UNIT II
IDENTIFICATION OF BLOOD

SECTION 3. HISTORY AND DEVELOPMENT OF MEDICO-LEGAL EXAMINATION OF BLOOD

There is a considerable literature concerning the history of legal, or forensic, medicine, but little mention is made of medico-legal examinations of blood and/or body fluids in most of the work. The National Library of Medicine's *Bibliographies of the History of Medicine* (U.S. DHEW, PHS, NIH, NLM, 1964–1969 and 1970–1974) contain some references to legal medicine; but Nemec's bibliography is perhaps the best and most complete single source (Nemec, 1974). One of the most well-known scholars of the history of legal medicine, the late Prof. R. P. Brittain, has done a useful bibliography of medico-legal works in English (Brittain, 1972), which contains some references to the medico-legal examination of blood and body fluids. Brittain was apparently engaged, at the time of his death, in the preparation of an extensive, closely-documented work on the origins of legal medicine. Some parts of this work have appeared as a series of papers in the *Medico-Legal Journal*; these papers deal with the medico-legal aspects of the *Constitutio criminalis Carolina* (Brittain, 1965a), the *Leges barbarorum* (Brittain, 1966a), the Assizes of Jerusalem (Brittain, 1966b), the *Capitularia* of Charlemagne (Brittain, 1966c), and the *Leges duodecim tabularum* of 449 B.C. (Brittain, 1967). In addition, there are papers on the origin of legal medicine in Italy (Britain, 1965b) and in France from the 12th to the 16th centuries (Brittain, 1966d). There are, however, no direct references in these discussions to the identification or individualization of blood or body fluids.

According to Kiel's interesting and well-documented review of the origins and development of legal medicine in China (Kiel, 1970), a Chinese doctor named Chich-ts'si wrote the first known treatise on forensic medicine. The work, entitled *Ming Yuen Shih Lu,* was written in the 6th century A.D. Around 1250 A.D., a more comprehensive and well known treatise on legal medicine, known as *Hsi Yuan Lu,* or "Instructions to Coroners," was compiled in China. Kiel notes that there are references in this work to the detection of bloodstains. It was said that an old bloodstain could be detected on a knife by heating the stain and treating it with vinegar, after which a brown stain, resulting from the formation of hematin crystals through the reaction of the hemoglobin with the acetic acid in the vinegar, would appear. A method for paternity testing was prescribed in the *Hsi Yuan Lu* as well. Apparently known as the "dropping test," this method called for the allegedly related persons, such as parent and child, to cut themselves and allow their bloods to drip into a single basin of water. Smooth mixing of the bloods was taken to mean relatedness, while clumping of the cells indicted incompatibility, and presumably, non-relatedness. Furuhata (1927) mentioned three other very early works in Chinese: *Sen-en-roku* (False charges cleared) in 4 volumes by Ji of the Sung dynasty (1247); *Kei-en-roku* by Cho of the Sun dynasty (year unknown); and *Mu-en-roku* (No false charges) by O Yo of the Gen dynasty (1308). The last was translated into Japanese in 1736 by Naohisa Kawai with the title *Mu-en-roku-jutsu* (Interpretation of no false charges). The "dropping test" was mentioned in these works, as was some method for determining affiliation between the living and the dead by dropping the living person's blood onto the bones of the deceased. While these methods were unreliable by any present standards, as noted by Wiener (1960), they are interesting precursors to present methods and ideas. The vinegar test for blood may have been a crude Teichmann crystal test.

Rosner (1973) has described an interesting passage in the *Talmud,* written in the 2nd century, which elaborates a method for the differentiation between bloodstain and a dye stain on a garment. According to the *Talmud,* seven substances were to be applied to the stain in a specified order; stains which then faded away or became fainter were bloodstains, while those which did not fade had originated from dyes. The seven substances, in order, were; tasteless spittle, the liquids of crusted beans, urine, natron (native sodium carbonate), *borith* ((lye, or perhaps sulfur, or a type of plant or grass), *kimonia* (Cimolean earth, or a type of clay, or salt, or pulverized dried grass), and *eshlag* (lion's leaf, said to be "found between the cracks of pearls, and . . . extracted with an iron nail," or possibly an alkali or mineral used in soap). In various sections of the *Talmud* and in various Talmudic commentaries, according to Rosner, the nature of these seven substances is discussed and specified. But, as will be clear from the parenthetical notes above, differences in translation and in various commentators' opinions have led to some confusion as to precisely what they were in all cases.

A few references are made to blood and body fluid examinations in Thomas' interesting and readable review of the highlights of the development of forensic science and medicine in Europe from the Renaissance to World War II (Thomas, 1974). This article contains some interesting material on M. J. B. Orfila, about whom we shall have more to say shortly. The contributions of Jules Bordet, Paul Uhlenhuth and Karl Landsteiner are discussed as well, and these, too, will be discussed in appropriate, subsequent sections of this Sourcebook.

It would be of interest to know when and where the medico-legal identification of bloodstains, using relatively modern chemical techniques, originated. It seems quite reasonable to draw a distinction between the ancient and medieval methods and those of more recent times on the basis

of the fact that the latter rely on at least some knowledge of the chemcial composition of blood. The fact that the knowledge of blood's chemical composition was very incomplete, and characterized by notions that have not stood the test of time, may make this distinction somewhat arbitrary. But it seems possible, nevertheless, to distinguish between the pre-19th century commentaries which are of purely historical interest, and those methods which appeared after 1800, and were based on what most people would regard as chemical priniciples, rather than on purely empirical observations having no theoretical framework whatsoever. One of the oldest books in English having to do with bloodstains is W. D. Sutherland's *Blood-Stains: Their Detection, and the Determination of Their Source* (Sutherland, 1907). The book is, in fact, one of the few in any language up to its time which treats what we now call forensic serology (in its broadest sense) exclusively. Sutherland, a Major in the British Indian Medical Service at the time of the book's appearance, was obviously something of a scholar, in addition to the fact that he had forensic casework responsibilities. The Preface of the book begins with the comment: "As there does not exist in any language of which I have knowledge a compendium of the modern tests by which the detection of bloodstains and the determination of their source may be carried out . . . ." From this remark, and from the nature and extent of his bibliographic reference list, it seems likely that Sutherland read a number of modern languages and probably Latin in addition of course to English. An interesting man, he later became a Lieutenant Colonel, and apparently stayed in India until he died in 1920. A brief obituary appeared in the *British Medical Journal* **2:** 189 (1920). He was very interested in the precise origins of medico-legal examinations of bloodstains, noting at the beginning of the second chapter:

> I have endeavoured to ascertain when the medico-legal importance of blood-stains first came to be recognized, but without success. The older medico-legists, whose works are cited in the bibliography, treat of most things from miracles to slight wounds, but none make special mention of bloodstains. And, as we see, even so late as 1834 the fourth edition of a popular German textbook contained no special reference to them, although in 1817 Orfila had dealt with the chemistry of the blood in his textbook of medical chemistry, and in 1808 Jacopi is said to have managed by the aid of the microscope to distinguish bovine from human blood. It is from the discussion which took place at the Academie Royale de Medecine in 1828 that I infer that for a considerable time the French experts had busied themsleves with the question of the detection of bloodstains.

The discussion to which Sutherland referred may be one which appeared in the *Journal generale de medecine de chirurgie et de pharmacie* in 1828 (Raspail, 1828a; Orfila, 1828a; Raspail, 1828b; Raspail, 1828c). It was based on a serious disagreement which had arisen between Raspail and Orfila over the value which was to be attached to the results of a series of chemical tests proposed by Orfila (1827a) for the identification of blood in medico-legal investigations. Mathieu-Joseph-Bonaventure Orfila was one of the most prominent medical scientists of his time. Born in Spain April 24, 1787, Orfila began his studies in that country, but completed them in France, receiving his doctorate at Paris in 1811. He remained in France until his death in 1853. He enjoyed a brilliant career, had considerable prestige, and his opinions and pronouncements carried a good deal of weight in the scientific community. Orfila was primarily a toxicologist; Prof. Dr. Muñoz, in a Spanish-language biographical tribute to Orfila, published in 1956 in Quito, Ecuador, called him the "founder of modern toxicology". A biographical sketch of Orfila's career appeared at the time of his death as well (Chevallier, 1853). There is no doubt that Orfila was one of the important figures in the development of legal medicine. At various times he focused his attention on blood and body fluids, and his contributions in this area are among the earliest systematic studies that can be found in the published literature.

