty-four hours at 0° with 0.05 cc of other A blood (S.V.) from the acetic acid. After centrifugation:

Absorbed serum + fresh A cells (S.V.) = moderate agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

The decanted serum is, for a third time, absorbed for twelve hours at 0° with 0.05 cc of other A blood (S.V.) from the acetic acid. After centrifugation:

Absorbed serum + fresh A cells (S.V.) = weak agglutination

Absorbed serum + fresh B cells (P.G.) = slow, moderate agglutination

The three sediments are combined and 0.4 cc of fresh 372 serum ab (S.G.) is added. After some hours at 0° the sediment is washed three times with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = very strong, rapid agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

(b) 0.05 cc of blood with A cells (S.V.) is dropped onto a microscope slide and kept suspended for twenty-four hours in a closed container containing acetic acid. It acquires a red-brown yellowish color. Then, it is left 1–2 days to dry at ambient temperature. This results in a small, rough, thin layer that smells slightly of acetic acid. It is put in a small tube, and crushed carefully for a long time with a small glass rod, but one obtains a coarse material rather than a powder, afterwards it is washed repeatedly with physiological saline solution and 0.1 cc of fresh serum ab (S.G.) is added. After an hour at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = medium agglutination

Absorbed serum + fresh B cells (P.G.) = slow and strong agglutination

The decanted serum is absorbed again for some hours at 0° with 0.05 cc of A blood (S.V.) as above. After centrifugation:

Absorbed serum + fresh A cells (S.V.) = weak agglutination

Absorbed serum + fresh B cells (C.C.) = slow, moderate agglutination

The decanted serum is absorbed for a third time for some hours at 0° with 0.05 cc of A blood (S.V.), as above. after centrifugation:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = slow, weak agglutination

The three sediments are combined and 0.4 cc of fresh ab serum (S.G.) is added. After some hours the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of the same and extracted for 15 min at 45°, than centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = rapid, very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

/373 3. Sodium hydroxide

(It is impossible to have cellular sediment from concentrated or dilute solutions of NaOH, whether in water or in physiological solution, since red blood corpuscles are hemolyzed, if not immediately (concentrated solution), then during the washing operations).

0.05 cc of blood with A cells (S.V.) are dropped onto a microscope slide and 0.05 cc 0.1N NaOH is overlaid and lightly mixed. It is left twenty-four hours to dry out at ambient temperature. Then the NaOH is neutralized by overlaying a drop of 0.1N HCl and the preparation left to dry out for another twenty-four hours at ambient temperature. This results in a small, hard, fragile layer that is placed in a small tube and pulverized, then is washed repeatedly with physiological saline solution (that of the first wash is slightly colored by hemoglobin. 0.1 cc of fresh ab serum (S.G.) is then added. After few hours, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = rapid, very strong agglutination

Another 0.1 cc of fresh ab serum (S.G.) is added and after twenty-four hours at 0°, the sediment is thrice washed with physiological saline solution at 0°; resuspended in 0.02 cc of physiological saline solution, extracted for 15 min at 45°, and centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

4. Ammonia

(It is impossible to have cellular sediment from concentrated or dilute ammonia solutions, whether in water, or in physiological saline solution, because the red blood corpuscles are hemolyzed.)

0.05 cc of blood with A cells (S.V.) are dropped onto a microscope slide and kept suspended for 3–4 days in a closed receptacle containing ammonia. The stain acquires a brown color and smells of ammonia. Then it is kept for twenty-five hours to dry out at ambient temperature. One obtains a small, hard, fragile layer that no longer smells of ammonia. This is placed in a small tube, and is pulverized. It is then washed repeatedly with physiological saline solution and 0.1 cc of fresh ab serum (S.G.) is added. After twenty-four 374 hours at 0° it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

0.1 cc of other fresh ab serum (S.G.) is added and after twenty-four hours at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of physiological saline solution, extracted for 15 min at 45°, then centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

5. Mercuric Chloride

0.05 cc of blood with A cells (S.V.) is washed twice; the sediment is treated for four days with 2 cc of 5% sublimate. Macroscopically, brown homogeneous suspension. Under the microscope the cells show altered form: lance-shaped biscuits; but they are all isolated from one another. The sediment is washed until all trace of reaction of the Hg disappears from the washing liquid, and is then suspended homogeneously in physiological saline solution. This suspension treated with sera a and b shown to contain the specific isoagglutinable property.

The absorption test on 0.05 cc of such A blood (S.V.) from the sublimate, kept for twelve hours at 0° with 0.1 cc of fresh ab serum (S.G.) and then centrifuged, gave the following result:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

0.2 cc of the same ab serum is added and after twenty-four hours at 0°, and being centrifuged:

Absorbed serum + fresh A cells (S.V.) = almost negative agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

The sediment is washed twice with physiological saline solution at 0°, resuspended in 0.03 cc of the same, kept for 15 min at 45° and centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = rapid, very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

Identical results were obtained in absorption tests with cells having remained for two months in 5% sublimate solution, and with *fresh ab serum* (S.G.).

In other experiments in which I employed cells that had

been left four days in sublimate, and *old ab serum* (L.L.), kept in a vial, but still strongly agglutinating for A and B cells in control experiments, the results were a little bit different in that they revealed a diminution in the specificity
375 of the absorption, not, however, confirmed by the test for extraction of the agglutinins. In fact the A cells showed an absorption of the b agglutinin (for which they do not normally have any affinity) but to a minor degree, without, however, yielding it up in the absorption test.

6. Silver Nitrate

0.05 cc of blood with A cells (S.V.) are washed twice; to the sediment is added 2 cc of 1% silver nitrate. Macroscopically: shiny, coarse suspension. Under the microscope: spherical cells reunited in large clumps.

After repeated washing, first with distilled water, then with physiological saline solution, is added 0.1 cc of fresh ab serum (S.G.). After twenty-four hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = slow, medium agglutination, more marked at the borders

0.1 cc of fresh ab serum (S.G.) is added and after some days at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of physiological saline solution, extracted for 15 min at 45°, and centrifuged while hot:

Extract from the sediment + fresh A cells (S.V.) = very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

7. Potassium permanganate

0.05 cc of blood with A cells (S.V.) are washed twice; to the sediment is added 2 cc of 2.4% potassium permanganate: coarse suspension.

After repeated washing with physiological saline solution is added 0.1 cc of fresh ab serum (S.G.), and after twenty-four hours at 0° it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = negative agglutination

0.1 cc of other fresh ab serum (S.G.) is added and after some hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = negative agglutination

0.3 cc of other fresh ab serum (S.G.) is added and after
376 24 hours at 0°, the sediment is thrice washed in physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and is centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = negative agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

8. Potassuim dichromate

0.05 cc of blood with A cells (S.V.) is washed twice; to the sediment is added 2 cc of 6.7% potassium dichromate (in other experiments, 3% was used with identical results). Macroscopically, a shiny, finely granulated suspension. Under the microscope, spherical cells are reunited into large clumps, simulating agglutination. After repeated washing with physiolgical saline solution is added 0.1 cc of fresh ab serum (S.G.). After 12 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

0.1 cc of other fresh ab serum (S.G.) is added, and after some hours at 0° the sediment is washed three times with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract of the sediment + fresh A cells (S.V.) = strong agglutination

Extract of the sediment + fresh B cells (P.G.) = negative agglutination

9. Osmium tetroxide

0.03 cc of blood with fresh A cells (S.V.) is washed twice; to the sediment is added 2 cc of 1% osmium tetroxide. Macroscopically homogeneous suspension of brownish color after a few minutes, and a few clumps. After 24 hours, the cells are washed three times with physiological saline solution. Under the microscope the cells appear in various forms: Lance-shaped and faceted but perfectly isolated from the other. Treated separately in hanging drops with serum a and serum b, loss of the specificity of isoagglutination is observed, in that both the sera agglutinate the cells intensely, though the a serum does so in greater measure. The absorption test, however, of 0.05 cc of such blood with A cells (S.V.), treated as above, kept for 24 hours at 0° with 0.1 cc 377
of fresh ab serum (S.G.) and centrifuged, gave the following results:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

0.1 cc of other fresh ab serum (S.G.) is added, and after 24 hours at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = negative agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

I obtained identical results after fractional absorption

with two equal portions of cells, each corresponding to 0.05 cc of blood, using blood fixed with osmium tetroxide vapors, according to the following technique:

0.05 cc of blood with fresh A cells (S.V.) is dropped onto a microscope slide, kept suspended for twenty-four hours in a vessel containing some 1% osmic acid then allowed to dry out for twenty-four hours. This gives a thin layer that is ground up in a small tube, washed three times with physiological saline solution and added to the absorbing serum.

10. Formaldehyde

0.05 cc of fresh blood with A cells (S.V.) is twice washed; to the sediment is added 2 cc of 5% formalin in physiological saline solution. Macroscopically, homogeneous suspension of brownish color after a few minutes. The cells thus fixed for six hours, and then washed three times with physiological saline solution, appear under the microscope to be perfectly preserved in their form. Treated separately in hanging drops with a serum and b serum, the specific isoagglutinability has been preserved, and is of an intensity similar to that of fresh cells. For the absorption test, 0.05 cc of formalin-treated blood with A cells (S.V.), three times washed, is added to 0.1 cc of fresh ab serum (L.L.). After some hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = rapid total agglutination

After some hours of incubation in another 0.2 cc of the same serum, by the end of which the a agglutinin is com-
378 pletely absorbed, the sediment is washed twice with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = almost total agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

I obtained identical results with cells kept in fixing solution for twelve days, or else fixing the cells with vapors of formaldehyde in the following way:

0.05 cc of blood with fresh A cells (S.V.) is allowed to coagulate on a microscope slide that is kept suspended for three days in a vessel containing some formalin. Then it is dried out for 24 hours, in order to facilitate the pulverization of the stain. This done, it is washed twice with physiological saline solution and the absorption and extraction of agglutinins carried out using techniques and obtaining results the same as the preceding.

11. Ethyl Alcohol

0.05 cc of blood with A cells (S.V.) is twice washed; to the sediment is added 2 cc of alcohol at 95°. Macroscopic appearance: large clumps. To this sediment, having remained in alcohol 48 hours and then having been washed three times with physiological saline solution is added 0.1 cc of fresh ab serum (S.G.). After 60 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = strong agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

The decanted serum is added to another 0.05 cc of A blood (S.V.) which was washed twice and kept for four days in 2 cc of alcohol, then washed three times with physiological saline solution. After 24 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = moderate agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

The decanted serum is added again to another 0.05 cc of A blood (S.V.) which was twice washed and treated for six days in 2 cc of alcohol, then washed three times with physiological saline solution. After 24 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = moderate agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

The three sediments are combined and added to 0.2 cc of fresh ab serum (S.G.). After twenty-four hours we proceeded with the usual extraction:

Extract from the sediment + fresh A cells (S.V.) = 379
very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

Such experiments were repeated even with cells which remained in alcohol for a month and prolonging for up to several days the contact of the absorbing serum with successive fractions of cells. The results were the same as those mentioned above, in the experiments in which fresh ab serum (S.G.) was employed. In two other experiments, on the other hand, in which old serum (L.L.) was employed, like that used in the experiments with the sublimate (see above), a diminshed degree of specificity of absorption was also shown, the A cells having absorbed and yielded up both the agglutinins, but in different measure: the a agglutinin totally but the b agglutinin only partially, as was shown by the marked difference in the intensity of agglutination with the A and B test cells.

12. Ethyl ether

0.05 cc of blood with A cells (S.V.) are washed twice; the sediment is added to 2 cc of ether. Macroscopically, the cells appear attached, in amorphous clumps of a pale brown color, to the walls of the small tube. After 24 hours, it is washed repeatedly with physiological saline solution, and 0.1 cc of fresh ab serum (S.G.) is added. After 24 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = very strong agglutination

There is added 0.1 cc of fresh ab serum (S.G.) and, after

some hours at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = almost total agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

I obtained identical results with cells exposed in the following way to the vapors of ether:

0.05 cc of blood with A cells (S.V.) is dropped onto a microscope slide and kept for three days in a closed container containing some ether. Then it is left to dry 24 hours at
/380 ambient temperature; a thin, hard, fragile layer in obtained, which is placed in a small tube and pulverized. It is washed repeatedly, and the experiments on the absorption and extraction of the agglutinins are carried out by the usual technique.

13. Chloroform

0.05 cc of blood with A cells (S.V.) is washed twice; the sediment is treated for twenty-four hours with 2 cc of chloroform. Macroscopically, coarse suspension. It is washed repeatedly with physiological saline solution, and 0.1 cc of fresh ab serum (S.G.) is added. After 24 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = rapid, very strong agglutination

0.1 cc of other fresh ab serum (S.G.) is added and after some hours at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = strong, agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

I obtained identical results with cells exposed to chloroform vapors in the following way:

0.05 cc of blood with A cells (S.V.) is dropped onto a microscope slide, and kept for twenty-four hours in a closed container containing chloroform. Afterwards it is dried out for four days at ambient temperatures yielding a thin, fragile layer that is placed in a small tube and pulverized. It is washed repeatedly and the absorption and extraction of agglutinin experiments are performed in the usual way.