The earliest methods for medico-legal identification of blood were chemical ones. Lassaigne is said to have published a paper on the discrimination of bloodstains and rust spots in 1825. This paper could not be located, though there is no doubt of its existence. In 1827, Orfila published a systematic series of tests for the recognition of bloodstains on various substrata, and their discrimination from rust spots, iron citrate, and a number of red dyes (Orfila, 1827a). He mentioned Lassaigne's work, but without giving a specific reference. Orfila said that, in order not to be accused of scientific plagiarism, he should point out that he had been engaged in this work since 1823.

Orfila's tests were based primarily on the solubility of bloodstain components in water, and the behavior of the aqueous extract toward a number of reagents, litmus paper, and so forth. Raspail disagreed that Orfila's criteria were sufficient to establish the identity of blood in a medico-legal investigation, and said that a stain could be constructed from ovalbumin and a red dye which would give a completely convincing set of false positive results using Orfila's tests. He noted further that there might exist other materials which would give results similar to the ones given by blood. Orfila responded to these objections in a point-by-point refutation which appeared in the discussion cited above (1828a), as well as in a separate paper (Orfila, 1828b). Orfila was convinced that he was correct, but Raspail did not accept the refutations as is clear from the discussions in the Royal Academy. It appears that Orfila's opinion on the matter prevailed to a large extent, at least in terms of acceptance by judicial tribunals. In 1835, Orfila and his colleagues J.-P. Barruel and J. B. A. Chevallier published a detailed report on a case in which they had been consulted. Three brothers named Boileau and a fourth man named Victor Darez were accused of having murdered a rural constable named Hochet. A number of suspected bloodstains were involved in the case, and the Court was interested in knowing whether certain of these stains were of human or animal origin, and

whether certain of them came from the same individual, assuming it could be established that they were stains of human blood. The experts established that a number of the stains were bloodstains using Orfila's chemical tests as criteria. They could not shed any light on the questions of species of origin, nor of common origin, nor on the question of the time interval which had elapsed since the deposition of the stains.

Around this time, some authorities were considering microscopical examination of bloodstains as a means of identification of blood in medico-legal inquiries. In some minds, microscopical results were more certain, and were preferable to the chemical methods. This subject is discussed in section 5.3. It was also recognized that carefully conducted microscopical observations of blood cells from bloodstains could, under some circumstances, help in diagnosing the species of origin. The blood of animals having nucleate red cells could, at least, be distinguished from mammalian blood. Some authorities, aware that the red cells of different species differed in size, thought that carefully conducted microscopical measurements of the red cells could, in some cases, serve as a basis for species determination, even among mammalian species. This subject is discussed in section 15. Orfila (1827b) looked into microscopical methods for blood identification and species determination. He was unable to obtain satisfactory results, and concluded that the chemical methods were much more reliable. Around 1836, Prof. Persoz at Strasbourg introduced the use of hypochlorous acid as a reagent for discriminating bloodstains from other red stains, especially older ones which were not very soluble in water. Orfila evaluated this technique quite thoroughly in 1845, and found it unsatisfactory if used alone, but admitted that it might be useful as an auxiliary method in certain types of cases.

A number of the early papers on the subjects discussed in this section may be read in their entirety in the translations (Unit IX). There is not much doubt that the earliest scientifically systematic attempts to employ physical and chemical methods to the medico-legal examination of blood and body fluids are attributable to the French scientists in the early years of the 19th century. Some of the earlier papers on the identification of seminal fluid, other body fluids, menstrual blood, and so forth, are discussed in appropriate sections and some have been included in the translations as well (Unit IX).

SECTION 4. CRYSTAL TESTS

4.1 Structure and Nomenclature of Porphyrins and Hematin Compounds

The discussion of crystal tests and of spectrophotometric and spectrofluorimetric methods which follows will involve many terms which refer to porphyrin and hematin compounds. The history and development of nomenclature of these materials is somewhat complex and can lead to considerable confusion. Interested readers are referred to the specialized works by Lemberg and Legge (1949), Falk (1963) and Marks (1969). Most standard biochemistry texts also carry a discussion of the subject (e.g. White *et al.*, 1973; Pritham, 1968; Mahler and Cordes, 1971). The present discussion is designed primarily to provide an outline of the nomenclature, and give some indication of the different meanings that may be associated with various terminology.

Porphyrins are derived from the cyclic ring compound *porphin*, a structure with four pyrrole-like rings linked by methylene bridges. Represented in Figure 4.1 in two different ways, the structures (a) and (b) are identical, neither is "correct" or "incorrect", and neither is preferable for any particular reason. Both are encountered in the literature.

The porphyrins which occur in nature are all compounds in which side chains are substituted for some, or all, of the hydrogens at positions 1 through 8 in Figure 4.1. It is convenient in giving structural representations of these compounds to use a "shorthand" notation for the porphyrin nucleus, omitting structural detail, but allowing the different side chains to be shown in their correct positions. Figure 4.2 (a) and (b) shows the shorthand representations corresponding to the structures in Figure 4.1 (a) and (b), respectively.

Introducing side chains into the molecule gives rise to a large number of different structural isomers. If, for example, the eight numbered hydrogen atoms are substituted with four methyl- and four ethyl- groups, four isomers are possible. These are shown in Figure 4.3 according to both "shorthand" conventions, corresponding to Figure 4.2 (a) and (b), respectively. Only compounds deriving from structures I and III, Figure 4.3, are found in nature, those from III being more important. In representing the isomers according to the shorthand notation, it is usual to number the positions as has been done in Figure 4.2. Not all authors number the positions in the same way, however, and while any sequentially numbered system is interconvertible to any other by rotation in the plane of the paper, it eliminates confusion, in my opinion, if the numbering convention is clearly stated. Some of the different, naturally occurring porphyrins are indicated in Table 4.1. Only the type I and type III isomers (Figure 4.3) are given. If the number of types of substituent side chains is increased, then clearly the number of possible structural isomers increases as well. In the case of protoporphyrin, for example, with three substituent types, there are fifteen structural isomers. All the porphyrins derived from hemoglobin and naturally occurring hematin compounds are of protoporphyrin type III, Table 4.1. The molecule is more commonly referred to as protoporphyrin IX, since it was the ninth in a series of isomers listed by H. Fischer (White *et al.*, 1973). Protoporphyrin IX is shown in shorthand notation in Figure 4.4.

Porphyrins possess the ability to combine with many metals, the most important biologically active molecules being those in which the porphyrin is combined with Fe or Mg. These compounds are collectively referred to as metalloporphyrins. The hematin compounds, which are the only metalloporphyrins of major interest to this discussion, are all iron-protoporphyrin compounds. The iron atom in an iron-protoporphyrin complex is coordinated to the four pyrrole nitrogen atoms in a planar configuration (Figure 4.5), replacing the two dissociable hydrogens of the porphyrin nucleus. The detailed properties, including the nature of the coordinate binding and thermodynamic stability, or iron and other metal porphyrins were discussed by Phillips (1963). The iron complexes readily add two additional ligands, which coordinate to the metal forming an octahedral structure and in which the metal is then hexacoordinated.

The valence of the iron atom is specified by the prefixes *ferro-* (for Fe^{2+}) and *ferri-* (for Fe^{3+}) in naming the various compounds. *Heme* is *ferroprotoporphyrin. Heme* is spelled *haem* in some countries, the spelling variation carrying over to other terms derived from the word *heme*, e.g. hemoglobin/haemoglobin, hematin/haematin, hemochromogen/haemochromogen, etc. *Ferriprotoporphyrin*, obtained as the chloride, is called *hemin chloride*, or *hematin chloride. Ferriprotoporphyrin hydroxide* is simply call *hematin.* The use of the term *hemin* is restricted by some authors to ferriprotoporphyrin halides, especially the chloride, (Lemberg

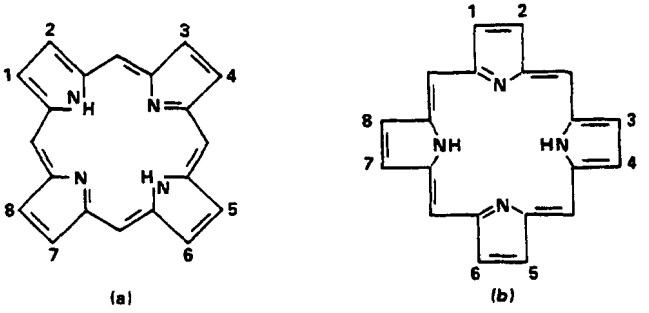


Figure 4.1 Porphin

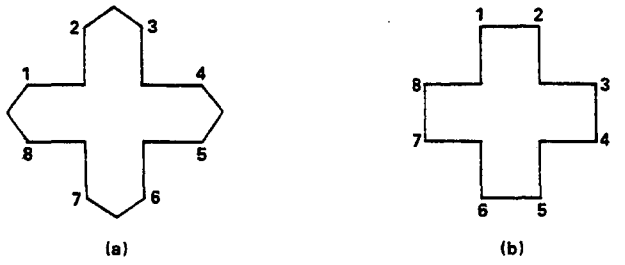


Figure 4.2 Porphin - Shorthand Notation

chromogens, and the terms *hemichrome* and *parahematin* have been applied to *ferrihemochromes.* Table 4.2, a modification of Table I, Chapter V, of Lemberg and Legge (1949), gives a comparison of some of the different nomenclatures.

Another important consideration, which is not really a matter of nomenclature, but which may be worthy of brief discussion here, is that of the interconvertibility of the various hemoglobin derivatives. Both the crystal and spectral tests for the presence of blood in stains rely on these conversions. There are, in addition, a number of methods designed to determine the age of bloodstains which rely on the

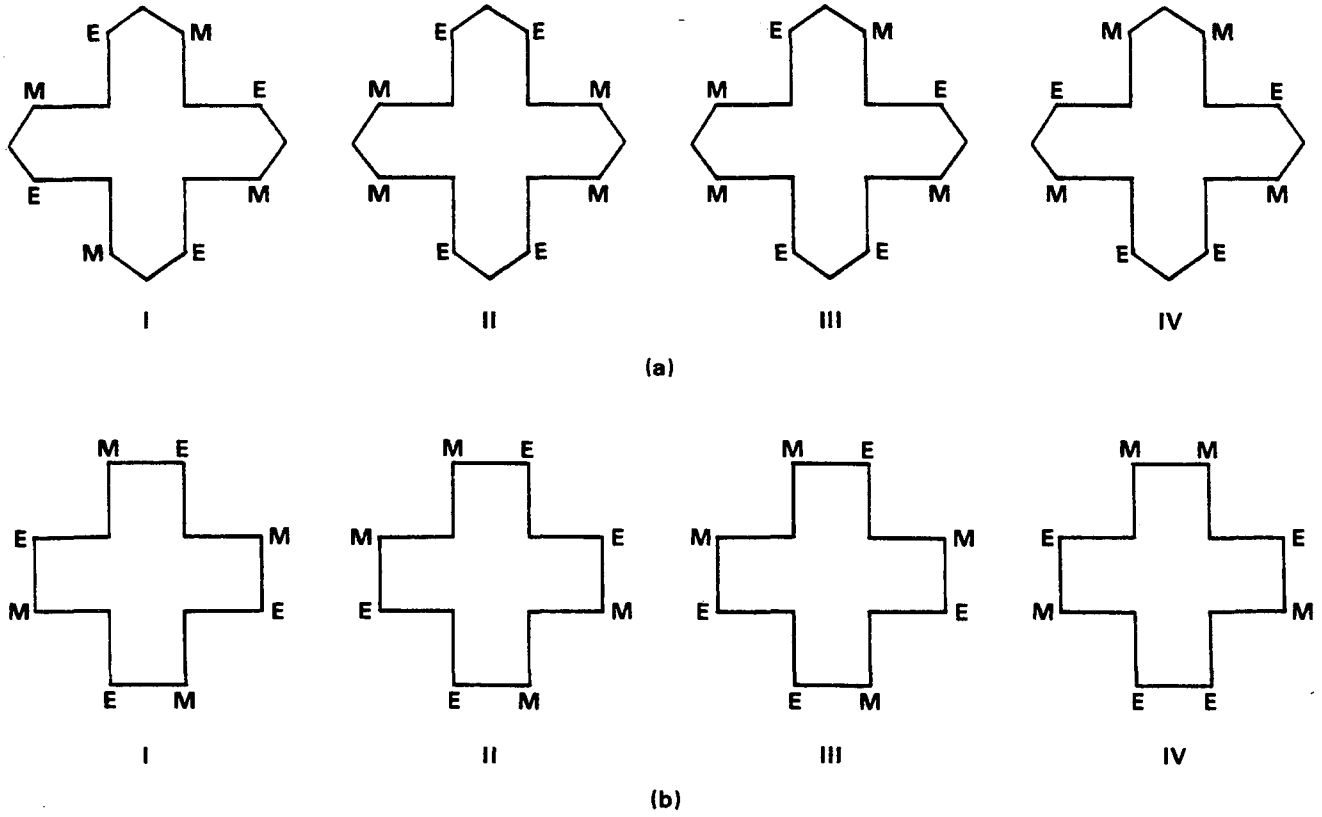


Figure 4.3 Structural Isomers of Etioporphyrin - Equivalent Representations

M - methyl E - ethyl

and Legge, 1949; Phillips, 1963; White *et al.,* 1973). Marks (1973) seems to be suggesting that the term *hemin* be reserved for ferriprotoporphyrin halide crystals (see Teichmann, 1853), and that it should not be used in place of the term *hematin. Ferroprotoporphyrin* may be called *ferroheme,* as *ferriprotoporphyrin* may be called *ferriheme* (or *ferrylheme*). In compounds in which the fifth and sixth liganding molecules are nitrogenous bases, the term *hemochromes* is often applied. The names *ferrohemochrome* and *ferrihemochrome* may be used to specify the valence of the iron atom. *Ferrohemochromes* have long been called *hemo*-hemoglobin-methemoglobin interconversion. These are discussed in a later section.

The structure of hemoglobin will not be discussed here, but in a later section dealing with the determination of genetically-determined hemoglobin variants. Suffice it to say that native human hemoglobin is a tetrameric molecule, consisting of two α and two β polypeptide chains, having one heme per peptide chain, or four in the intact molecule, and a molecular weight of about 68,000. The iron atom is divalent in hemoglobin. Oxidation of the iron atom to the ferric state gives rise to methemoglobin (hemiglobin; fer-

Table 4.1. Side Chain Structures of Some Porphyrins

Porphyrin	Substituents	Type I	Type III
Etioporphyrin	4 M, 4 E	1,3,5,7-M 2,4,6,8-E	1,3,5,8-M 2,4,6,7-E
Mesoporphyrin	4 M, 2 E, 2 P	1,3,5,7-M 2,4-E 6,8-P	1,3,5,8-M 2,4-E 6,7-P
Protoporphyrin	4 M, 2 V, 2 P	1,3,5,7-M 2,4-V 6,8-P	1,3,5,8-M 2,4-V 6,7-P
Coproporphyrin	4 M, 4 P	1,3,5,7-M 2,4,6,8-P	1,3,5,8-M 2,4,6,7-P
Uroporphyrin	4 A, 4 P	1,3,5,7-A 2,4,6,8-P	1,3,5,8-A 2,4,6,7-P
Deuteroporphyrin	4 M, 2 H, 2 P	1,3,5,7-M 2,4-H 6,8-P	1,3,5,8-M 2,4-H 6,7-P
Hematoporphyrin	4 M, 2 HE, 2 P	1,3,5,7-M 2,4-HE 6,8-P	1,3,5,8-M 2,4-HE 6,7-P

Abbreviations used in the table: Numbering corresponds to Fig. 3.2. M- methyl E- ethyl P- propionic acid V- vinyl HE- hydroxyethyl A- acetic acid H- hydrogen

rihemoglobin). Methemoglobin does not bind oxygen, but will bind a number of other ligands, such as hydroxide, cyanide, azide and nitrite (Kiese, 1954). Figure 4.6 summarizes the interrelationships between the various derivatives (Lemberg and Legge, 1949; Pritham, 1968).

4.2 Crystal Tests

4.2.1 Introduction.

Parkes (1852) reported that, in examining microscopically the residual matter in a bottle which had contained partially putrefied blood, had been rinsed with water, and allowed to stand for a time, needle-like crystals could be observed in abundance. These crystals were insoluble in water and in strong acetic acid, but soluble in what I presume to be KOH. The crystals could be reprecipitated with strong acetic acid, but were less satisfactory and less abundant than the original crystals. Parkes noted that around this same time Funke had reported similar crystals from blood-water mixtures using horse spleen blood and fish blood. Drabkin (1946) has suggested that Funke may have been looking at hemoglobin crystals. Kölliker (1853-1854) reported that he had observed crystals in dog, fish and python blood in 1849. These were soluble in alkali and in acetic and nitric acids, and he said they were identical to Funke's crystals. Parkes subsequently attempted to prepare crystals similar to those which he had discovered by accident, but did not again obtain them in the same quantity. He did note that a number of different types of crystals are obtainable from putrefying blood, but could not identify them. He did not think they were identical to the hemoglobin crystals of Virchow nor to the albumin crystals of Reichert.