14. Acetone

0.05 cc of blood with A cells (S.V.) are twice washed; the sediment is added to 2 cc of acetone. Macroscopically, coarse suspension. After 24 hours, it is washed repeatedly with physiological saline solution and 0.1 cc of fresh ab serum (S.G.) is then added. After 24 hours at 0°, it is centrifuged:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (P.G.) = total agglutination

0.1 cc of other fresh ab serum (S.G.) is added and after 381/
some hours at 0°, the sediment is thrice washed with physiological saline solution at 0°, resuspended in 0.02 cc of the same, extracted for 15 min at 45°, and centrifuged in the heat:

Extract from the sediment + fresh A cells (S.V.) = very strong agglutination

Extract from the sediment + fresh B cells (P.G.) = negative agglutination

I obtained identical results with cells exposed to acetone vapors in the following way:

0.05 cc of blood with A cells (S.V.) is dropped onto a microscope slide and kept for twenty-four hours in a closed container containing acetone. Afterwards it is dried out for four days at ambient temperature: a fragile, reddish-white, thin layer is obtained, which is placed in a small tube and pulverized. It is repeatedly washed and the absorption and extraction of the agglutinins is then carried out with the usual technique:

III. Blood Devoid of Isoagglutinins

Finally I carried out experiments on dried blood containing the two agglutinable substances A and B, namely, belonging to the group which never shows any isoagglutinins in fresh stains.

0.05 cc of dried AB blood is pulverized and suspended in 0.3 cc of fresh ab serum (L.L.). After ten hours:

Absorbed serum + fresh A cells (S.V.) = negative agglutination

Absorbed serum + fresh B cells (C.C.) = negative agglutination

The sediment is thrice washed with physiological saline solution at 0°, and extracted at 45° with 0.05 cc of physiological saline solution:

Extract from the sediment + fresh A cells (S.V.) = strong agglutination

Extract from the sediment + fresh B cells (C.C.) = moderate agglutination

As controls for some of the various experiments described, I even tried to keep dried A blood in contact with serum b, and I could not, as was to be expected, observe any absorption; and even from the washed sediment I could not recover 382/
any trace of agglutinin.

IV. Old Blood

I had the opportunity to apply these experiments to a practical case of determining the isoagglutinable substances in old human bloodstains seen in connection with a crime.

In these stains, dating back 18 months, Professor Lattes had been successful, some months before, in demonstrating the a agglutinin, now strongly attenuated. The demonstration of a specific isoagglutinable substance would constitute in this case an effective, appropriate control.

The multiple stains were crusted on the material of a beret: several of them were scraped off, carefully pulverized, and suspended in 0.1 cc of fresh serum (L.L.), diluted 1:3 in physiological saline solution. After ½ hour, it is centrifuged:

Absorbed serum + fresh A cells = total, immediate agglutination

Absorbed serum + fresh B cells = nearly negative agglutination, with some small groups from cells lying on one another

(The unabsorbed ab serum immediately and intensely agglutinated the A and B cells).

The test of heat extraction of the sediment, washed at ordinary temperatures, did not yield results; nevertheless, with only the above-mentioned test the presence of a substance in the stain able to absorb the b agglutinin could be demonstrated, consistent with the report of a agglutinin previously obtained. The blood under examination could thus be assigned to group aB.

Conclusions

From the above experiments results the biologically interesting fact that the antigens of the blood, to which the isoagglutinins are bound, preserve this property, unaltered, after baking at 100° (by analogy to certain microbial antigens with respect to specific agglutinins), and even when the dried out blood has undergone the prolonged action of time.

/383 Further, in examining the action of various chemical agents, such power is conserved. Thus it is so with the following substances: hydrochloric acid (1N solution), sodium hydroxide (0.1N), ammonia (vapor), mercuric chloride (5%), silver nitrate (1%), potassium dichromate (3-6.7%), osmium tetroxide (1% and vapor), formaldehyde (5% and vapor), chloroform (100% and vapor), ethyl ether (100% and vapor), acteone (100% and vapor).

Some substances (acetic acid and ethyl alcohol) although not to completely abolishing the selective absorption, weakened it, although with the acetic acid it was not possible to stabilize (see the experiments) that part of the decay due to the modification of the isoagglutinable substance nor the cellular destruction (acetic acid in the liquid state) nor the coarseness of the pulverized material (vapors).

Still other substances (2.4% potassium permanganate) completely abolish all selective absorption.

The diminution of the degree of specificity of absorption recounted in some experiments (sublimate, alcohol) is not attributable to the denaturing treatment on the cells, but seems more properly to have to do with the serum.

In fact, the phenomenon occurs consistently every time old serum in employed. In the very few cases in which the same fact is seen with fresh serum (silver nitrate and acetic acid vapors) it could be due rather to true absorption with damage to the agglutinins, due to the prolonged contact with cells loaded with denaturing substance.

Indeed, completely negative results were always obtained in such experiments (in spite of the accuracy of the technique and the previous addition of ab serum in excess) with the opposite test, that of the extraction of those agglutinins, apparently fixed in part to the cells in the absorption test in a way contrary to the group specificity.

From a practical point of view, the possibility emerges of being able to identify the isoagglutinable substance in dried blood, which has been rendered insoluble by age, excessive heat or the influence of numerous chemical substances.

The best results are obtained by allowing the least possible quantity of a fresh ab serum to act upon the pulverized blood substance (most appropriately for several hours, shaking frequently) and sampling, after centrifugation, the residual isogglutinating properties. Blood A absorbs the a agglutinin, blood B the b, blood AB, both. When, after prolonged absorption for twenty-four hours, the two agglutinins were still active, but to a different degree (this a sign of incomplete absorption) it was possible, where there is enough of the stain, to render the test completely specific, by a second absorption with a new dose of pulverized blood. A confirmation of the specific absorption, though less constant for the sensitivity of the technique, can be achieved by extracting the agglutinins at 45°, which were fixed in the substance of the stain in the previous test, and by then determining their nature by reaction with fresh cells of a specific group (A and B). In order to facilitate this test it is necessary to suspend the pulverized stain in an excess of ab serum and to carry out the necessary washing with physiological saline solution at 0°. It is advisable to use fresh ab serum, with which more precise and specific results are obtained.

In conclusion, even when one does not find isoagglutinins in the stains, either because of the group to which the blood belongs, or because of their destruction, the demonstration of their individual orgin, it is nevertheless very often possible to demonstate their individual origin (their group), by means of identification of the isoagglutinable substance contained in the stains.

I thank Professor Lattes for the suggestions he gave me and for the constant assistance rendered me during the execution of this research.

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A Simple Procedure for the Determination of Groups in Dried Blood by Agglutinin Binding*†

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/445 Whenever one writes or speaks about the importance of blood groups in forensic medicine, it immediately gets down to the question of determining blood groups from dried blood traces. In fact, that ought to be one of the most important problems for the forensic physician. Though the problem is solved now, the situation is unfortunately still bad. This results from the fact that the number of communications concerning this problem is in crying disproportion to its importance, and many of these studies misunderstand the other problems in the area of blood group research.

We ought not to assume that few researchers have tackled this question; the explanation lies rather in the old observation that one does not report on his failures. We have earnestly striven here to make some progress, and we believe that we can report today on some essential successes.

The identification of the characteristics of blood corpuscles and serum usually causes no difficulties in the case of fresh blood. If the dried blood is not too old, the substances are indeed present on which the peculiarities of the groups are based, but here we are faced with the difficulty that the encrusted blood corpuscles can no longer be separated as the unsuccessful experiments which Biffi set up in this direction have demonstrated. Thus, we are no longer able to detect agglutination.

In order to base a blood group identification on these blood spots, a whole series of processes have been offered, all of
/446 which rest on two fundamental ideas. The one is to get the agglutinins in solution by dissolving the blood spot and then to test this liquid in the same manner as serum with test blood cells known to belong to a certain group. On this principle rest the processes of Landsteiner and Richter, de Domenicis, and Lattes. Landsteiner and Richter have claimed that, with this test, the blood group can be determined from a dried blood stain up to four months old.

Though the picture of clumping about the blood flake is a neat one, nevertheless, in the majority of cases this method has failed. Even with blood stains no more than a week old, clumping failed to take place in half the cases. With older stains we had only isolated cases of success.

The assertions of Müller of the Zurich surgical clinic, together with Brunner, concerning the agglutinin enrichment process seemed to us most enticing.

He produced extracts stronger in concentration than in the original blood and thought thereby that he could extract a smaller quantity of more powerful substances from a larger quantity of inferior-grade initial substances. According to Goroncy's report, Muller leached the blood stain at a temperature of 0° in order to eliminate the autoagglutination. Dried swabs served for the experiment.

To dissolve the dried blood he recommended a weak, saline solution of 0.2 to 0.3 percent, more than distilled water, observing that better leaching was to be obtained with a saline solution. He took the solution in an ample quantity and concentrated it in a vacuum at 16° to 19° to the consistancy of syrup. He stored the concentrated residue in an icebox until he was ready to carry out the test. If the concentration goes too far, as can easily happen, the syrup can be diluted again somewhat in order not to have a salt content which could disturb the test. Müller especially stresses that only lecithin blood corpuscles are to be used. Since in the case of weak agglutinins the clumping often makes its first appearance after a long period, Müller keeps the slides in a moist chamber. Experiments to free extracts from hemoglobin by means of animal charcoal were unsuccessful in that Müller thereafter produced no clumping. In order to test the authenticity of the clumping, Müller depends not on a cover slip as Lattes did, but he tests with O-blood corpuscles whose clumping proves autoagglutination.

According to their report Müller and Brunner have been able to establish the original blood group in seventy percent of eighty dried samples which were up to eighteen months old. Thus, age definitely plays no role in this test. As Popoff reports, Serebrjaikow, whose work was not available to me, has described a procedure very similar to that of Müller.

With great hopes we tried Müller's process with our collection of swabs. Though we followed exactly the prescrip- 447/
tions we were unsuccessful.

* Translation of: "Ein einfaches Verfahren zur Gruppenbestimmung an vertrocknetem Blut durch Agglütininbindung".

in *Deutsche Zeitschrift für die Gesamte Gerichtliche Medizin* 16: 445-458 (1931).

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† Delivered at the 19th meeting of the German Society for Legal and Social Medicine in Königsberg, November 12, 1935.

We returned again to the properties of the blood corpuscles. Since in the agglutination of blood corpuscles agglutinins are taken out of the blood fluid, it is a closely related concept that the agglutinogen in dried blood can be identified by its ability to bind with a specific agglutinin.

There exist a number of reports, especially in the treatices, concerning the methods to identify groups, though, as mentioned by way of introduction, very little is said about the employing of such processes. Schiff and Higuchi provided the most exact prescriptions.

Compared to the processes of distinguishing the blood group of fresh blood, these are certainly intricate and lengthy so that false notions about the difficulty of the undertaking and about the quantity of blood required for the test frightened us at first, as it probably did many others.

The process is based essentially on the following: a serum of group O with the agglutinins α and β or a serum β and a serum α are treated separately or in a mixture with the dried blood stain whose group identity is unknown. Afterwards the sera are tested to see if their agglutinin content has remained the same or has altered. If agglutinins are used up, then the corresponding agglutinogen was present in the blood spot. In this process the strength of the agglutinins of the test serum, a strength which varies extraordinarily from person to person, plays a decisive role.

After we had learned in experiments with fresh blood corpuscles about the necessary quantitative relationships to produce a sufficient absorption, we went on to experiments with dried blood. Blood in a dried state is estimated in general to weigh about ⅙ that of fluid blood. Schiff estimated that, to absorb an agglutinin, ⅝ of its weight in fresh blood is necessary. If we hold to his estimate, then, given that blood dries to approximately ⅙ of its weight (Hammarsten's and our own experiments) and given that the agglutinogen does not essentially decrease in the drying process, a quantity of dried blood from ⅛ to 1/10 of the serum mass would be sufficient for absorption, i.e., for 0.1 cc serum, approximately 0.01 g of dried blood. This hypothesis, derived from calculations, was completely confirmed by experiments.

From pulverized dried blood 1/15 of the serum mass (0.02 g dried blood to 0.3 cc serum) is sufficient to take up almost all
448 the agglutinin. Even 1/20 to 1/30 are still clearly able to reduce the agglutinins.

After many experiments the following process proved to be the best to determine blood groups from dried blood samples. Finally we use it exclusively.

In small test tubes 6 cm long and 5 to 6 mm wide, 10 mg of dried blood are placed. If blood on swabs is involved, then from a selection of thoroughly blood-soaked swabs, the same amounts are weighed out in the same fashion — the weight of the fiber is not to be considered at all. Then the serum, which has been measured out before the experiment, is introduced into the test tubes with a graduated syringe. Using a syringe has the advantage of speed over filling with a graduated pipette.

All the test tubes in the test series are shaken hard once and then placed for twenty-four hours in the icebox. Thereafter, these are centrifuged and a small amount of the liquid is siphoned off with fine, hand-drawn pipettes for the new evaluation. As the best test sera, we select such serum as contains almost exactly the same quantity of agglutinins α and β compared to our constant test blood sample. The essential part of our method is the type of evaluation of the serum and the preparation of the dilutions which are required for such evaluation.

The evaluation was carried out on glass plates with eight concave depressions such as those introduced for the evaluation of sera in the institute by my director, Professor Meixner.*

In carrying out these experiments our need for plates increased greatly. We were able sometimes to meet our needs by cementing to a standard 9 × 12 glass plate four microscope slides, each with two concave depressions, which also reduced our costs considerably. It is easier and cleaner, of course, to work with ready-made plates.