The preparation, microscopical and spectroscopic examination of crystalline forms of hemoglobin derivatives have occupied a great deal of attention in the development of methods for the medico-legal identification of blood. Many methods have been devised, and most are based on the preparation of either hematin or hemochromogen crystals. Many authorities have regarded crystal tests as methods of certainty in the identification of blood in stains (Beam and Freak, 1915; Brunig, 1957; Bertrand, 1931; Casper, 1861; Chiodi, 1940; Derobert and Hausser, 1938; Gonzales et al, 1954; Guarino, 1945; Lopez-Gomez, 1953; Lucas, 1945; Mueller, 1975; Olbrycht, 1950; Rentoul and Smith, 1973; Schleyer, 1949; Smith and Fiddes, 1955; Sutherland, 1907). Others who have considered the crystal tests in detail have been less explicit about the issue of whether the tests should be considered certain or not (Ziemke, 1938; Kirk, 1953). Dalla Volta (1932) took the position that the microscopical methods used to examine the crystals in routine forensic practice were inadequate to insure proof. Rather more sophisticated crystallographic analysis than would be routinely practical would be needed, in his view, to establish with certainty the presence of blood by these methods.

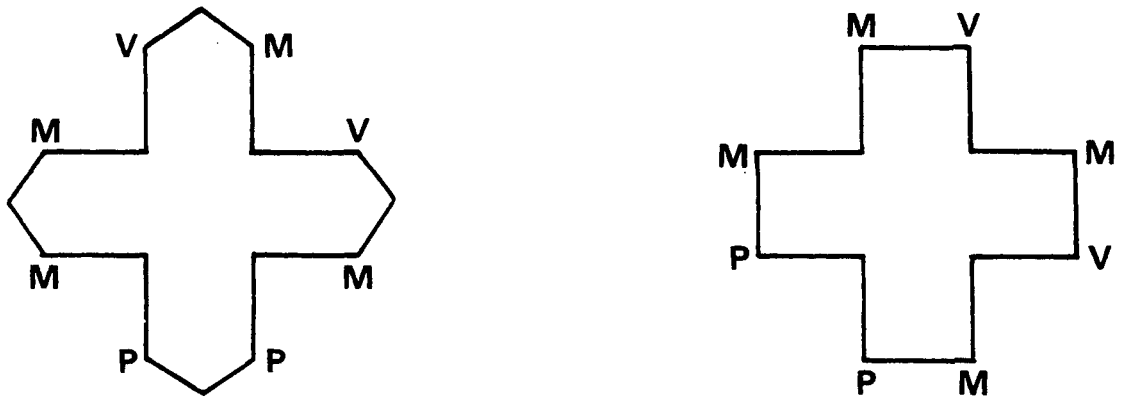


Figure 4.4 Equivalent Representations of Protoporphyrin IX

M - methyl V - vinyl P - propionic acid

4.2.2 Hematin crystal tests

In 1853, Teichmann published an extensive study of the formation of crystals in blood; this paper, still occasionally cited, formed the basis of the use of crystal tests for identification of blood in bloodstains. Teichmann's name has subsequently become synonymous with the hematin crystals which he obtained as well as with his own, and various modifications of the method for doing so. Teichmann's crystals are obtained by treatment of blood with glacial acetic acid in the presence of small quantity of salt and gentle heating. He called these crystals hemin, but they are now known to be hematin chloride. He suggested that the ability to form them reliably would provide the basis for medico-legal identification of blood in stains, as indeed it has done. Sutherland (1907) regarded a positive Teichmann crystal test as *"a sure proof of the presence of blood in the suspected stain"* (emphasis his own).

The extensive literature which developed on the subject has had mainly to do with the following matters: (a) development of different (hopefully improved) reagents for obtaining the crystals, including the use of halides other than chlorides, and of acids other than acetic; (b) variations in the way the test is carried out; and (c) experiments to determine the different conditions and substances that could interfere with the test, cause it to fail, or yield a result in the absence of blood. Casper (1861) in his Handbook mentions the test, noting that it is of value only when positive. The older literature on hematin crystals and crystals from blood in general was reviewed by Bojanowski in 1862. Table 4.3 is a modified version of one which was prepared by Lewin & Rosenstein (1895) in their review and reproduced by Sutherland (1907).

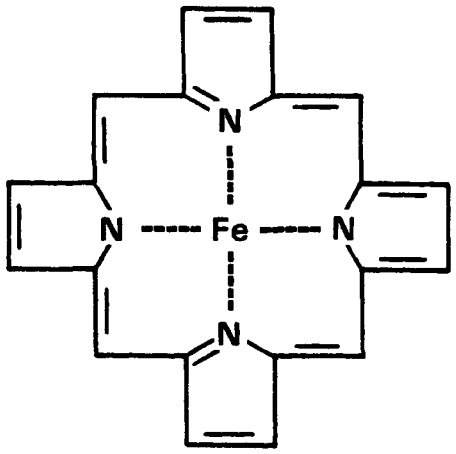


Figure 4.5 Iron Protoporphyrin

From it, one can get an idea of the different variations of the hematin crystal test in the older literature. It should be noted that some authorities felt that it was unnecessary to add salt because blood contains salts, which are present in sufficient quantity to yield crystals.

Henocque (1875) reported that an investigator named M. C. Husson had prepared hematin iodide crystals from the blood of a number of species. Sarda (1910) noted that Husson's work was reported in 1875, and that it involved both the iodide and the bromide of hematin. He reported very good results of his own with the potassium and ammonium salts of bromide and iodide.

Table 4.2. Comparison of Nomenclature of Hematin Compounds

Coordinating Ligands in iron protoporphyrin IX	Charge on over-all coordinate complex	Valence of Fe	Old Names	Nomenclature of Pauling & Coryell (1936) and Guzman Barron (1937)	Nomenclature of Clark (1939), Clark et al. (1940) and Drabkin (1938 and 1942a)	General Nomenclature
four pyrrole N	0	2	reduced hematin; heme	ferroheme	ferroporphyrin	heme
four pyrrole N, two additional N of nitrogenous base	0	2	hemochromogens; reduced *hemochromogens*; reduced hematin	*ferrous or ferro-* hemochromogens	base (e.g. dipyridine—, nicotine—) ferroporphyrin	hemochromes
four pyrrole N, water and OH^-	0	3	hematin; hydroxyhemin; oxyhemin	ferriheme hydroxide	ferriporphyrin hydroxide	hematin
four pyrrole N	yes★	3	hemins, e.g. chlorhemin, bromhemin	ferriheme chloride, bromide etc.	ferriporphyrin chloride, bromide etc.	hemins (Cl, Br, etc.)
four pyrrole N, two additional N of nitrogenous base	yes★	3	parahematins; oxidized *hemochromogens*	*ferri- or ferric* hemochromogens	base (e.g. dipyridine—, nicotine—, etc.) ferriprotoporphyrin	hemichromes

★ Charge depends on pH.