Dilutions of the serum, proceeding according to the power of two, is produced in the depressions of the glass plates in the following way. First, in all the cavities four drops of physiological saline solution are added with the same fine pipette. Then four drops of the serum to be evaluated are dropped into the first niche and are subsequently mixed carefully by repeatedly drawing up and ejecting the fluid with the pipette, while at the same time stirring the fluid. Then we carry over four drops of this mixture into the next tray; the rest is ejected back into tray one. So, in the same way, the dilution is carried through until, in the eighth cavity, we have reached a dilution of 256. Thereupon, from each niche of this first plate, half of the contents, i.e., two drops, 449
are transferred into the corresponding cavities of the second plate. The one plate serves for the test with A-blood corpuscles, the other for the test with B-blood corpuscles. From a two-percent suspension of these we add a drop to each depression. We label the one plate with A, the other with a large B. We have accustomed ourselves always to place the A plate on the right in order to avoid mix-ups. Having reached a level of dexterity through practice, I now require five minutes at the most to set up the evaluation experiment from the introduction of the saline solution to the addition and stirring of the test blood corpuscles. With good pipettes I need only three minutes.

Immediately after mixing in the test blood corpuscles, the reading times are notes in ink or wax crayon on the plate.

The first reading is taken after ten minutes, reckoning from the time the test blood corpuscles were added, the second after 30 minutes (Fig. 1 [not reproduced in the translation]).

In the determination of endpoint titer the same process is followed. Naturally, the same blood corpuscles must be used, since blood corpuscles also display a widely varying sensitivity, so that some are easily agglutinated while others

* The plates can be obtained from the Siebert Co. in Vienna for 5 schillings.

are not, a fact which numerous experiments have confirmed.

Occasionally the final yield is too small, forcing an experiment with half the quantity of serum. Quite often the tubes were placed again into the icebox and the experiment repeated after several days. In the icebox, very little of the fluid is lost through evaporation. We also noticed that an overgrowth of germs, which was indeed present in the dry samples, impaired the results.

In every test series, a tube filled only with serum was also placed in the icebox and was evaluated together with the
450 others in order to reveal any reduction in titer from another cause. By this means we found in a few cases an unimportant reduction, at the most a single dilution. Such a small reduction, however, was not sufficient to prove agglutinin binding.

Our experiments were conducted at first with samples of dried blood whose blood group had already been determined beyond a doubt as fresh blood. The blood samples came for the most part from the clinics where in most cases we immediately soaked a swab with blood; some samples I collected myself. 330 such tests were undertaken. In addition, we examined old, dried blood samples whose blood group was not established in the fresh blood, among which were samples over fifty years old. We have conducted 100 such experiments to date. We will discuss these later. Here let us emphasize that a clear reduction in binding capacity could not be established in cases of these old blood stains.

Table 1 shows a section from an experimental series. The cases have not been selected for any particular reason, but they are reproduced as they were examined. We are dealing here with samples, all of which were at least eight months old. The series was set up with a serum of group O of small and unequal titer. The bold face numerals† indicate a reading taken after thirty minutes, those enclosed in parentheses the reading taken under the microscope, a process carried out with all the samples, producing values one dilution higher. The decision regarding the blood group was reached only through a comparison of the final titration with the initital titer, and afterwards, the results were compared with the blood group determined by tests with fresh blood. The table shows that the group determined with dry samples is in complete agreement in these cases.

† Values in boldface type in the original article are italicized in the translation.

Table 1

	O Serum, Reinstadtler, Alois Sept. 3, 1929					Initial Titer		Reduction in titer in dilution steps		
						10	4(8)	8(32)		
						30	*4*(16)	*16*(32)		
Dried Blood	Serum (cc)	Person's age (yr)	Age of blood (mo)	Weight (mg)	Blood Group (determined in fresh blood)	Time of reading (min)	Final Titer A	Final Titer B	with A	with B	Conforms to blood group
Josef H.	0.1	30	8	15	AB	10	0(0)	0(0)	2	3	AB
						30	*0*(2)	*2*(4)			
Albin V.	0.1		9	15	B	10	4(8)	0(4)	0	4	B
						30	*4*(16)	*0*(2)			
Franz K.	0.1	51	8	15	O	10	8(16)	16(32)	0	0	0
						30	*8*(16)	*16*(64)			
P . . . (app.)	0.1	5	8	15	A	10	0(0)	16(32)	2	0	A
						30	*0*(2)	*16*(64)			
Müller J. .	0.1	30	8	15	A	10	0(0)	16(32)	2	0	A
						30	*0*(4)	*32*(64)			
Johann Ko.	0.1		8	15	B	10	4(8)	0(0)	0	4	B
						30	*4*(8)	*0*(4)			
Siegfried H.	0.1	16	8	15	O	10	4(8)	16(32)	0	0	O
						30	*4*(16)	*32*(32)			
Adolf N.	0.1	16	8	15	A	10	0(2)	16(16)	2	0	A
						30	*0*(4)	*16*(16)			
Frau X.A.	0.1		8	15	A	10	0(2)	16(32)	2	0	A
						30	*0*(2)	*32*(64)			
Wilhelm St.	0.1	34	8	15	A	10	0(0)	16(32)	2	0	A
						30	*0*(2)	*32*(32)			
Wilhelm Tö	0.1		9	15	O	10	4(8)	16(16)	0	0	O
						30	*4*(16)	*16*(32)			
Franz Wi. .	0.1	46	8	15	B	10	4(8)	2(4)	0	3	B
						30	*4*(16)	*2*(4)			
Franz E	0.1	81	8	15	O,	10	4(8)	16(16)	0	0	O
						30	*4*(8)	*16*(32)			
Johann Dob.	0.1	45	8	15	O	10	8(8)	8(16)	0	0	O
						30	*8*(16)	*16*(32)			

Table 2

O-Serum, Müller, Fr. October 8, 1929							Initial Titer		Reduction in titer in dilution steps[1]		
						10 30	16(32) *32*(32)	16(32) *32*(64)			
Dried Blood	Serum (cc)	Age of person (yr)	Age of blood (days)	Weight (mg)	Blood Group (determined in fresh blood)	Time of reading (min)	Final Titer A	Final Titer B	with A	with B	Conforms to blood group
Olga K	0.1	—	8	10	A	10	0(0)	16(16)	5	1	A
						30	*0*(0)[1]	*16*(32)			
Heinr. Pr.	0.1	—	8	10	O	10	16(16)	32(64)	1	1	O
						30	*16*(32)	*32*(64)			
Marcia Gr.	0.1	—	8	10	B	10	16(16)	2(4)	1	4	B
						30	*16*(16)	*2*(4)			
Maria Hirz.	0.1	23	8	10	B	10	8(16)	0(2)	1	5	B
						30	*16*(16)	*0*(2)			
Maria K..	0.1	21	8	10	B	10	16(16)	2(4)	1	4	B
						30	*16*(16)	*2*(4)			
Herbert Ph.	0.1	—	8	10	A	10	0(0)	8(16)	5	2	A
						30	*0*(0)	*8*(16)			
Josef Kner.	0.1	—	8	10	O	10	*16*(16)	16(16)	1	1	O
						30	*16*(16)	*16*(32)			
Schreyer	0.1	—	8	10	B	10	16(16)	2(4)	1	4	B
						30	*16*(16)	*2*(4)			
Kohlh.	0.1	—	10	10	A	10	2(2)	32(32)	4	0	A
						30	*2*(2)	*32*(32)			
Johanna Gr.	0.1	—	10	10	O	10	8(16)	32(32)	1	0	O
						30	*16*(16)	*32*(64)			
Gretl B	0.1	—	10	10	A	10	0(0)	16(32)	5	0	A
						30	*0*(2)	*32*(64)			
Sauter	0.1	—	10	10	B	10	8(16)	4(4)	1	3	B
						30	*16*(16)	*4*(8)			
Mttre	0.1	—	10	10	O	10	16(16)	32(32)	1	0	O
						30	*16*(32)	*32*(64)			
Karl H.	0.1	—	10	10	A	10	0(0)	4(8)	5	2	A
						30	*0*(0)	*8*(8)			
Unterg	0.1	—	10	10	O	10	16(16)	16(32)	1	0	O
						30	*16*(32)	*32*(64)			

[1] Since we started with a dilution of one-half, zero means that clumping was no longer visible by the second dilution step
[2] Reading by naked eye after 30 min.

Table 2 comes from an experimental series conducted with dried blood in its second week. Here the experiment was carried out with higher-potency serum having almost identical titers for A and B corpuscles. When using lower-grade serum the binding sites of a dried blood sample are frequently not completely saturated. When more potent serum is used, the binding sites can be completely saturated, and thus more decisive results are obtained, thereby bringing about greater certainty of interpretation. Even if the weak sera were completely deprived of agglutinins and the stronger serum, either in 1:2 dilution or undiluted, still agglutinated, the lowering in the case of the latter was still more pronounced than in the case of the weak serum with its binding capacity completely used up. Still more exact experiments were set up in the following manner. Eight drops of sera with various high titers (unfortunately no especially potent sera were at my disposal) were each mixed with one drop of washed blood corpuscles which were as free as possible of liquid. These are shaken and left to stand for a certain length of time at room temperature. They are then shaken once more and centrifuged. Then the serum is measured against the identical quantity of the same test blood corpuscles. Here again it is clear that extremely low-potency sera 2(4), 4(8), were as a rule completely deprived of agglutinins, and were no longer capable of causing agglutination. Sera, however, with higher titers, 16(32), 64(64), were after saturation so exhausted that they only agglutinated further at full or half strength. With weak sera the binding capacity of the blood corpuscles added does not fully come into play. This is shown by special experiments where blood corpuscles, treated with weak serum and then added to fresh serum, still extracted agglutinins from the fresh serum. 451/ 453/

By combining the results of all the binding experiments, the following picture emerges. Agglutinins of titer 8 were reduced by a blood corpuscles on the average 2.7 dilution steps, by B blood corpuscles on the average 2.4 dilution steps; agglutinins of titer 16, on the other hand, were reduced on the average 3.5 degrees of dilution by A and 2.8 degrees of

dilution by B corpuscles. It is, therefore, recommended that high-potency sera be used for agglutinin binding, though, of course, the quantity of serum in these cases ought not to be too large.

As is apparent from the above figures, we found a stronger agglutinin binding produced by A than by B. Several unsuccessful attempts with samples of dried blood also corroborated this finding. Thus, out of seven samples of dried blood which Professor Lattes kindly gave to us, we incorrectly identified the 2 B bloods as O. Even repeating the experiment, this time leaving the samples for a longer time in the icebox, produced only a small weakening of β.

We achieved better success with eight samples which were sent to us from the Vienna Institute of Forensic Medicine. Of these eight we were able to identify all of them correctly.

With 56 samples on filter paper which also were kindly given to us by the Vienna Institute, we made twelve incorrect identifications, i.e. 21.4%. In one of these cases the binding of β by B failed to occur, whereas eleven times a false binding of α without A occurred, and one time a false binding of β without B.

The most unwelcome false reaction was the binding which made one think of a binding caused by the substratum. We undertook experiments to examine this problem, using various types of flour, potatoes, rice starch, other parts of plants, sand, cloth, cotton, different types of paper, and similar objects. Using filter paper, these experiments, in fact, produced insignificant binding of the agglutinin. Recently we obtained relatively strong binding with blood-soaked mud, while less contaminated samples of the same blood did not produce false reactions. Cloth and cotton-wool have so far shown themselves to be harmless. In all cases one must be cautious and should always test the substratum for its binding capacity.

All together we tested 387 cases, including the dried samples of Lattes and those from Vienna. We correctly identified 366 samples, which represents a success rate of over 90%.

/454 Finally, with the method here presented we tested old blood stains which were found among the *corpora delicti* of our institute. These blood stains were on tools of every sort, and also on bullets, clothes, parts of plants, and earth. Finally, there was dried blood which had been stored for other reasons years and even decades earlier. Here, too, the results of the experiments were suprisingly good in that we obtained, in a large number of cases, a clear binding of one or of both agglutinins. Naturally, it was not possible to show whether this binding corresponded to the blood group. One should notice, however, that the binding occurred in the same strength and in the same time as with samples which were not as old. Moreover, though only 100 cases were tested, the comparative numbers of the blood groups determined by the test agreed pretty well with the distribution of blood groups in our population (see in Table 3).

Table 3. Distribution of Blood Groups

Results of examination of 100 old samples		Percent in roughly 2000 diagnoses in Innsbruck based on double determinations
group	*percent*	*percent*
O	38	41.95 (42)
A	28	43.15 (43)
B	12	10.22 (10)
AB	10	4.68 (5)
uncertain	12	—

The examination of several blood stains on a cudgel which was used forty years earlier in a robbery-murder, produced a varied binding, which indicated blood of different groups. This result was explained without further ado since two criminals, one of whom carried this club, had killed four people in the attack.

In agreement with Siracusa who reported that it is still possible to demonstrate blood groups when dealing with blood treated with alcohol and formalin, we were successful in using our procedure on pieces of a collection which had, for the most part, been treated following Kaiserling's method. We intend to follow this line still further by checking the blood group diagnosis of old *corpora delicti* by determining the blood group of the corresponding cadaver sections which have been preserved.