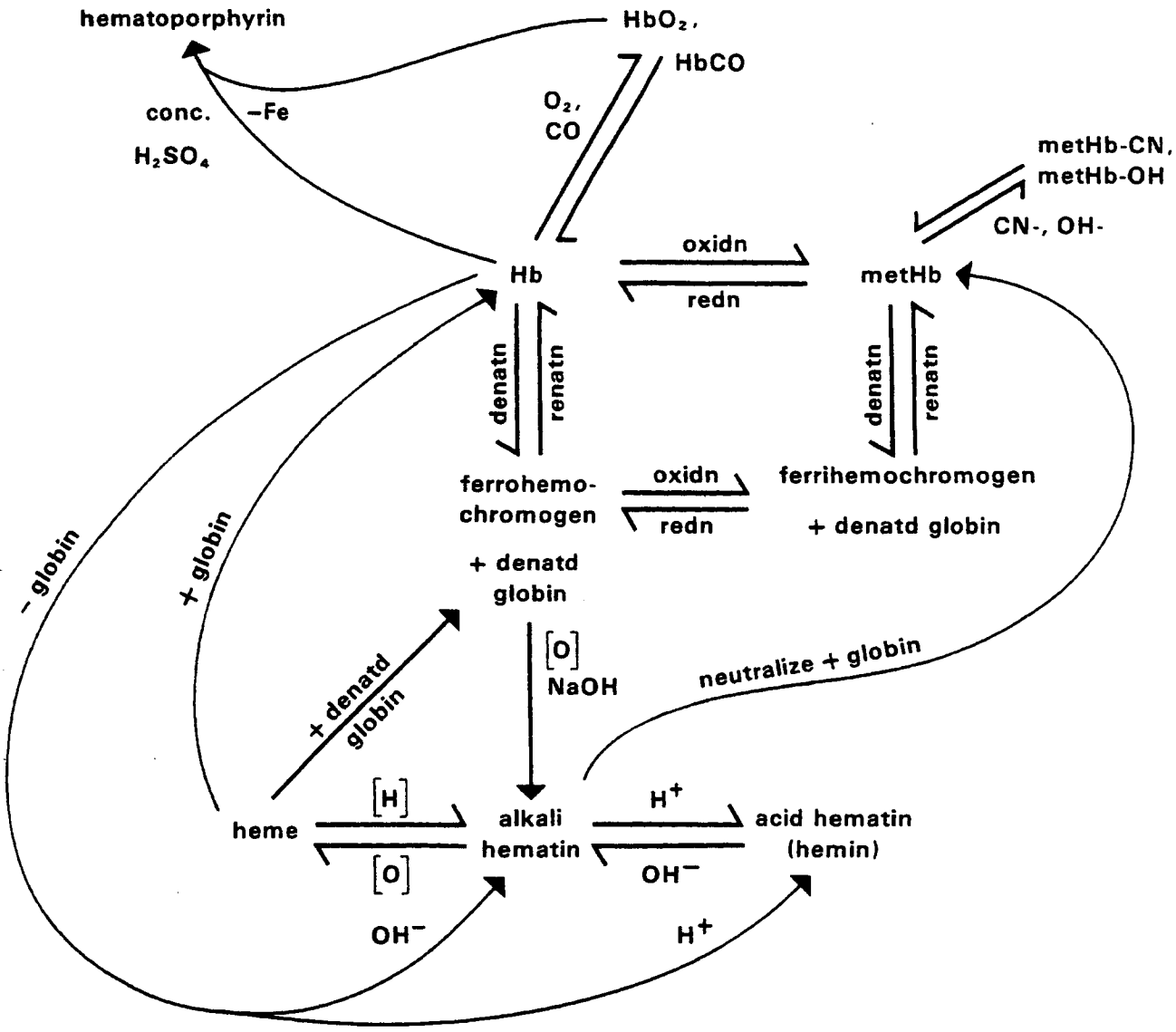


Figure 4.6 Interrelationships Among Hemoglobin Derivatives

Abbreviations used: Hb = hemoglobin HbO_2 = oxyhemoglobin Hb CO = carboxyhemoglobin metHb = methemoglobin metHb-CN = cyanomethemoglobin metHb-OH = hydroxymethemoglobin denatn = denaturation renatn = renaturation denatd = denatured [H] = reducing agent [O] = oxidizing agent oxidn = oxidation redn = reduction H^+ = acid OH^- = base conc = concentrated

Table 4.3 *Some Modifications of the Hematin Crystal Test (modified from Lewin and Rosenstein, 1895)*

Blood Preparation	Acetic Acid	Other Acid	NaCl	Other Salt	Heat or Temperature (°C)	Reference
dried,	much	oxalic, tartaric citric lactic	–	–	25 - 62.5	*Teichmann (1853)*
dried, fluid	slight excess	–	only if none in blood	–	cold or 40 - 60	Büchner and Simon (1858)
dried	to fill space under cover slip	–	yes	–	heat in flame	Virchow, 1857
–	yes	–	no	–	gentle heat or spontaneous evaporation	Morache, 1881
dried	few drops	–	no	–	heat until bubbles appear	Janert, 1875
dried, fluid	yes	–	few drops salt soln	–	*heat in H_2O bath*	Brücke, 1857
dried	yes	oxalic, tartaric *in alcohol*	yes if blood Cl free	NaBr, KBr NH_4Br, NaI KI	–	Bikfalvi, 1886
sediment prepared by copper sulfate pptn and extraction with alcoholic sulfuric acid	yes	–	yes	$BaCl_2$, SrCl, KCl LiCl, $CaCl_2$, NH_4Cl $MnCl_2$, $SnCl_2$, $FeCl_2$ $MgCl_2$	–	Teichmann, 1856
dilute solutions of blood pigment, add ammonia, tannic acid, then acidify with acetic acid, get *hematin tannate precipitate*	yes	–	no	NH_4Cl	–	Struve, 1880
alcoholic extract of dried precipitate by sodium carbonate pptn of defibrinated blood	yes	–	yes	$CaCl_2$	–	Gwosdew, 1886

According to Oustinoff (1929), who advocated the iodide crystals, Strzyzowski had prepared hematin iodide in 1902. Guarino (1945) employed iodoform in alcohol for the preparation of the crystals, and Lopez-Gomez (1953) reviewed, in some detail, the use of bromide and iodide salts in hematin crystal test reagents. Lopez-Gomez and Cantero (1942) are said to have carried out systematic studies on bromide and iodide crystals. Gouillart (1939) studied the formation of hematin iodide crystals in great detail. There is some confusion in the literature regarding the preparation of hematin fluoride crystals, Welsch and Lecha-Marzo (1912a) seeming to advocate their use, while Leers (1910 and 1912) believed that other halogens were greatly preferable. In any case, there does not seem to have been much subsequent use of fluoride-containing reagents. A number of authorities have recommended solutions containing 0.1g each of KCl, KBr and KI in 100 mℓ glacial acetic acid for the production of Teichmann crystals (Nippe, 1912; Kirk, 1953; Smith and Fiddes, 1955). Fiori (1962) said that there is no particular advantage to preparing hematin crystals from halogens other than chloride.

An extremely thorough study of the formation of both hematin and hemochromogen crystals was published by Mahler in 1923. He studied bloods from different species, including human, mostly as dried stains under a variety of different conditions and using a number of different methods. In these expriments, Mahler compared among other things the method of Wachholz (1901), utilizing alcoholic solutions of strong acids, with that of Nippe (1912) which called for a solution that is 0.1% (w/v) in KI, KBr and KCl in glacial acetic acid. Mahler got his best results from a combination of the two methods, wherein the blood-stained fragment was first warmed with alcoholic glacial acetic acid, and then warmed again following the addition of the Nippe reagent.

A similar set of comparative experiments was done by Kerr and Mason (1926). Some of the methods they studied were the same ones that Mahler had considered, but there were some differences. They preferred the method of Sutherland (1907), according to which a drop of saline is evaporated by heating on a clean slide, the stained fragment then being placed on the residue, and a drop of glacial acetic acid added. After applying a cover slip, the preparation is heated gently until bubbles just appear. It is then set aside and crytallization allowed to proceed.

Blood or bloodstains heated to temperatures in excess of 140° to 145° will not yield Teichmann crystals (Katayama, 1888; Hammerl, 1892; Wood, 1901). Bell (1892) discussed the hematin test in his review, and described the techniques then being used in this country by Formad (Formad, 1888) and by Prof. Tidy. Wood (1901) discussed his own experiences with the test in a paper read to the Massachusetts Medico-Legal Society. Any substance or condition which causes hemoglobin or hematin to form its decomposition product, hematoporphyrin (iron free hematin), he said, will interfere with crystal formation. Heat, long exposure to sunlight, and some organic solvents often cause difficulty. It is to be noted, nonetheless, that Muller *et al.* (1966) reported a positive Teichmann test from bloodstains on clothes that had been dry cleaned. Schech (1930), in his studies of the effects of ironing bloodstains on cloth on the subsequent ability to detect and analyze the bloodstain, noted that hematin crystals might be obtained if the iron had not been applied directly, but through a wet cloth for example. Rust and exposure, and especially the combination of these, interfere with the test (Sutherland, 1907).

Among the many modifications of the test that have been proposed, a few others will be mentioned. Oustinoff (1929) thought that the incorporation of gum arabic into the reagent improved the results. The heating step in the procedure, if done, is very critical. Bertrand (1931) discussed this point, noting that it is possible to err either in the direction of overheating or of underheating. He recommended a solution containing glycerol, apparently to lower the volatility of the reagent. Wachholz (1901) recommended a solution of a concentrated acid (lactic, sulfuric or acetic) in 95% alcohol because this boils easily, and reduces the chances of overheating. Sottolano and DeForest (1977) have described a technique utilizing Kirk's (1953) solution for hematin crystals which involves placing the test slides on a rack within a pressure cooker. Crystal formation is facilitated by the increased pressure, and the danger of total evaporation is overcome. The technique is applicable to the formation of hemochromogen crystals using Takayama's solutions (see Section 4.2.4) as well. Beam and Freak (1915) proposed a technique which, they stated, rendered crystal formation much more certain. The essential ingredient of this modification is very slow evaporation. The material to be examined is placed in the bottom of a flat, arsenic sublimation tube. A few drops of glacial acetic acid, containing 0.01 to 0.1% NaCl, are added, and a fine cotton thread is placed in the tube such that its lower end contacts the solution and its upper end is near the top of the tube. The thread is moistened if necessary to insure that it is everywhere in contact with the side of the tube, and evaporation is allowed to proceed at its own rate, without heating. The process which may require from 12 hours to more than a day, is accompanied by capillary movement of the solution within the thread, the crystals forming along the thread's length. This technique, recommended by Lucas (1945), is capable of giving large crystals suitable for crystallographic analysis (Fiori, 1962), but may not be very practical for routine work because of the investment of time required.