An especially important question in the absorption process is the significance of the test results. We repeatedly saw that even blood corpuscles of group O reduced their titer by a small amount, although usually only one degree of dilution. So too we saw that blood corpuscles of group A and B were able to weaken slightly the other agglutinin, which was confirmed by the experiences of Thomsen and Worsaae.* Thomsen not infrequently observed a reduction of the other agglutinin by half through the action of the opposite agglutinogen, especially in the case of sera of not very high titer. 455/

This relationship likewise struck us. One can easily explain this, as Thomsen and Worsaae indicate, in the following manner. To reduce the agglutinin content by a half in the case of a high-potency (agglutinin-rich) serum, much more agglutinogen is necessary than with weak (non-agglutinin-rich) serum. For this reason high-potency serum is certainly to be preferred, serum in which the binding of the opposite agglutinin makes a difference of only a fraction of a degree of dilution. By examining this relationship, Thomsen and Worsaae have found that the "connection of the two agglutinins in the O serum is only an apparent one, that rather the heterologous agglutinin is secondarily bound to the complex of blood corpuscle and homologous antisubstance".

A small reduction in titer, therefore, allows no conclusion. Occasionally, we have not taken notice of a two-dilution step reduction, especially when the other agglutinin was greatly reduced.

If one has enough blood, one can endeavor to clarify the

*Oluf Thomsen and E. Worsaae, Über die Möglichkeit eines Zusammenhanges zwischen den im Serum der O-Gruppe enthaltenen Isoagglutinen Anti-A (α) und Anti-B(β). *Z. Rassenphysiol.* 2, No. 1 (1929).

results in doubtful cases by repeating the experiment with different sera or by allowing the mixture to stand for a longer time.

If the determination of the blood group based on the binding to agglutinogen is possible in the case of fresh blood only with a certain error rate, we ought not to be surprised that the error factor is greater with altered blood. Our goal must naturally be to get at the agglutinin in old blood. In a large number of cases, therefore, we have also carried out the Lattes-test along side of the process described here. Unfortunately, as we have already stated, this test was mostly without success.

Hôdyo maintains that faeces binds agglutinin in a similar way as blood does, which would naturally be of great significance. Experiments which we set up to examine this effect, however, have shown no correspondence with the blood; rather they have indicated an irregular and non-specific binding, which constantly changes even in the case of stool samples from one and the same person. Sometimes they have displayed a total disturbance of the agglutinins.

In any case we want to examine this question still further.

Several investigators, Schiff, Lattes and others have suggested a confirmation of the binding test by means of an elution experiment, and Schiff is reporting successes with dried blood. We have had no success with it. This failure
456 seems understandable to us. An elution such as that after the binding by fresh corpuscles in only conceivable, if the agglutinin is fixed for the most part to the reticulum of the blood corpuscles and remains bound to the reticulum even in the case of altered blood. Schütze has observed binding with stroma, and there are also claims made concerning the binding capability of hemoglobin. I myself was able to obtain binding peculiar to a group with hemoglobin, which had been dissolved and freed from the stroma, just as I could with dried blood. I succeeded in doing this also with pure hemoglobin which had been dried and stored for a long time.

In separated serum, however, we always have after absorption hemoglobin which had dissolved. This could explain the failure of the separation experiments. If namely the binding of the agglutinin with dissolved hemoglobin does not take place accompanied by precipitation, then the separation of the agglutinin through the usual separation process is understandably not possible. The question of whether, in the case of the binding of the agglutinin by the agglutinogen, a precipitation takes place, is not easy to answer. First, it is possible to separate out precipitate from apparently pure sera with high-speed centrifuges; on the other hand, it is very difficult, especially when working with small quantities, to obtain blood solutions to produce binding which are completely free of corpuscular constitutents. Experiments, which we set up to clarify this problem, argue for precipitation.

The agglutinin which is bound by fresh blood corpuscles, can be completely freed again at a temperature of 45°. When dried blood is added to the serum, however, the hemoglobin goes into the solution. The hemoglobin does not now precipitate out when the agglutinin is bound as do the blood corpuscles. Even when it is possible by warming to separate the agglutinin from the dissolved hemoglobin, we cannot separate again the dissolved hemoglobin by means of centrifugation as we can the blood corpuscles, and at a temperature at which the agglutinin can be identified by agglutinating of the test blood corpuscles, it would most probably be bound immediately a second time by the hemoglobin present.

One should think of the possibility of separating the hemoglobin at a temperature of 45° by using chemicals and thus again of obtaining the agglutinin.

In summary, we ought to say that the receptors A and B are constant, in any case much more permanent than the agglutinins, and they make possible an identification of the blood groups through binding of the agglutinin even in the case of old, dried blood stains. The process we have used and described above is relatively simple and demands no special preparations. It fails in a very small percentage of cases; we were clearly successful in over 90% of our attempts.

Errors result most easily through false binding or through the failure of binding in which case, again, the receptor B is more likely to go unrecognized than the receptor A. Still in 457
the area of the natural sciences there are scarcely any experiments which do not contain some possibility of error.

In any case the method we have utilized has produced significantly better results than all those processes to date concerning which there have been rather exact reports available.

A principal advantage is that it can still be used with very old samples as well as with blood stains as small as two mg.

Concerning the success in experiments with cadaver sections which have been preserved in the ordinary fashion as exhibit samples, we are in need of a comprehensive overview, though here the process can also be used.

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Investigations on the Medico-legal Usefulness of the Secretion of Blood Group Substances. (Preliminary Report)*[1]

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234 At first, after the discovery of blood groups, attention was directed only to the blood corpuscles themselves, but by 1910, experiments were begun concerning group substances in the organism besides those in the red blood corpuslces (von Dungern and Hirschfeld, Halpern).

235 After conducting experiments with semen and saliva, Yamakami along with Shirai published their results in the *Journal of Immunology* **12,** 185 (1926). In the same year in the same journal Landsteiner and Levine reported their experiments with semen, after they had worked with this problem for a long time. While these two authors examined semen samples with group-specific immune sera from rabbits, the Japanese workers were testing sperm and cell-free seminal fluid with isoagglutinins. The experiments showed clearly a group-specific inhibition.

There followed reports concerning blood group substances in organ cells (Kritschevsky and Schwarzmann; Witebsky; Witebsky and Okabe; Yosida Kan-Iti), and in leucocytes and in lymphocytes (Thomsen).

In 1924 Schiff demonstrated the presence of group substances also in cell-free serum using group-specific precipitins. Group substances were identified further in saliva, urine, seminal fluid, stomach fluid, amnionic fluid, milk, tears, and so forth (Yamakami, Yosida Kan-Iti, Brahn and Schiff, Schiff, Thomsen, Putkonen, Hirszfeld, Hamburger, Lehre, *et al.).* Group substances were also determined in vaginal secretions (Shirai, Yamakami).

In 1931 Schiff's doctoral dissertation was published by Fischer in Jena with the title, "Concerning Group-specific Substances of the Human Body." In it, Schiff reported the presence of group substances in organs and body fluids, and, of greatest significance, he tested accurately the A substance by means of the sensitive technique of hemolysis inhibition. In these tests Schiff also confirmed the difference between weak A and strong A, in that extracts from organs in the case of weak A were much weaker than such extracts in the case of strong A (Schiff and Akune; Schiff in *Zentralbl. Bakter.* **98,** 91, (1930)). Continuing these studies Schiff, sometimes together with Sasaki, and sometimes with Akune and Weiler, studied the secretion of group substances further, and also analyzed them more exactly. He speculated that the secretion of serological group characteristics O, A, and B was dependent on a simple, Mendelian pair of factors, S and s, without regard to the blood groups, Schiff and Sasaki supported their supposition with observations of 144 twins and of 68 families consisting of 351 persons. In the same study, the authors also noted that non-secretors were more numerous in group O, and that they also found differences in type in infants. Schiff and Sasaki further determined that secretion was dominant over non-secretion.

While Schiff, in the studies of group O, used mostly anti-O agglutinins from normal cattle sera after absorption with AB blood, E. Eisler reported in several studies on the use of 236
heterologous immune agglutinins, which were obtained from goats by immunizing them with Shiga's bacilli (*Bacillus dysenteriae*). These were much more effective according to the reports, and Schiff confirmed this after testing two samples of such serum. Moreover, nothing needs to be absorbed out before the serum can be used.

These important results were impetus enough to test the usefulness of the sera in medico-legal questions, especially since Schiff had already touched on this area.

My Own Experiments

Our experiments had a multitude of objectives.

1. Could the secretion or non-secretion of group substances in various persons be observed continuously over a rather long period to make possible a judgment concerning the persistence of this characteristic.

2. Could it be tested whether, in the case of secretors, there exist constant relationships between the amount of group substance secreted in the stomach contents and the time elapsed since the last intake of food.

3. Whether secreted group substances resist decay better than those substances do in the blood, and thus make possible diagnosis of blood group in highly decomposed corpses.

Procedure: In order to satisfy practical demands our effort is directed at the identification of all four blood groups. Therefore, from the outset only the agglutinin inhibition procedure could be considered. Despite their higher sensitivity, the complement fixation reaction and the hemolysis inhibition procedure had to be abandoned, since only the A characteristic could be identified in those ways. (Schiff, Hirszfeld).

To meet the requirements of legal medicine, our type of examination must be adapted to testing small quantities and cannot be too difficult to execute. After many attempts, the following procedure has proven itself practical and generally useful.

With a capillary pipette the fluid to be tested is progressively diluted 1:2:4:8:16 on glass plates with concave depressions, such as those we use for serum evaluation. The last well contains a drop of pure, physiological saline solution, equal in volume to the other solutions, to serve as a control. Then, a drop of test serum (agglutinin) is added to each well and mixed thoroughly (beginning at the left and proceeding to the higher dilutions). Finally, the drop of saline solution is mixed thoroughly with a drop of serum. After five minutes, one drop of a 3% blood-corpuscle suspension of the same size is added to each well, and all the samples are
237 again stirred thoroughly in the same fashion.

In numerous comparative experiments, it has turned out that it normally makes little difference whether the addition of the test blood corpuscles takes place immediately after the adding of the serum. One gains some time by this method and also avoids any drying. (The experiment certainly is somewhat more sensitive if one places the dilutions in tubes rather than on plates, and if one allows the mixture of liquid and serum to stand before the addition of the blood corpuscles).

A reading of the test using plates was taken after ten minutes. An observation was made whether, and to what degree of dilution, agglutination failed to take place, i.e. was inhibited. The agglutination in the saline control solution served as a standard of comparison. As testing sera, A, B or even O sera can be used; in the testing of the O substance anti-0 sera can also be used, confirming the hypothesis of Schiff, Sasaki, and Eisler. The selection of the sera is not without its effect on the amount of inhibition. If the sera are of very high potency and contain a great quantity of agglutinins, these agglutinins are sometimes not removed in the first dilutions of the material to be tested to a sufficient extent to cause an inhibition effect in the agglutinating of the test blood corpuscles. In the case of weak sera, on the other hand, there is the danger of non-specific inhibition (compare Schiff, *Die gruppenspezifischen Substanzen des menschlichen Körpers,* Jena, 1931). One ought, therefore, to choose sera of a middle strength.

When testing native saliva, its viscous quality makes mixing to homogeneity difficult (often this disturbance is still noticable in a dilution of 16). In addition the formation of streaks simulates a false agglutination or masks real agglutination. I have, therefore, gone over to the idea that saliva should be heated before carrying out the tests (½ hour in boiling hot water) to make it thin enough to flow. One can do this without any risk, since the group substances resist heat and, as Schiff has shown, the saliva can be kept at a temperature of 126° for two hours or at 150° for one hour without damaging its inhibition effect. The heating of the saliva has still another special purpose which we must mention here. As Schiff and Weiler (*Biochem. Zeitschrift,* **225,** 454, 1931) discovered, feces and at times also saliva, have the peculiar property of destroying blood-group substances. By heating for five minutes at 100° this disintegration effect is hindered and the agent destroyed (Schiff and Akune, *Münch. med. Wschr.,* **78,** 657, 1931). Recently a repeated attempt was made to characterize more closely the so-called blood-group ferment. Witebsky and later Sievers succeeded in "culturing" the effective principle. While Sievers was unsuccessful in isolating or enriching the bacteria with the enzymatic effect, Schiff reported (*Klin. Wschr.* 1935, 750) that he discovered several strains of gangrene bacilli which 238
were able to destroy the A as well as the B substances in saliva. On these grounds the heating of the saliva is completely justified. In dealing with strongly acidic gastric juice, it has also been recommended that it be neutralized beforehand. One can combat injurious influences due to hypotonicity by means of a corresponding addition of saline solution.

Now a few words about anti-0 sera. Anti-0 sera can be produced by immunizing rabbits with human blood of group 0. Although producing such sera is not easy, it has been constantly successful (Landsteiner, Wiener, Schiff). By immunizing with Shiga's bacilli, Eisler has also obtained such sera which have the advantage of not needing to be cleaned up before they are used, and which, in addition, are very effective. Anti-0 agglutinins can be more easily extracted from certain cattle sera as Schiff and Sasaki have recommended. By absorption with A, B blood corpuscles, the cattle sera lose the agglutinin directed against the foreign type (human blood corpuscles in general), as well as any anti-A or anti-B present. After this treatment, however, suitable cattle sera still possess an agglutinin against O and A_2 blood corpuscles which is, to be sure, often only a weak agglutinin. Such sera are used in the same way as anti-A and anti-B. Among the cattle sera obtained from the slaughter house many are found to be usable, in complete agreement with Schiff's claims.

Experiments to produce an inhibition of anti-M and anti-N sera with saliva or gastric juice have been negative. The antiserum removed for testing was in no way influenced, which also agrees fully with most of the reports to date on the absence of M and N in organs and body fluids.[2]

Our experiments are organized in the following way:

1. Tests concerning secretion in living persons.
2. Tests in corpses.

Saliva was first examined for secretion, then urine, and in some cases seminal fluid, and less often, siphoned gastric juice.

Saliva of different persons was tested, as well as from persons of the same families and from mother-child combi-

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* Translation of: "Untersuchungen über die gerichtlich-medizinische Verwertbarkeit der Ausscheidung von Blutgruppensubstanzen."

in *Deutsche Zeitschrift fur die Gesamte Gerichtliche Medizin* 28: 234–248 (1937).

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nations. Obtaining saliva from adults creates no problems. In order to avoid as much as possible any admixture of blood or epithelial cells, we ask the people to collect the saliva, with their mouths slightly open, without sucking, onto a folded
/239 piece of waxed paper and put it into a tube.

In the case of small children and babies, obtaining saliva samples was at first considerably more difficult. After some attempts I tried a simple suction device (Figure 1) with which it was possible to collect an ample quantity of saliva without the least difficulty even in the case of new borns.† One puts one tube in the child's mouth and begins to suck lightly on the other tube. The children are immediately pacified, when the small tube is put into their mouths. They stop crying and begin to suck, whereby the saliva secretion is stimulated. The saliva collects in a test tube which is inserted between the baby's tube and that of the researcher. The saliva can be immediately heated in this tube and used for the experiment without being transferred.

As experiments have shown, a small admixture of blood has no great importance in terms of disturbances (this is true for saliva and for stomach contents.[3]). This was also confirmed in the case of two corpses which had swallowed quantities of blood and even breathed some in. This caused only a comparatively small disturbance.

Saliva Experiments

Of 116 persons whose saliva was tested according to the method presented here, 97 were found to be secretors and nineteen to be non-secretors. Table 1 provides a summary. As Table 1 demonstrates, most of the saliva samples were still effective even at a dilution of 1:128 to 1:256. These few tests, as well as the repetition of the test on saliva samples from one and the same person, show that, as a rule, it is possible to distinguish between S and s in the first experiment. It also shows, however, that there are cases in which the weakness of the inhibition effect renders it doubtful whether one is still dealing with a secretor or whether the 240/
slight inhibition is due only to admixtures of cells.

We, therefore, took saliva samples for examination from seventeen persons belonging to different groups, repeating the test in some cases as many as sixty times on different days over a lengthy period of time. A summary of these experiments is given in Table 2.

† Figure 1, not reproduced in the translation, shows a simple device consisting of a test tube with a two-hole stopper; a glass tube through one of the holes is connected to the suction, and a glass tube through the other to a piece of tubing, the end of which could be put into the youngster's mouth.

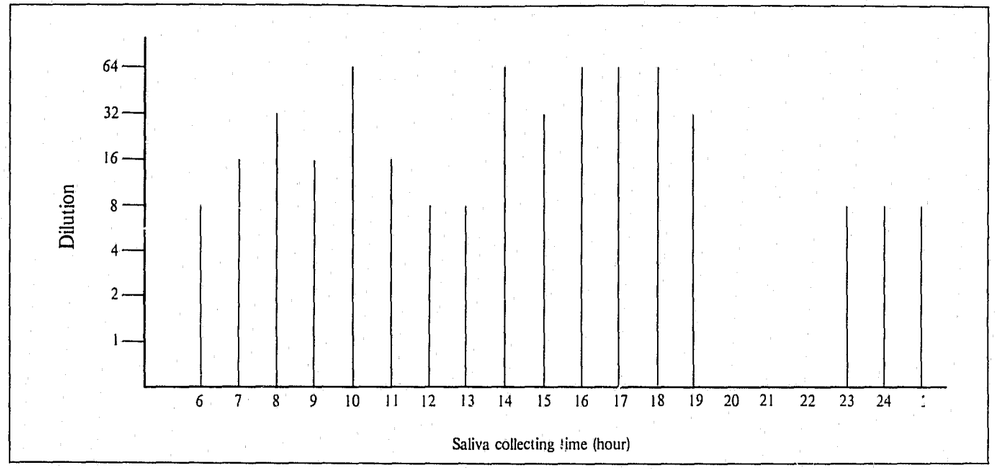
Table 1. Summary of the saliva tests

Blood Group	Secretors S	Dilution 1	2	4	8	16	32	64	128	256	512	1024	2000	4000	8000	Non-Secretors s
0	6	–	–	–	2	1	1	–	1	–	–	–	–	–	–	3
A	74	–	2	–	6	9	7	13	15	8	8	4	2	–	–	12
B	12	–	–	–	–	1	1	–	4	4	–	1	1	–	–	1
AB	5	–	–	–	–	2	–	1	–	2	–	–	–	–	–	3

Table 2. Summary of the Repeated Saliva Tests

	Blood Group	Secretor S	Dilution 1	2	4	8	16	32	64	128	256	512	1024	2000	4000	8000	Non-Secretor s
V.S.	O	16	2	3	–	1	2	1	3	2	2	–	–	–	–	–	1
M.S.	A	35	1	1	2	16	2	3	9	1	–	–	–	–	–	–	
K.T.	A	26	2	1	4	9	8	2	–	–	–	–	–	–	–	–	–
K.L.	A	23	–	1	1	4	2	5	6	3	1	–	–	–	–	–	3
K.V.	A_2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	34
Ho.	A_2	54	4	1	3	12	13	8	6	6	1	–	–	–	–	–	6
Sche.	A_1	15	1	1	–	1	–	2	1	3	4	–	2	–	–	–	
Ki.	A_1	23	–	1	–	1	1	3	5	4	4	4	–	–	–	–	–
Me.	B	19	–	–	2	2	1	1	2	3	3	3	1	–	1	–	–
Fri. E.	A_1B	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	27
Lä.	AB	7	–	–	–	–	–	1	–	2	2	–	1	–	–	–	–
K. Ge.	A_1	6	–	–	–	–	1	–	–	–	1	–	1	1	1	1	–
Pan.	O	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	10
V. Bi.	A_2	5	–	–	–	1	2	1	–	1	–	–	–	–	–	–	–
M. Bi.	A_2B	5	–	1	–	–	–	3	–	1	–	–	–	–	–	–	–
K. Bi.	B	5	2	–	–	–	–	–	–	2	–	1	–	–	–	–	–
Mö.	O	2	–	–	–	–	–	1	–	–	–	–	1	–	–	–	–

Figure 2.



It is interesting to notice that in this series there are negative samples even among the secretors. On the other hand, it turns out that, in the case of non-secretors, inhibition never takes place despite many repetions of the test (up to 34) and hourly extractions of saliva. In the case of a secretor from whom saliva was extracted every hour for twenty-four hours,
/241 three successive samples were negative, but thereafter, fairly abrupt and clear inhibition effects could be observed (compare Fig. 2). Although the experiments are still small in number, and we must still test further to what extent group enzymes play a role here, the question already can be posed whether there exists another group of humans between the secretors and the non-secretors, a group whose members sometimes secrete and sometimes do not.[4]

Moreover, we have the impression that the intake of food, and the time elapsed since the intake, has some sort of influence, but we do not understand the effect from our quantitative experiments to date. More experiments are required on the relationship of different physiological and pathological conditions to the test results.[5]

Perhaps it would be possible with more sensitive methods to identify inhibition in some samples which were evaluated as negative with the procedure described here.

As we said above, in judging the variations in the content of group substance in saliva, one should also think of a disturbance caused by blood group enzymes, such as those which appear in the intestine. In order to prevent this as much as possible from the outset, it would be expedient to heat the saliva immediately after its extraction.

Our experiments with families agree totally with Schiff and Sasaki, in that secretion was dominant over non-secretion. The designation "Secretion-type" S was very appropriately introduced by Schiff.

In view of the reported findings, we must urgently warn against the premature use of this fact in paternity cases. A setback due to hastiness could lead to a serious decrease of confidence on the part of the courts in the classical blood groups and in the M and N factors. In 1928 Cuboni says in his summary that secretion was not consistent and that its systematic use in legal medicine seemed to be improbable. 242/

Meanwhile, false determinations could be greatly reduced by means of repeated experiments. Thus, if the dominance of S could be further confirmed, the secretion characteristic could serve to corroborate the improbability of descent demonstrated in other ways. Still this method of testing should not be considered of too great an importance in paternity cases, since the secretion type S predominates to a great extent over the non-secretor so that the possibility of an exclusion is reduced.

The use of secretion of group substances to diagnose the group of human secretions, such as urine, saliva, vomit, semen, from wet or dry stains, is not disputed. Repeatedly, different authors (Schiff, Lattes and others) have confirmed that it is useful in these cases. We have even been able to use successfully the test of saliva stains in legal cases.

In conducting tests with seminal fluid, parallel to the saliva tests, we showed that group substances in fact appear in seminal fluid even where there is a scarcity of spermatozoa. It seems though, that these substances are inferior to those in saliva with regard to the inhibition effect. Group substance was also identified in urine, agreeing completely with what is presently known of secretors, although considerably less than in the respective saliva. In the course of these experiments, A saliva and B saliva were repeatedly tested with anti-O liquid. It was shown in these tests that some A and some B saliva produced a clear inhibition effect, but that AB saliva never does, whether it comes from secretors or non-secretors.

Table 3 (Feb. 29, 1936) presents examples of such reactions in cases of saliva samples of all the groups, and it shows that, in the case of A persons and B persons, a secretion of O never is found without a secretion of A and B.[6] This suggests that the secretion of O together with A and B depends on the presence of an O gene. While inhibition with Anti-O sera could, in the case of A-secretors, be conditioned also by an A_2, this is not so in the case of B secretors. Possibly, the way is opened here to distinguish homozygous and heterozygous A and B, a distinction which would be of great practical meaning in regard to questions of descent. Now it is well known that A and B blood corpuscles are able also to absorb anti-O sera. According to Schiff (*Zeitschrift* /244 *Immun.forsch.* **82,** 302, 1934) in experiments with Shiga bacillus immune serum from goats the A and B secretors can almost always be distinguished from the non-secretors by the inhibition of O-agglutination. The inhibition, however, was often noticeably less than it was in the case of saliva of the O-group. Schiff, therefore, thought that one must ascribe a certain amount of the so-called O factor to the blood corpuscles A_1 (as well as to the other groups). Morzycki (*Zeitschrift Immun.forsch.* **84,** 80, 1935) assumes that the anti-O sera react with such elements which are present in varying amounts in all individuals. Thus, he and Hirszfeld conceive of the so-called O-receptors, first of all, as species receptors and assume that they are present in some individuals only in very small quantities or that they are lacking completely.

Table 3

No.	Boiled Saliva	Group	Anti-	Dilution 1	2	4	8	16	32	64	128	265	500	1000	2000	4000	8000	16000	NaCl control
1	V. Sch. Feb. 22, 1936 ..	O	A	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	−	−	−	−	−	++	++	++	++	++	++	++	++	++
2	M. Feb. 22, 1936	A	A	±	+	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	±	±	++	++	++	++	++	++	++	++	++	++	++	++
3	K. T. 2/22/36.....	A	A	−	−	±	±	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	−	−	±	++	++	++	++	++	++	++	++	++	++	++
4	K. I. 2/22/36	A	A	−	−	−	±	+	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	−	−	±	±	++	++	++	++	++	++	++	++	++	++
5	K. V. 2/22/36.....	A	A	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
6	Kirch	A	A	−	−	−	−	−	−	−	±	±	+	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	−	−	++	++	++	++	++	++	++	++	++	++	++	++
7	Merkl	B	A	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	±	+	+	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	±	++	++	++	++	++	++	++	++	++	++	++	++	++	++
8	M.H.	B	A	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
9	K. H. El.	AB	A	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
10	Lä	AB	A	−	−	−	−	±	±	+	++	++	++	++	++	++	++	++	++
			B	−	−	−	±	+	++	++	++	++	++	++	++	++	++	++	++
			O	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
11	Ho.	A	A	−	−	−	−	±	++	++	++	++	++	++	++	++	++	++	++
			B	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++
			O	−	−	±	±	++	++	++	++	++	++	++	++	++	++	++	++

O-Serum, 2/13/36: Anti-A 4(8); Anti-B 4(8)
Anti-O Serum, 2/5/36: Anti-O 2(4)

If, after all, one may express himself only with the greatest reserve and ought not to have too great hopes, it still seems desirable to carry out further tests in the direction which has been taken.

After our experiments concerning the secretion of group substances in the stomach fluid had completely confirmed the reports of earlier authors (Schiff and Akune, Hirszfeld), the experiments which followed were arranged, proceeding from the obvious question regarding the constant relationship between the time elapsed since the last food intake and the amount of secreted group substances in the contents of the stomach.

Shortly after a monitored breakfast, gastric juice samples, taken with a stomach pump, were examined in one experiment together with saliva, which served as a control. But to this day I have only a modest number of these experiment results. In this experiment the inhibition was also small in the case of the secretors, a result that may have been connected with the short duration of the reaction (the stomach was pumped after twenty minutes). Further experiments, and especially self-examinations with the stomach tube, should increase the number of our observations. With one exception, saliva was more effective than gastric juice in the cases tested so far.