Although a number of materials and conditions interfere with the formation of hematin halide crystals, the age of the stain alone does not seem to be deleterious. Haseeb (1972) got a positive Teichmann test on a 12-year old stain kept on the laboratory bench in the Sudan. Beam and Freak (1915) obtained crystals from 10-year old human and 12-year old ovine bloodstains, as did Mahler (1923) from human stains over 20 years old. Dervieux (1911) mentioned that he had obtained hematin-iodide crystals from a 4000 year old bloodstained cloth from a mummy.

4.2.3 Acetone chlor-hemin crystal test

In 1935 Wagenaar recommended the preparation of acetone chlor-hemin crystals. A few drops of acetone are added to a fragment of bloodstain, followed by a drop of dilute mineral acid. Crystals form quickly at room temperature, even when the stains are old or the blood partially putrefied (Wagenaar, 1937). Dérobert and Hausser (1938) discussed this technique in their reference book. Chiodi (1940) carried out extensive studies on it with human, and different animal bloods, and stains exposed to adverse conditions. He recommended that it replace the Teichmann test. In 1949, Schleyer showed that the test could detect as little as 2 to 8 μg hemoglobin, and that methemoglobin and putrefied blood would give a positive test. Hematoporphyrin and blood heated above 200° do not give the test. Apparently, crystals are obtained from bile as well (due perhaps to the bilirubin content), but not from the urinary or fecal pigments (probably stercobilin, urobilin and urochrome) (Schleyer, 1949). Stassi (1945) reported that the test did sometimes fail in the presence of blood.

While the Teichmann and Wagenaar crystal tests are at least valuable, if not conclusive, tests for the presence of blood in stains, the crystals are not always easy to obtain. Even in experienced hands, these tests sometimes fail in the undoubted presence of blood (Sutherland, 1907; Corin, 1901; Dalla Volta, 1932; Olbrycht, 1950; Stassi, 1945; Mahler, 1923).

4.2.4 Hemochromogen crystal test

Hemochromogens are those compounds of ferroporphyrin (i.e., Fe^{2+}) in which the fifth and/or sixth positions of the hexacoordinate complex are occupied by the N atom of an organic base, such as pyridine, histidine, pyrrolidine, or various amines.

Hemochromogen was first prepared by Stokes in 1864. He obtained this material, which had a very characteristic spectrum by treating hematin with a reducing agent in alkaline solution. Stokes called the substance "reduced hematin." Hoppe-Seyler took up a number of further studies on the pigment finding, among many other things, that it bore as close a resemblance to hemoglobin as it did to hematin. He proposed that it be called "hemochromogen" (Hoppe-Seyler, 1877, 1879), this name having essentially supplanted that given the compound by the original discoverer. In 1889, Hoppe-Seyler prepared crystals by exposing hemoglobin to 100° temperatures in basic solution. These crystals have often been referred to in the literature as the first example of hemochromogen crystals. Leers (1910) noted that Hoppe-Seyler's student, Trasaburo-Araki reported obtaining similar crystals in 1890. Gamgee (1898) said that Hoppe-Seyler had not in fact obtained hemochromogen crystals, as had been reported in the textbooks for a number of years:

> It is quite erroneous to state, as is asserted in all textbooks, that Hoppe-Seyler succeeded in separating haemochromogen in a crystalline condition. He only succeeded (*at most*) in obtaining crystals of the CO-compound, and concluded that haemochromogen itself must be a crystalline body, but he never asserted that he had actually obtained the crystals, and a promise made in 1889 to describe the assumed crystalline haemochromogen, though implying that he had already obtained the body in this condition, was never fulfilled. Moreover, in the last systematic account of haemochromogen which he published in 1893 [Hoppe-Seyler and Thierfelder, 1893] Hoppe-Seyler does not refer to its being crystalline, but, on the contrary, speaks of it (as he had done in 1889) as separating in the form of a violet-grey powdery precipitate.

Copeman (1890) observed that hemochromogen crystals form from hemoglobin crystals upon long standing. Menzies (1895a) noted that allowing blood to stand in a water bath for some days in the presence of the chloride, bromide or iodide salts of potassium gave rise to a substance which could be converted to hemochromogen upon addition of a reductant, $(NH_4)_2S$. He further showed that ammonium sulfide would convert acid hematin to hemochromogen (Menzies, 1895b). Hüfner (1899) used hydrazine hydrate to convert alkaline hematin to hemochromogen.

Donogany (1893a) working in Budapest was the first investigator to note that pyridine reacted with hemoglobin to form hemochromogen crystals, and that these formed within a few hours if the material was placed on a microscope slide and sealed with a cover slip. He showed that this reaction occurred with dried blood, and suggested its application to the problem of medico-legal blood identification. In a subsequent paper (1897), Donogany suggested that the pyridine hemochromogen test would be useful for the determination of blood in urine, noting at the same time that he had first published his observations on pyridine hemochromogen in the Hungarian literature four years earlier (Donogany, 1893b).

Hemochromogen crystals may be obtained from either acid or alkaline solutions. Fiori (1962) stated that crystallization from acid solution was first carried out using acetic acid, pyridine and pyrogallol (1,2,3-benzenetriol), quite possibly a reference to the papers of Welsch and Lecha-Marzo (1912) or of Lecha-Marzo (1907), the latter of which was cited by Gisbert Calabuig (1948) who proposed the use of an improved solution: 0.5 mℓ glacial acetic acid, 1.5 mℓ pyridine and 1 mℓ 2% ascorbic acid.

It is far more common in the literature, however, to find descriptions of crystallization from alkaline solution. The older methods consisted of treating the stained material with pyridine and ammonium sulfide (Bürker, 1909; De Dominicis, 1902 and 1911), following upon the work of Menzies (1895a). Lochte (1910) suggested a modified version of the reagent containing NaOH and alcohol. Kürbitz (1909) and apparently Lecha-Marzo (1908) recommended extraction of the stain with alcoholic iodine solution prior to adding the pyridine-ammonium sulfide reagent. Leers (1910) used this method, but apparently employed an aqueous iodine solution. Alkaline solutions of pyridine containing hydrazine hydrate (Mita, 1910) or hydrazine sulfate (De Dominicis,

1909; Puppe, 1922) as reductants were described as well, being logical in view of Hüfner's earlier observations (1899). Nitrogenous bases other than pyridine will participate in hemochromogen crystals formation. Cevidalli (1905) successfully employed piperidine solutions, and Lochte (1910) noted that piperidine or picoline could be substituted for the pyridine.

The most comprehensive study of hemochromogen in the early literature is almost surely that of Dilling, published in 1910 in both German and English. This volume records the results of Dilling's extensive experiments carried out in Prof. R. Kobert's laboratory. Hemochromogen crystals were prepared from blood, hematin and other derivatives of hemoglobin utilizing pyridine, piperidine and a number of other nitrogenous compounds, with ammonium sulfide, hydrazine hydrate, and ammonium sulfide in NaOH as reductants. In each case the microscopic and spectral characteristics of the crystals obtained were described, as well as any problems encountered in the course of applying a particular technique. As nitrogenous bases, Dilling tested pyridine, piperidine, nicotine, methylpiperidine, ethylpiperidine, coniin (2-propylpiperidine), conhydrine (2-(α-hydroxypropyl) piperidine), and its isomer pseudoconhydrine, 2-methyl- and 3-methyl-pyridine (α- and β-picoline, respectively), α-dimethylpyridine (α-lutidine), as well as trimethylpyridine (collidine)† and tetramethylpyridine (parvoline), the two last mentioned probably consisting of mixtures of the isomers. Pyridine and piperidine were found to be the most satisfactory of all these compounds for crystal formation from blood. In addition to presenting his results, Dilling gave a good, comprehensive review of the pre-1910 literature. Somewhat less comprehensive reviews have been given by Kalmus (1910) and by Kurbitz (1910).