A second series of tests was undertaken with twenty-six corpses. Among these were two certain non-secretors and one questionable secretor. The secretors were in a very clear majority here. The stomach contents of these corpses was tested, and, where possible, also the saliva, serum, urine, bile, and the walls of the duodenum, small intestines, and the colon. In order to see how much group substance was contained in the stomach and intestinal walls, the pieces were extracted with a saline solution. The process was uniformly the following: After washing, a piece almost 1 sq. cm. is stamped out (the method of stamping is very easy while at the same time being sufficiently exact). The sections are then placed in small test tubes and mixed with ½ cc of physiological saline solution. After being shaken they are allowed to stand for a rather long time, usually overnight, but sometimes throughout the day, and are shaken several times dur- /245 ing this period. Then the tubes are centrifuged. The agglutinin-inhibition reaction is carried out in the ordinary manner with the supernatant fluid. Usually, it turns out that, despite the rather great dilution produced by adding the saline solution, the extracts still cause a rather strong inhibition effect, often up to a dilution of 1:16. It also turns out that large amounts of group substances are contained in the stomach wall (mucous membrane). It appears, moreover, that the place from which one chooses to take a sample of the stomach membrane is relatively unimportant. While the effective action of the extract from the stomach wall ceased between dilutions of 8 and 32, the stomach contents frequently caused a clear inhibition effect in a dilution of 2000.

There are, thus, considerable differences between the inhibition range of the stomach contents and that of the extracts from the stomach lining. Just as group substances could be leached from individual sections of the lining, so the stomach of the corpse produced such substances in the liquid with which it was filled. At post-mortem examinations, the stomach contents were collected from the excised stomach. The stomach was then thoroughly rinsed and filled with physiological saline solution corresponding to the amount of stomach contents extracted. Then, at various intervals fluid was extracted through a test puncture. From the test, it turned out that a very considerable quantity of group substance was in fact transferred to the saline solution, and in a short time too. For example, the fluid extracted after six hours had an inhibition effect even in a dilution of 1:16 to 1:32. After 40 hours this had certainly increased somewhat, and after 70 hours an inhibition effect could be observed in dilutions of 1:64 to 1:128. In natural stomach contents which have been left in the stomach, concentrations of group substances grow somewhat stronger from the time of the post-mortem examination, as samples taken by puncture have demonstrated. Experiments in this direction are still continuing. The leaching experiments after death show that, indeed, group substances are still present in large quantities but that the fluid never again reaches the degree of saturation of the original stomach fluid. More experiments on persons who have died shortly after filling their stomachs are now being considered.

In most of our cases we also observed the behavior of the bile. Group substances were also secreted in the bile in rather considerable quantities, though as a rule less than in the stomach mucous membrane. As a control, in testing whether a person is a secretor, the bile is quite important since in dealing with corpses it can be difficult to extract saliva. The stringy quality and viscosity of the bile, however, sometimes create quite a considerable disturbance in the agglutination inhibition, as they do in hemolysis. Here one must, of course, keep hemolysis and agglutination-inhibition separate. Moreover, in the case of bile, nonspecific inhibition appears to 246 take place more easily. With regard to serum and urine, our experiments with corpses have proven of very little value. Even in the case of secretors, one can often fail to recognize them from the urine of the corpse. Also, the pericardial fluid, which we tested at the same time, produced only a weak inhibition. Besides the stomach lining we also tested the duodenal, small intestinal, and the colon walls. Our records here confirm the decrease of demonstrable group substances toward the end of the digestive tract. A noticeable decrease can usually be noticed in the small intestine as well. The colon and rectum were usually found to be free of group substances. There exists the possibility that the secreted substances were reabsorbed. It has been proven, nevertheless, that the intestinal contents destroy group substances by means of an agent whose effective principle, as we already mentioned, is destroyed by cooking (Schiff and Weiler). It is an enzyme which is also active in germ-free filtrates (Schiff and Akune, and cf: Witebsky and Satoh). The finding of a large quantity of inhibiting substance in both the small and large intestines of a newborn, 40 cm long, macerated fetus is

in agreement with the thinking of Witebsky and Satoh (*loc. cit.*) that this inhibition enzyme is first formed in the first months of life. Group substances are already secreted in embryonic life (Schiff, Witebsky and Satoh). Even the secretion of O could be established with our tests on these corpses. As in the experiments with saliva, our observations regarding gastric juice, bile, semen and so forth, argued for a specific inhibition with respect to anti-O serum. We established this with secretors of A and B as well.

The secretion of group substances in the stomach can be used with advantage to secure a positive group diagnosis in the case of badly decomposed corpses.

Let the following case serve as an example. In a highly decomposed corpse of an old man, which had lain in water for more than forty days, the blood had completely hemolysed; the blood corpuscles had completely vanished. The Landsteiner-Lattes test with A blood cells caused an uncertain clumping. The diagnosis with the absorption method produced an incontestable absorption of anti-B. At the same time, to serve as a control, bile, stomach contents, the stomach lining, and urine were tested following the agglutinin-inhibition procedure. In total agreement with the blood test, a definite, specific inhibition reaction, caused by the B substance, was demonstrated in these tests. In the gastric juice the inhibition effect continued up to a dilution of 1:4000.

In view of these results, we think that in all similar cases
247 such testing is valuable as a complement. Obviously, we can evaluate only a positive result, i.e., a clear inhibition, since, with a negative result, we might in fact be dealing with a non-secretor. We should also mention in reference to these cases, that, when possible, throat mucus and saliva should be collected and preserved.

Summary

In order to test secretion of group substances for medicolegal purposes, different experiments were set up on the agglutinin inhibition procedure.

The tests confirm the findings to date, indicating that the possible to distinguish secretors from non-secretors but that, occasionally, a secretor does not secrete or secretes so little that it is not detected by the test.

The test confirm the findings to date, indicating that the secretion type is a dominant hereditary trait. For use in paternity cases, however, the secretion type can, for the time being, only be employed with the greatest reservations and only after repeated testing.

Experiments on the relationship between the amount of secreted group substance in the stomach and the time elapsed since the last intake of food, in this case the time of death, did not produce any noteworthy results.

The secretion of group substances can serve to corroborate the group diagnosis in highly decomposed corpses.

The inhibition of anti-O serum by saliva from secretors of groups A and B may indicate a way of recognizing the presence of an O gene and thus, of distinguishing homozygous and heterozygous A and B carriers.

Footnotes

1. Delivered in abstract form at the Conference of Physicians and Researchers in the Natural Sciences in Dresden, September 1936.
2. Schiff was able to produce an anti-M agglutinin by immunizing rabbits with human saliva. He, therefore, concluded that the M characteristic appears as a true antigen in the saliva (Schiff, F., *Über die gruppenspezifischen Substanzen des menschlichen Körpers*, Jena, 1931, p. 42).
3. Compare also the experiments of Matson and Brady (*J. of Immun.* 30, 444, 1936)
4. Almost the same as Hirszfeld's method, according to which he divides the organs into "absolute," "facultative," and "negative" group carriers.
5. In Thomsen's laboratory Fog-Möller (*Z. Immun. forsch.* 84, 359, 1935) tested the secretion of group substances in cases of disease, namely pernicious anemia, and they found here no deviation from the norm.
6. Tests with gastric juice produced similar results (see below).

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The Current Status of Blood Group Serology and Its Forensic Importance*†

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Classical Blood Groups

416 In the first fifty years since the discovery of blood groups by Karl Landsteiner[1] understanding of and experience with the four classical blood groups has become so well established that their identification now rarely causes difficulties in clinical and forensic medicine.

In the last few years, however, researchers have written much about the subgroups and widened our knowledge of these.

Subgroups of A

On the technique of differentiating strong from weak A, a series of articles has appeared, written by Thomsen,[2,3] Thomsen, Friedenreich, and Worsaae,[4] Lauer,[5] Lehmann-Facius,[6] Ottensooser and Zurukzoglu,[7] Holzer,[8] Krieger,[9] Wolff and Jonsson,[10] Blinov,[11] Ponsold[12] and others.

Among A types a weaker A_2 was distinguished. Dahr[13,14] tested a seven-month old child with a very slight A-agglutinability and considered a division of the clearly-defined cases of weak A into A_3, A_4, and A_5 as still premature; Gammelgaard and Marcussen[15] on the other hand, thought that they had a sound basis for proposing the existence of the additional subgroups A_4, A_5, and so forth, because of the presence of clear, quantitative differences and distinct hereditary transmission.

A question which is still not completely clarified is that touched on by Friedenreich[16,17] in 1931, namely that concerning the conversions between the blood groups A_1 and A_2.

Dahr[18] also admitted the existence of intermediate A forms. Witebsky[19] even claims that a new division (reclassification) into A_1 and A_2 is necessary. Of 100 samples 75 could be identified as A_1, fifteen as intermediate A, and ten as A_2. The intermediate group was almost totally separated from the A_2 group.

Since the communication of Laguna[20] and the case of 417
Haselhorst and Lauer,[21] special attention must be given to the weak A on the part of researchers, particularly the investigators in paternity suits.

Of interest in this connection is Boltz's[22] observation at the Vienna Forensic Medical Institute of a phenotypic latency and late manifestation of blood group A_2B in the case of a young blood donor, who was at first determined to be a B with a weak α, and only two years later was diagnosed as an A_2B with an irregular α_1. This girl has donated blood fifty-one times, sometimes as an AB, without any complications.

Such observations make comprehensible a certain reluctance in the evaluation of A_1/A_2 exclusions, a reluctance which one sees in the literature, for example, in the work of Andresen[23] and others.

For subgroups of A, Dahr does not assign the high-probability percentage of 99.8%, i.e., the obviously impossible. The conclusion in assessing exclusions based on the subgroups ought, therefore, not to imply that the subgroup excluded is absolutely impossible, but rather that it is highly unlikely.

The American authors Davidsohn, Levine, and Wiener,[24] write in a recent report concerning the forensic use of blood tests that the subgroups of A, though theoretically of use in cases of disputed paternity, cause problems in practice in distinguishing the subgroups, especially with newborns. Thus, the Committee of the American Medical Association for Forensic Medical Problems claims that tests based on the subgroups of A are not yet to be trusted for forensic medical use.

In contrast to this caution on the part of the American authors is the positive evaluation of the demonstrated capabilities of such tests, expressed by other researchers.

Formaggio (personal communication) found no exceptions.

Wichmann,[25] Mayser,[26] and especially Ponsold[27] emphasize both the reliability and the value as evidence of the hereditary aspect of the subgroups, even in exclusions for paternity cases where the interpretation "clearly impossible" can be made.

Ponsold is a man who has thoroughly dedicated his efforts to the A_1A_2 problem, especially by using the capillary

* Translation of: "Der gegenwärtige Stand der Blutgruppenserologie und deren forensische Bedeutung."

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method and the absorption identification by means of "exhaustion." Ponsold[28] has shown with the aid of an extremely instructive case, clearly identical with one described by Bohmer and Greiner[29] in 1951, that the scientific question of the value of an elimination based on A subgroups is affected not only by the state of the experiment, but just as much by the selection of the expert and the test which he employs.

Subgroups of B

418 In the case of the blood characteristic B there are scarcely any difficulties or mistakes.

P. Moureau reported at the Congress for Blood Transfusion in Paris concerning the appearance of a weak B. Although quantitative differences among the various subgroups of B appear (Mudguti,[30] Honde[31]), they are not so pronounced as between A_1 and A_2, as Formaggio,[32] a man from the Lattes school, asserted in a recent article.

Basing their arguments principally on the results collected by Matta,[34] Schiff and Boyd[33] believe that the forensic medical use of the subgroups of B still lacks sufficient foundation.

Various authors (Killer,[35] Schiff,[36] Zitzman,[37] Pietrusky,[38] Manz,[39] and Jungmichel[40,41]) have indicated the possibility of indirect exclusions in the case of deceased subjects, and of additional indirect exclusions with living subjects.

In 1943 at the Robert Koch Institute in Berlin, Werner Fischer[42] thoroughly treated the different possibilities in a very careful study.

Fischer considered that the chances were good for successful exclusions when the parties in the dispute were dead. Fischer's statements justify a greater use of indirect blood-group diagnosis in judicial investigations.

The indirect method of determining the heterozygote hereditary type is all the more important since, despite our many-sided efforts, we still do not possess a trustworthy serological method to recognize recessive O in the case of heterozygotes.

In 1938 Dahr[14] set up valuable experiments in this direction; some were confirmed, and some were refuted.

According to Dahr[14] the identification of the heterozygosity of A and B blood is fundamentally possible since the inherited O trait is not completely repressed by the A and B trait inherited at the same time. Boorman, Dodd, and Gilbey[43] speak of a co-dominance of the blood-group gene O with A and B.

It is questionable whether the idea which I expressed in 1937,[44] namely the possibility of recognizing heterozygosity by testing saliva from A and B persons with anti-O agglutinin, can, indeed, be realized. Dahr's co-workers, Manz and Altenberg, recently tried this method.[14] On the basis of his experiments Formaggio[45] considered it to have no prospects, since he observed a secretion of O substance both in A_1A_1 and A_1B individuals.

Secretion in the ABO System

419 In his latest experiments concerning the secretion of blood-group substances in 2000 persons tested in Pavia, Formaggio[45] found a statistically good correlation in the division of secretors from non-secretors, which we found and described in the literature.

In the group AB, he found that sometimes A and B were secreted, sometimes only A or only B, and sometimes absolutely no substance.

Consequently, to test for S or s it would not be enough in the case of AB to test for A or B in saliva, although until recently Dahr[14] thought that it was.

Relationships were established between the amount of saliva and the quantity of group substance. If a great quantity of saliva is secreted, the group substance in this saliva is diluted; if the saliva increases rapidly in amount, the secreted group substance can decrease almost to zero, so that such a person could appear as a non-secretor in only a single test.

In complete agreement with Wiener and Kosofsky,[47] Formaggio[46] found no difference between A_1 and A_2 with respect to secretion.

While Wiener advised caution in using secretion in the blood group O because the sera against O are difficult to obtain, Formaggio found the reactions with good anti-Shiga bacilli serum to be trustworthy and clear, even for paternity questions. He presented his conclusions with the aid of the table drawn up from his experiments.