A great many authorities since 1912 have preferred to prepare hemochromogen crystals using the reagents described by Takayama in that year (Akaishi, 1956; Brünig, 1957; Gonzales *et al.*, 1954; Greaves, 1932; Hunt *et al.*, 1960; Kerr and Mason, 1926; Kirk, 1953; Lopez-Gomez, 1953; Mahler, 1923; Olbrycht, 1950; Rentoul and Smith, 1973; Thomas, 1937; Ziemke, 1924). Takayama's name has come to be used to describe not only the reagent he devised, but also the procedure and the hemochromogen crystals thus obtained, in much the same way as did Teichmann's name in the case of hematin halide crystals. The original paper in 1912 is cited in several different ways in the literature‡ and some difficulty was encountered in locating it. The paper, written in Japanese, appeared in *Kokka Igakkai Zasshi.*

Curiously, Takayama's paper was published in 1912 in Japan, but it does not appear to be mentioned in the European literature until Strassmann's paper appeared in 1922. It would be of interest to know how the information got from Japan to Germany. Takayama was in Germany around the turn of the century, but before 1912 (see in Unit IX, Translations). We had some correspondence on this point with Prof. Dr. Hiroshi Hirose of Kyushu University in Fukuoka, Japan. After some extensive searching in the early literature, Prof. Hirose discovered the paper by Strassmann (1922), and kindly shared the fruits of his search with us. His letter to me, in which the historical details are given and fully documented, has now been published (Hirose, 1979). Strassmann learned of the Takayama test from Prof. Fujiwara who was a student of Takayama's, and who was in Europe from 1920–1923. Takayama had noted that Reagent I required that the preparation be warmed for best results, while Reagent II did not require warming. Perhaps for this reason, many authorities preferred the second reagent, even in Japan where the test was widely used before its introduction in Europe. As mentioned in the footnote, the citation of the original paper was not correct in the German and English literature. Neither we nor Prof. Hirose could find any journals with the titles given in the incorrect citations. Takayama proposed two solutions, II being a sort of improved version of I.

Solution I:		Solution II:	
10% dextrose	5 mℓ	Saturated dextrose solution	3 mℓ
10% NaOH	10 mℓ	10% NaOH	3 mℓ
pyridine	10–20 mℓ	pyridine	3 mℓ
water	65–75 mℓ	water	7 mℓ

The crystals, obtained by treating a small amount of blood or stain fragment with these solutions are shallow rhomboids, salmon-pink in color. Gentle heating was needed with the first reagent, but not with the second.

As mentioned previously, Mahler's (1923) extensive study of blood crystals included hemochromogen crystals as well as hematin crystals. He compared a number of different methods and solutions for obtaining the crystals from a large number of different types of dried blood specimens. Kerr and Mason (1926) discussed the test in some detail as well, concluding, as had Mahler, that Takayama solution II was the most reliable reagent for obtaining hemochromogen crystals. The speed with which the crystals appear depends on temperature, being almost immediate if the material is heated, somewhat slower at room temperature (1 to 6 minutes), and slower yet if in the cold (e.g. if the reagent has been kept in the refrigerator). The reagent is stable for 1–2 months, crystals taking somewhat longer to form with older reagent. Greaves (1932) recommended the use of Takayama solution II for hemochromogen crystals as the method of choice for blood identification. He preferred not to heat the material, and suggested waiting up to several hours if necessary before deciding that the result is negative. Puppe (1922) also noted that good results were obtained without heating. Oustinoff (1930) suggested that gum arabic be included in the reagent, 1 part to 3 parts pyridine.

There are a number of advantages to the hemochromogen

† 2,4,6-trimethylpyridine is now called γ-collidine, while α-collidine is 4-ethyl-2-methylcollidine and β-collidine is 3-ethyl-2-methyl-collidine.

‡ Kerr and Mason (1926) and Greaves (1926) cited the paper as having appeared in the Japanese Journal of Toxicology. Ziemke (1924) and Wagenaar (1929) cited it as having appeared in Japan. Zeitschrift für Staatsarzneikunde, this last having probably been taken from Strassmann (1922).

test, as compared with the hematin test. A technical advantage is that heating is not required to obtain results within a reasonable amount of time; and even if one does prefer to apply heat, the test is not subject to being ruined by overheating. The test also yields positive results under some of the circumstances where the Teichmann test fails. Thus, Mahler (1923) obtained a positive Takayama test in cases of various 22 year old bloodstains on linen and of stains on rusty knives up to 23 years old, which failed to give Teichmann crystals. Similarly, Kerr and Mason (1926) showed that stains on linen and glass which had been heated to 150° for 30 minutes, stains (relatively fresh) washed in hot water, and stains on rusty metal surfaces up to 45 years old, all yielded hemochromogen crystals but did not give hematin crystals by the Sutherland technique.

According to Kirk (1953), both the specificity and sensitivity of the hemochromogen test are about the same as those of the hematin crystal test. Kerr and Mason (1926) and Greaves (1932) say that the hemochromogen test never failed in their hands in the known presence of blood. Mahler (1923) got some failures, but with solutions other than the Takayama II. The proof value of a positive hemochromogen test, with spectroscopic confirmation of the identity of the product, is not widely disputed. A negative crystal test, however, should not necessarily be interpreted as meaning that blood is absent (Kirk, 1953; Olbrycht, 1950). That bloodstains which have been exposed to heat, or are old or weathered, become increasingly insoluble has been known for a long time (Katayama, 1888; Hammerl, 1892). In cases such as these, solubility can be a problem in itself; if it is not possible to solubilize any hemoglobin or hemoglobin derivatives, it will obviously not be possible to obtain a positive crystal test.

Comparisons of sensitivity present some difficulty because various different authors express sensitivities in different ways. It is not always possible to convert one set of units or reference frame to another. This problem is encountered in many of the identification and serological and biochemical tests used in this field.

As to hemochromogen crystal tests, Greaves (1932) mentions only that the fragment of stain or stained material should be very small, only just large enough to be seen and manipulated onto a slide. Antoniotti and Murino (1956) noted that the test is still positive with 1 $\mu\ell$ of blood or about 0.1 mg hemoglobin, while Hunt *et al.* (1960) could obtain crystals from a stain fragment containing only 0.2 $\mu\ell$ of blood. Akaishi (1965) stated that a positive test could be obtained from a stain made from a 1:30 dilution of whole blood, but did not state how much stain was taken for the test. Miller (1969) thought that the Takayama test was considerably more sensitive than the Teichmann test. He could obtain Takayama crystals from 5 $\mu\ell$ of a 1:1000 dilution of whole blood, provided that the material was dispensed onto a slide in 10 separate 0.5 $\mu\ell$ aliquots in order to keep the area occupied by the test material as small as possible.

Not long after Kerr and Mason (1926) published their article on the Takayama test, Dilling (1926), in a letter to the Editor of the *British Medical Journal,* suggested that the hemochromogen test should not supplant the hematin test, but rather be used as to supplement it. He brought up two other points: (1) Kerr and Mason had said that the only discussion of hemochromogen tests in English prior to their paper was Sutherland's discussion, and Dilling correctly pointed out that he had published an extensive study of the subject in 1910; and (2) he believed that the purpose of the sugar in the Takayama reagent was to decrease the solubility of hemochromogen, and not, as Kerr and Mason had suggested, to serve as a reductant. Kerr (1926a) replied to the letter, saying that he agreed with Dilling's interpretation of the mechanism of action of the sugar. He further said that there was no prior account of the Takayama method in English, and that he still believed it to be much superior to the hematin test for medico-legal work.

Recently, Blake and Dillon (1973) have investigated the question of false positive hemochromogen crystal tests. They correctly note that the question has received virtually no attention in the medico-legal literature. Of particular interest was the issue of whether other iron-protoporphyrin containing substances, such as the enzymes catalase and peroxidase, would give misleading false positive crystal test reactions. Three presumptive tests were also carried out on the material, including the benzidine and phenolphthalin catalytic tests (sections 6.3 and 6.4) and the luminol test (section 6.7). A number of microorganisms were tested, since these are known to be particularly rich in catalase and peroxidase activities. Pure samples of both the enzymes gave Takayama crystals, those formed with catalase being virtually indistinguishable from the crystals obtained with blood. These materials also gave positive results with all three presumptive tests. The effectiveness with which different bacteria reacted with benzidine, in a two stage test, was directly related to the catalase content of the cells. One drop (about 0.05 mℓ) of a suspension of Citrobacter having a cell concentration of 400 $\times$ 10^6/mℓ gave a positive benzidine reaction, and several microbial suspensions which had been dried as spots on filter paper gave positive benzidine reactions after 2 months. Heating a number of different bacteria at 100° for 30 min or at 150° for 15 min did not abolish the benzidine reaction. Although pure catalase and peroxidase could give a positive crystal test, none of the bacteria tested contained sufficient concentrations of these enzymes under the test conditions to give a false positive Takayama test. Blake and Dillon cautioned that great care should be used in the interpretation of catalytic and crystal tests, and in combinations of them, since it is not only possible, but even likely in some situations, that case material will be contaminated with bacteria.