Formaggio[46] has indicated the important potential use of secretion to recognize the especially weak A (namely in the so-called "defective" O).

Even when the A characteristic is so weak that it cannot be demonstrated in the blood corpuscles either by agglutination or by absorption, the testing of the saliva from such secretors showed that A substance was clearly secreted. Thus, especially weak A may still be identified.

M and N

Iso-antibodies against M and N are uncommonly rare. According to Wiener[48] only seven cases with anti-M in normal human serum were described up to 1946.

In May of this year Wiener[49] described the seventh and eighth cases of a natural anti-M in the sera of male Negro twins, both belonging to the blood group BNss Rh_0.

Numerous studies concerning the technique of MN identification have appeared.

While Schiff[50] recommended the absorption procedure in 420 doubtful cases, Wiener[48] (p. 226) believes it is better to test with more serum and to repeat the experiment. Moreover, Boltz[51] recently pointed out that the absorption experiment is, in general, less reliable than the agglutination test. One must characterize the absorption experiment as fundamentally more cumbersome, which in unclear cases, ought not to be used again.

Since the discovery of a weak N characteristic by Crome,[52] and its confirmation by Pietrusky,[53,54] there have been other similar observations reported by Friedenreich,[55]

Lauer,[56] Pietrusky,[57] Dombrowsky,[58] and Langenberg.[59] There is currently a wide-ranging discussion of this subject. Dahr[14] (p. 106) has pointed out the difference between the weak N described by Pietrusky and the weak N reported by Friedenreich and Lauer. In their report the weak N displayed approximately ¼ of the agglutinating capability of normal N.

A third allelomorphic gene N_2 was assumed.

According to Andresen's[23] report (1947), only eight cases, including the four cases reported by Friedenreich, were observed during a ten-year period at the Forensic-medical Institute in Copenhagen among 20,000 paternity cases.

In 1948 Krah[60,61] described cases of weak N, two of which resembled more closely the cases described by Pietrusky than those described by Friedenreich and Lauer. He indicated that, besides the increase in serum titer, the qualitative character of the sera played an essential role in identifying weak N, since sera of the same titer do not behave in a uniform way. Freshly produced sera are better suited to attach to this weak N receptor than are older, used sera of the same titer.

The absorption test in the normal manner usually fails in the cases first described because the reduction in titer is too small. From his observations Krah reached the conclusion that to recognize the weak N receptor it was necessary to have freshly extracted, high-potency, strongly specific anti-N sera with the greatest possible reactive range.

Since there exist not only weaker N types but also defective types, the terms Nd (defective) and Ns (weak; German: *schwach*) were recommended.

In addition to a weak N, observations concerning a weak M have been published in the last few years. Friedenreich and Lauridsen[62] issued the first report in 1938.

In 1943 Pietrusky[63,64] described in a sample of MN blood a weak M receptor—he called it M_2—with a clear but nevertheless weaker absorption than that of MN blood. Dahr[65] at that time called attention to the possibility of clarification by quantitative methods and the mosaic-like complex of the human M and N agglutinogens.

In 1943 Pietrusky and Hausbrandt[66] issued a report on a 421 certain type M_3 with the advice that, in view of the difficulties of the MN system, superior expert advice ought to be introduced in all paternity decisions based on the MN system.

Jakobowicz, Bryce, and Simmons[67,68] have observed, in addition, a qualitatively deviant M form.

We ought not to overlook the confirmation of Dahr[69] in 1944 that a considerable quantity of anti-N agglutinin was released from human M-blood corpuscles which had been brought together beforehand with a specific anti-N serum (decanted for testing). Kindler[70] also confirmed this when he continued the experiments at Dahr's institute. He was able to separate complete N antibodies from crude anti-N serum by means of absorption with M blood corpuscles.

In any case we need to devote special attention to the M and N experiments in the future.

The last work I was able to obtain was one by Walter Boltz[51] which recently appeared concerning the deviant forms in the M-N system. It contains an interesting report concerning a weak M in the case of a child and the defendant. Here the blood of both persons was "surprisingly" similar regarding the M characteristic. One could designate the two samples of blood as M(s)N.

In this case the relationships were as follows:

mother	O	N	
child	A_1	Ms	N
defendant	A_1	Ms	N
witness	A_1	N	

One could thereby not only choose the adulterer, but one also had an important indication of the actual paternity as a result of the rare weak-M characteristic in both the child and the defendant.

This work from the Vienna Institute rightly emphasized the following:

False testimony on the part of witnesses in paternity suits is an everyday occurrence, and it does not compare as a source of error to the dwindling error factor resulting from an unrecognized blood-type variant.

Although one must still carefully evaluate a decision made on the basis of the subgroups of M and N, nevertheless, almost all authors and experts are in agreement that the same weight should be given to a decision based on M and N which was reached by experienced investigators, and confirmed by superior expert advice, as to that based on the classical blood groups.

In these cases the judge is sometimes unable to reach a decision, or sometimes, when a judgment based on hereditary biology argues for parentage from simple probability or possibility, it can go against the MN judgment. Such cases, however, should not be held against the method and the scientific evidence.

The S Characteristic

In 1947, Dr. Walsh and Miss Montgomery[71] discovered in 422 Sydney, Australia, in the serum of an rh negative mother with a dropsical stillborn an agglutinin which did not fit into the systems to date.

In the case of the gene designation S, Race and Sanger,[72] as they confess, overlooked the fact that the letter S was already assigned to the secretion type. They[73] looked into the inheritance of the S characteristic themselves, and spoke of four allelomorphs, MS, Ms, Ns, and NS.

Up to 1950 only seven examples of anti-S agglutinin were discovered.

Experiments conducted by Mourant and Ikin[74] in immunizing rabbits to S were unsuccessful up to that time.

Manz and Orbach[75] by chance possessed the same group constellation as had been present in the case of Walsh and Montgomery in the discovery of the anti-S; they wanted to produce the antibody, anti-S, by means of self-immunizations. Antibodies against Rh showed that the test person was, in general, well suited to build antibodies. The

experiment, however, was not successful in identifying any anti-S, even in traces.

If the routine identification of the S characteristics were made possible by the easier availability of anti-S, then the possibility of making an exclusion in the MN system would be greater. The chances of an exclusion would be even better if the anti-s were available to be used for forensic blood tests, which is not yet the case (Wiener[76]).

Statements concerning blood-group changes have disappeared from the more recent literature.

In view of the enormous number of transfusions which take place today throughout the world, one must think about the possibility of a mistake due to the group characteristics of transfused blood corpuscles in the recipient.

As Schwer-Körner and Kim[77] in Dahr's institute reported in 1948, incompatible blood can cause false determinations regarding MN for a long time—up to fifty-three days.

The experienced researcher, however, will notice that only some of the blood corpuscles were clumped and thus protect himself from an incorrect determination.

P

The first tests of Landsteiner and his co-workers have already demonstrated that the factor P is developed in varying strengths.

How are these differing reports to be evaluated, when the one is P+++ and the other P+?

In his experiments with his anti-P sera from pigs Jungmichel[78] has divided the P into three groups.

423 Wiener[48] as well as Race and Sanger[72] mention the appearance of the P characteristic in varying strengths.

The determination of the strength of P in the case of both monozygotic and dizygotic twins was made by Dahr[79] as well as by Schmidt and his co-workers, as a result of which it seems likely that the varying strength of the P characteristic is conditioned by heredity.

Henningsen[81] distinguished four classes of P according to the strength of the P gene: strong P, middle P, weak P, and P minus. He discovered, in conducting family studies, that in the case of P+ × P− pairs, the offspring cannot exhibit a stronger P than that of the P-positive parent. This corresponds to several different genes which produce the P antigen of different strength.

These observations of Henningsen should be expanded. If they are confirmed in large series of tests and in family studies, we could expect a further development for paternity cases here as in the case of A_1 and A_2; the test could demonstrate the improbability of generation, even if all three persons tested are P-positive.

Because of the difficulty of its nature, P is not yet used for forensic medical purposes in America according to the report of the committee[24] although Levine and Wiener have had access from the first to special experience through their experiments.

Krah and Harter[82,83] recently occupied themselves with the difficulties of determining P and of obtaining animal P anti-serum. They succeeded in obtaining high-potency, anti-P sera from normal pig serum.

Concerning the use of blood groups in criminalistics, there have been in the last few years no fundamental innovations or new methods, as Formaggio[84] admits in a summary report which appeared in 1950.

In general, the old methods are still used and are tried on the new blood-group characteristics.

To better dissolve the agglutinins for the agglutinin identification in dried blood, Faraone[85] recommended warming for thirty minutes to a temperature of 40° to 50° in a hanging drop.

Some very fine and successful results have been obtained from the methods to date. Thus, Moureau[86] was successful in 1948 in achieving an interesting criminalistics group identification on the sweat band of a hat.

Muller and Christiaens[87] were able to convict a thief by identifying N substances in blood stains.

The experiments concerning the identification of Rh in blood stains and secretions are still in the experimental stage.

In the case of Rh identification, bacterial decomposition 424 of the sera makes its appearance as a disturbance during absorption in the heat. For this reason Formaggio mixes in merthiolate.

Rh Groups

Since the discovery of the classic blood groups, the discovery of the Rh groups by Landsteiner and Wiener[88] has been the most important development.

Thorough monographs such as the one by Fanconi, Grumbach and his co-workers,[89] those by Formaggio[90] and by Edith L. Potter,[91] and that by Hill and Damashek,[92] as well as those of several others, have appeared which deal chiefly with the clinical and serological problem, as have congresses dedicated especially to the Rh questions (for example, in Turin, in Naples, and in Milan). These demonstrate the importance which the Rh factors have achieved today.

The original anti-Rh is now called anti-Rh_0 or anti-D.

Wiener and Landsteiner[93] had assumed three major genes, R_1, R_2, and r. Fisher[94] introduced for the Rh genes the symbols Cc, Dd, and Ee.

Each of these genes can, under certain circumstances, stimulate the corresponding antibody.

Fisher's theory is today generally acknowledged.

Wiener,[95] Landsteiner and Wiener,[96] and Levine and his co-workers[97] have acknowledged since 1941 that different Rh types occur.

In addition to C and c Callender and Race[98] found in 1946 a third allelomorphic gene C^w. Later c^v and c^u were described.

In addition to D and d, Stratton[99] described a third gene D^u, probably identical to Wiener's intermediate gene. According to van Loghem,[101] this gene can have an antigenic effect.

Armytage, Ceppellini, Ikin and Mourant[102] first described a third allelomorphic antigen E^v in addition to E and e. Mr. Schleyer from Bonn will report soon concerning reactions with various E-gene types.

Diamond[103] in 1946 and Hill and Haberman[104,105] in 1948 described the anti-d (anti-Hr_0).

While at first the anti-Rh sera were obtained by immunizing animals, the sera containing the Rh antibodies we use today come for the most part from humans immunized by transfusion or pregnancy.

On account of repeated stillborn babies due to the anti-Rh, Diamond[106] treated intravenously such anti-Rh sterilized women with varying amounts of Rh-positive blood and was able to produce with very small amounts of blood (0.1cc) an increase in the titer.

Moreover, Wiener (personal communication), Hill, Ha- /425 berman, and Orozco,[107] Callender and Race,[98] and others were successful with this method.

Wiener and Sonn-Gordon[108] injected Rh-negative donors with 4 cc of a 50% blood-corpuscle suspension, repeating the injection after four months. Then days after the second injection they obtained usable anti-Rh sera.

Van Loghem[101] succeeded, after thirteen to seventeen injections, in obtaining anti-C and anti-E in the case of professional donors, especially from those who react to vaccine injections. He was thereby able to confirm Diamond's[109] observations that, in the case of progressive immunization, the agglutinins effective in saline solution change to incomplete antibodies. If agglutinins effective in saline solutions are desired, he recommends that the immunization be interrupted at the right moment.

Maresch[110] and Speiser[111] observed too, how the agglutinins present at the beginning of the immunization were later replaced by univalent antibodies.

The Methods of Rh Testing

These are based on the antigen identification by means of agglutinins or conglutinins, complete or incomplete antibodies.

In order to be used with all groups, anti-A and anti-B sera, containing anti-Rh, must be purified before use with A_1B blood of the corresponding Rh genotype, with purified A and B substance, or with saliva of an A_1B secretor. In doing this, one must keep in mind what Cappell and McFarlane[112] found, i.e., that, after long storage, an unwanted anti-A and anti-B can again appear in absorbed serum. If the sera are strong enough, one can dilute them for use, according to Diamond,[103] most effectively in albumin.

A great number of studies in recent years have dealt with the Rh technique.

For Rh identification the incomplete, conglutinating antibodies are the most important and are predominantly the ones used.

The indirect and the direct Coombs tests serve more than any other method to identify incomplete antibodies.

An enzyme test, the trypsin test, was introduced by Pickles,[113] and Morton and Pickles,[114] a test which proved itself also in routine experiments according to the assertion of English authors.

In recent years the identification of incomplete antibodies by means of macromolecular substances (polyvinylpyrrolidon, dextran, etc.) has been used.

In 1950 Hummel and Hamburger[115,116] in Germany and Formaggio[117,118] in Italy tried a synthetic colloid (polyvinylpyrrolidon, periston) to identify successfully incomplete antibodies.

Formaggio's method, which has been preserved for us, is 426 the following:

One dilutes the serum with physiological saline solution. One drop of diluted serum and one drop of 2% blood-corpuscle suspension are mixed in a tube or in the hollow depression of a slide tray and left to stand for ten to fifteen minutes. Then, one adds a drop of a 12–13% solution of polyvinylpyrrolidon diluted with physiological saline solution. After the mixture is allowed to stand for an hour at 37°, a reading is taken with the naked eye.

When using known, incomplete antibodies, this method, applied in reverse, serves to identify Rh types and other antigens.

The method saves albumin or AB serum or the serum of the required blood-corpuscle suspension, as well as the anti-globulin serum.

Hummel and Hamburger,[116] however, emphasize the influence of weather on the sensitive colloidon test. This ought not to cause amazement since, in working with colloidon on stormy days, changes in the specific gravities—for example, milk coagulating promoted by foul weather—can be observed.

Fisk and McGee[119] report that the same conditions prevail in the gelatin test.

Concerning the gelatin-Rhesus test as a conglutinin test, we have favorable experiments to report. Prokop[102] from Elbel's institute in Bonn emphasized in 1951 both the economy of gelatin solutions and their high-quality, clear results. He warned, however, against dilutions of too great concentration.

According to the experimental results of most authors exclusion of paternity can be determined from the hereditary relationship of individual allelomorphic genes on the basis of C, c, D, E, and e.

The simplest rule runs: the antigens

D	C	E	c	c
Rh_0	rh′	rh″	hr′	hr″

can only appear in children, if they are present in one of the parents.

Formaggio[121] found repeatedly in his experiments that the Rh-characteristics in newborn babies are already very strongly developed.

Witebsky and Engasser[122,123], moreover, have shown that, when human immune anti-A and anti-B sera are used, the antigens appear in newborns and in adults at the same strength.

The reliability of the hereditary rules in the Rh system has

best proven itself in wide-ranging family investigations.

Wiener[124] and Levine have used the Rh characteristics for a rather long time in the USA. Also, the most recent report of the committee cites as dependable the test of Rh and even the test of the subtypes.

427 The further use of individual subtypes to ascertain the exact hereditary type is now hindered only by the difficulty in obtaining individual antisera.

The use of only anti-D (anti-Rh_o) offers a smaller chance of exclusion since according to Race[72] both supposed parents are Rh negative only in about 2.5% of the cases involving whites and in even fewer cases involving colored persons.

Although the establishment of the Rh hereditary type is so desirable and promising, especially for paternity cases, one will have to be satisfied with the testing of Rh-phenotypes for forensic purposes from time to time until anti-Rh subgroup sera are produced in larger quantities. Thus, Ponsold[27] considers the testing of the Rh hereditary type not yet ripe for forensic purposes.

The determination of Rh phenotypes, however, presents a far-reaching differentiation of the blood formula and in many cases an exclusion of paternity.

Blood Groups Besides the ABO, MN, and Rh Systems

In recent years a few more new blood-corpuscle characteristics and antibodies have been described. First, the Lutheran blood groups were discovered by Callender and Race[98] by identifying the corresponding antibody in the serum of a Lutheran patient who had often received transfusions.

Mainwaring and Pickles[125] succeeded in obtaining the anti-Lutheran antibody by means of transfusions of Lu (a+) blood. They distinguished two Lutheran types, a stronger and a weaker, which can be compared to the A_1 and A_2; they assumed three allelomorphic genes.

The Lutheran groups were introduced into testing processes by Race and his co-workers, and in the future they will perhaps still play a role in paternity suits.

At present, however, the serum is still quite scarce. Second, the Kell blood groups were discovered also in 1946 by Coombs, Mourant and Race[126] by identifying an antibody of incomplete type in the blood of a mother with a child suffering from hemolytic disease. One year later, Wiener and Sonn-Gordon[27] described a second case with an anti-Kell. Moreover, the Kell-characteristic is dominant in hereditary transmission. The genes are K and k, of which 10.17% have been observed as Kell+ and 89.85% as Kell−.

In 1948 Levine[128] discovered in the serum of a Mrs. Cellano an antibody which produced no reaction in only 0.2% of the blood tested. The Cellano characteristic can be conceived of as antagonistic to the Kell characteristic.

428 Third, the Lewis blood groups are of special interest; they were discovered also in 1946 in England by Mourant[129] and were named after the two donors Lewis.

In 1947 Andresen[130] reported that he and Friedenreich discovered antisera which agglutinated 21% of the blood of adults.

Andresen made the interesting observation that L-positive blood is more frequent among children than it is among adults and that adults of type L− can have children of type L+. From this he concluded that, in the case of adults, only LL homozygotes produce the L+ reaction while, in children, Ll heterozygotes also produce the L+ reaction.

Andresen[130] found a second antibody, anti-L_2. That only 42% of A_1 was agglutinated by anti-L_2 and that many reactions were weak or doubtful Andresen connected with the phenomenon of epistasis[1] concerning which Mr. Prokop from Bonn will offer a closer study in the future.

In 1948 Grubb[132] from Lund made the observation at the Lister Institute in London that practically all Lewis positives were also non-secretors of A, B, or H substance.

The Le (a+) antigen was demonstrated by Grubb and Morgan[133,132] to be present in the saliva of all Le (a+) persons. Moreover, the majority of the Le (a−) persons displayed a weaker anti-Le^a inhibition effect in saliva. Because in the first years of life the reactions are not so clear as in later years, the use of Lewis blood groups in paternity cases is limited.

Fourth, the Duffy blood groups were described in 1950 by Cutbush, Mollison, and Parkin[134,135] after discovery of an antibody in the case of a man who, on account of hemophilia, had received numerous transfusions during the previous twenty years.

The antigen was found in 64.9% of the blood samples. Genes: Fy^a and Fb^b; genotypes: Fy^aFy^a, Fy^aFy^b, and Fy^bFy^b.

Among the rare blood-group systems which remain to be mentioned are the Levay, the Gr and the Jobbins systems.

Callender and Race[72] discovered the Levay group and Graydon[136] the Gr group in 1946, a year most productive in finding new antibodies. The brother and the father of the blood donor Levay possessed the antigen. Graydon himself thought that the antigen Gr could possibly be identical with the Levay antigen. The rarity of both, however, makes this unlikely.

The Jobbins blood group with an incomplete antibody was 429
described in 1947 by Gilbey.[137]

In 1951 Orth[138] gave a comprehensive presentation on the new blood-group systems.

Landsteiner, Strutton, and Chase[139] had discovered in 1934 a peculiar factor in the case of Negroes, and that only in persons who exhibit either the N factor or the factors M and N. It seems as though this factor is related to the MN system.

[1] By *epistasis* one means the masking of a hereditary factor by another factor which does not belong to the same allelic order.

If a gene hinders the phenotypical expression of another gene which belongs to a different allelic order, one says that it is epistatic over the other. The gene, which has been hindered in its phenotypical expression, is called hypostatic.

In 1951 Ikin and Mourant[140] also discovered in rabbit immune serum an antibody which reacted in a special way with Negro blood. The rabbit, however, had been pretreated with M blood which argues against assuming that this antigen is identical with the one identified by Landsteiner, Strutton, and Chase, or that it was perhaps a variety of N.

Most recently, strongly individual consanguinity-related cell characteristics have been discovered, and are independent of all systems to date. Here we must place Elbel and Prokop's[141] discovery of the Becker antigen.

Finally, the procedure of Löns deals with the supposition of an individual hereditary gene structure of the body cells and with the existence of antigens closely related to consanguinity.

This short overview ought not to close without reference to the Löns procedure which is still at the stage where it is being tested and evaluated.

The procedure consists of the following. Blood from an especially large number of persons is injected subcutaneously into a goat. In carrying this out, Wassermann blood samples are used. A mixture of the smallest blood samples (0.01cc) from approximately 200 persons is injected twice during a week until the blood from a total of 1000 persons has been used for immunization. In this way serum is obtained which contains antibodies against (?) all possible known and unknown blood characteristics.

This serum is absorbed by the blood corpuscles of the mother and of the possible sire; the corresponding antibodies are bound. Since the child can only have such characteristics which are also present in the parents, the antibodies which could be effective against the child's blood are removed from the goat's serum by the blood of the parents. If the goat's serum, pretreated (absorbed) by the parents' blood, is added to the blood of the child, no agglutination (clumping) should take place. If it does occur, then the supposed father can not have produced the child.

This process is original and includes antigen blood characteristics which are unknown up to the present time.

If the process is confirmed, it means a giant step forward, not only for the exclusion of paternity, but also for its positive-determination capability.

430 Dahr[142] and Ponsold[143,144] have occupied themselves with a thorough and comprehensive testing of the process. Though there have been arguments advanced against the method up to now, as almost always takes place in the case of a process so complicated and still in a state of development, none have yet brought about its rejection.

It is unnecessary for us to pursue any further the Löns test and the results and follow-up experiments conducted up to now, since Schmidt, Dahr, Sachs, and Ponsold will communicate their personal experiences.

In agreement with Dahr, however, we regret that a process has been taken over by several courts before it has been in fact recognized as sound on the grounds of follow-up testing. We regret that conclusions which are drawn from the results can actually be appealed to as though they had the strength of the law.

If new processes are dependable, then it is not necessary that they be employed in practice until after general scientific recognition.

If, through follow-up testing, new methods are shown to be unsuitable after they were prematurely publicized, and put to use by the courts, this could easily discredit the use of biological testing methods in legal processes in general.

Even when judges want testing for the entire "alphabet" of the groups and factors, as recently happened, the researcher ought not to leave the sure ground of established methods.

It is here that the forensic physicians as assessors can contribute much to preserving the reputation of blood testing by exercising an essential caution.

I will never forget the words which Karl Landsteiner spoke thirty-five years after his discovery of the blood groups and seven years after his identification of the M, N, and P characteristics: "I am only happy that I have nothing to do with the practical application of blood groups; I could not endure the responsibility."

In the meantime, the use of blood groups has experienced an unexpected growth. The chances are considerably better of excluding a man who has falsely been declared as the father.

Calculations concerning the chances of an exclusion based on blood characteristics have repeatedly been set forth.

In a recent article concerning "The Directions and Perspectives of Blood-group Research for Determining Paternity Based on Forty Years of Application," the old master of serology and the co-creator of the first blood-group hereditary theory, Ludwig Hirszfeld,[145] coined the concept of *complete* and *incomplete* applicability.

Incomplete applicability corresponds to the situation 431
where a dominant characteristic has been established in the case of the child, a characteristic which is lacking in the mother, but must be present in the father.

In the case of complete applicability, the rule has been established that the homozygous DD male (the dominant characteristic is double) cannot be the father of a homozygous-negative child.

Incomplete applicability has as its maximum 8.19% when group frequency is 75%; complete applicability, its maximum as 18.75% when the group frequency is 75%.

Tables, based on the characteristics OAB, MN, Rh (CDE), permit us to predict the probability of exclusion. It is noteworthy that the applicability of the characteristic Rh E was found to be six times greater than that of the characteristic Rh D.

The individuality of the blood, established on grounds of the known blood characteristics, is today well-advanced. Speiser[146] recently published a table which took into consideration the characteristics A_1, A_2, A_3, O, B, M, N, P, Rh types, and secretor with a total of 1,728 combinations.

Race and Sanger[72] (p. 275, table 88) were able to exclude approximately 62% of all men falsely accused of being the

father by considering the most important blood group characteristics known to date. The following table (table 88) from Race and Sanger's book shows the individual exclusion possibilities.

Table 1. The possibility of excluding a man, falsely accused of being the father.*

	System	Through the individual system	Through the combination of systems
1.	ABO	0.1760	0.1760
2.	MNS	0.2741	0.4019
3.	Rh	0.2520	0.5526
4.	Kell	0.0421	0.5714
5.	Lutheran	0.0333	0.5857
6.	Secretion	0.0258	0.5964
7.	Duffy	0.0496	0.6164

* According to Race and Sanger ("Blood Groups in Man," Oxford, 72, p. 275, Table 88

The same book also includes the following extremely revealing table (76) with the phenotypes of the most important blood-group systems and the 29,952 possible combinations.

Table 2. Blood Group Determinations Which Can Be Made in Many Laboratories

Blood Group System	Obtainable sera	Number of Recognizable Phenotypes
A_1 A_2 BO	anti-A-B	6
M N S	anti-M-N-S	6
P	anti-P	2
Rh	anti-C-c-C"-D-E-e	26
Lutheran	anti-Lu^a	2
Kell	anti-K	2
Lewis	anti-Le^a	2
Duffy	anti-Fy^a	2
	Phenotype combinations	29,952

In these figures D^u, c^v, C^n, A_2, N_2, k, and Le^b are not included.

If all the antibodies mentioned by Race and Sanger [72] were used together, they would produce over one million phenotypes, an amount which is equivalent to a highly-developed individuality of the blood.

These possibilities illuminate at the same time the great progress which blood group serology has experienced in the fifty years of its existence, and they highlight its special importance for forensic medicine.

Andresen in Copenhagen has rendered the field a personal service in collecting and publicizing the titles of numerous new works in this field in his bulletin entitled "Blood-group News."

When we, as the experts, make use of the achievements of blood-group research in proving the truth before the court, we do not want to forget the tremendous pioneering work which has been done; we want to thank all those who have made efforts in the past and are doing so now in the interests of developing this science.

Our greatest thanks, however, transcends the grave to honor in the first place the man who, at the beginnning of this century, opened for us the door to this wonderful field of research and to its recognition and its practical world-wide use, a man who recognized its importance for forensic medicine fifty years ago, Karl Landsteiner.

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