SECTION 5. SPECTRAL AND MICROSCOPICAL METHODS

5.1 Spectroscopic and Spectrophotometric Methods

Spectroscopic and/or spectrometric analysis of hemoglobin and its various derivatives is considered to be among the best methods for the certain identification of blood in stains (Brünig, 1957; Dérobert and Hausser, 1938; Ewell, 1887; Gonzales *et al.*, 1954; Gordon *et al.*, 1953; Lopez-Gomez, 1953; Mueller, 1975; Olbrycht, 1950; Rentoul and Smith, 1973; Simonin, 1935; Sutherland, 1907; Walcher, 1939; Ziemke, 1924). The methods are not technically difficult in practice, but as with so many of the methods and tests, a good deal of care should be exercised in the interpretation of results. Most of the older workers employed hand spectroscopes or microspectroscopes or both, since spectrophotometers were not widely available until relatively recently. Some authorities have said that the identity of hemochromogen crystals (section 4.2.4) should be confirmed microspectroscopically.

While the spectral methods might be thought to have the singular advantage of being nondestructive, they really do not if the tests are carried out properly. It does not suffice in the opinion of most experts to determine the spectrum of an extract of a stain, and infer from that alone the existence of blood in the stain. Even in cases where one can be reasonably certain that the material being examined is pure, the identity of a substance should never be inferred solely on the basis of the observation of its absorption spectrum. Lemberg and Legge (1949) state this concept especially cogently:

> While it is certainly true that under identical conditions the same substance cannot have different absorption spectra, spectra which are apparently identical are insufficient evidence for chemical identity.
>
> . . . . . It is always necessary to demonstrate identical *alterations* in spectra when chemical reactions are performed, before identity of two substances can be considered in any degree certain.

This *caveat* must be considered especially relevant to medico-legal identifications, not only since there are a large number of porphyrin compounds in nature, many of which have common spectral features, but also since it can almost never be assumed that stain evidence is uncontaminated. Most of the spectral methods, therefore, involve the preparation of various hemoglobin derivatives, followed by verification that these have actually been obtained by measuring the spectra. This subject, along with the crystal tests and other chemical tests, has been excellently reviewed by Fiori (1962). I am indebted to this work for source material as well as for lucid explanations of many aspects of the various tests.

Since there is quite a large number of different hemoglobin derivatives that may be used in identification procedures, and a fairly extensive literature on their absorption spectra, it seemed most profitable to present a cross section of this information in tabular form (Lemberg and Legge, 1949; Fiori, 1962). When there are multiple absorption maxima, the bands are sometimes called I, II, III, etc., in going from longer to shorter wavelengths; another convention is to refer to the major visible bands as α and β, the α-band being at longer wavelength. A very intense band in the region of 400 nm, characteristic of all conjugated tetrapyrrole structures, is known as the "Soret band". Table 5.1 gives the absorption maxima reported by different workers for a number of hemoglobin derivatives. A composite representation of the spectra of some of the derivatives, as presented by Lemberg and Legge (1949), is given in Figure 5.1. The absorption bands for a number of derivatives, as seen in the spectroscope, are given in Fig. 5.2, as originally shown in the review of Hektoen and McNally (1923). Representations such as that in Figure 5.2 are still seen in textbooks of legal medicine.

Among the earliest papers on the spectral properties of the "coloring matter of blood" was that of Hoppe (1862). He observed and reported the absorption bands of hemoglobin and several of its derivatives in the visible range of the spectrum, and suggested that the spectral method be employed for the forensic identification of blood. More elaborate studies were done by Stokes (1864) who recognized the difference between Hb and HbO_2, described the spectral properties of hematin and, for the first time, of hemochromogen. Sorby (1865) independently studied the spectra of a number of hemoglobin derivatives and advocated the spectral method for the identification of bloodstains. In 1868, Herepath discussed the techniques in some detail. He was able, using the microspectroscope, to identify blood on the wooden handle of a hatchet which had lain exposed in the country for several weeks. In this case (Reg. v. Robert Coe, Swansea Assizes, 1866), the amount of blood remaining on the handle was very small. The technique was stated to be sufficiently sensitive to detect ". . . . less than one thousandth of a grain of dried blood, the colouring matter of which had been dissolved out by a drop and a half of distilled water." Bell (1892) noted that Dr. Richardson of Philadelphia had said he was able to detect the blood on an ax handle equivalent to $\frac{1}{3000}$ grain of blood in a case that he had had. Sorby (1870) described his own technique, and stated that it was equally applicable to medico-legal blood identification and to the clinical identification of blood in urine. These applications were based on his earlier studies of the spectra of the various derivatives (Sorby, 1865), in which he had first

Table 5.1 Summary of Absorption Maxima of Hemoglobin and Some Derivatives

Compound	Abbreviation	Method of Formation	Conditions/Remarks		Absorption Maxima (nm)	References ★
Hemoglobin	Hb	from HbO_2 by reduction with $(NH_4)_2S$, 10% dithionite, 0.2% hydrazine sulfate, etc.	–		270-280 430 555 755	6,7,17,20,21,24 26
Oxyhemoglobin	HbO_2	–	–		275 350 412-415 540-542 577 920-930	6,7,11,13,17,18,19,21,24,25
Carboxyhemoglobin	HbCO	CO readily replaces O_2 in HbO_2	spectrum similar to that of HbO_2		270-280 330-340 418 538-540 568-572	6,11,13,17,18,21,25,26
Methemoglobin	MetHb	oxidation of hemoglobin by $KMnO_4$, $K_3Fe(CN)_6$, etc. – forms in bloodstains upon aging	spectrum is pH dependent; the 540 and 577 bands increase in intensity as pH increases and the red band shifts to 600 and decreases – acidic compound is brown – basic one is red	acidic	266-275 404 500 570 630 (pH 7)	1,11,13,15,17,19,20,25
				basic	411 540 577 600 (pH 10)	
Cyanmethemoglobin	MetHb-CN, HiCN	addition of neutral solutions of cyanide to MetHb solutions	compound is red		412-421 540	7,18,25
Hematin	–	acid: addition of glacial acetic acid containing ethanol to solution of hemoglobin prolonged exposure of bloodstains yields this product	spectrum is pH dependent	acid	400 510 540 630-635 (in acetic acid)	11,21
					278 376 526 550-558 626 662 (in HCl)	11,22,25
		alkaline	pH dependent spectrum	alkaline	220 365 385 492 606-615 (NaOH) 585 (borate, pH 10)	2,6,10,11,12,13,14
					402 590 (alcoholic $NaHCO^3$)	
Hemochromogen (hemochrome)	–	from blood or hematin compounds by addition of reducing agent in presence of nitrogenous base	(Fe^{++}) 20% pyridine and pH 11.6 solutions		419-424 525-530 550-560	3,5,8,9,16,21
			(Fe^{++})		545 575	8
Cyanide hemochromogen (Dicyano ferroporphyrin)	–	from bloodstains heated with 10% KCN, then treated with reducing agent	–		430 537-540 565-570	4,5,10,27
Sulfhemoglobin	–	treatment of Hb with H_2S in presence of O_2	–		400 617-623	19,20
Hematoporphyrin	–	treatment of Hb, blood with conc. H_2SO_4	–	acid	404 553 577 598	23
		treatment of acid hematoporphyrin with KOH	–	alkaline	504 539-541 566 618	11

★ Numbers in reference list in Table refer to the following references:

1. Austin and Drabkin (1935)
2. Clegg and King (1942)
3. DeDuve (1948)
4. Drabkin (1942a)
5. Drabkin (1942b)
6. Drabkin (1946)
7. Drabkin and Austin (1935a)
8. Drabkin and Austin (1935b)
9. Falk (1963)
10. Guzman Barron (1937)
11. Hellmeyer (1933)
12. Herzfeld and Klinger (1919)
13. Hicks and Holden (1929)
14. Hogness et al. (1937)
15. Holden (1943)
16. Holden and Hicks (1932)
17. Horecker (1943)
18. Kennedy (1927)
19. Klese (1954)
20. Lemberg and Legge (1949)
21. Mahler and Cordes (1971)
22. Newcomer (1919)
23. Schmidt (1940)
24. Sidwell et al. (1938)
25. Van Assendelft (1970)
26. Winegarden and Boorsook (1937)
27. Ziemke (1910)

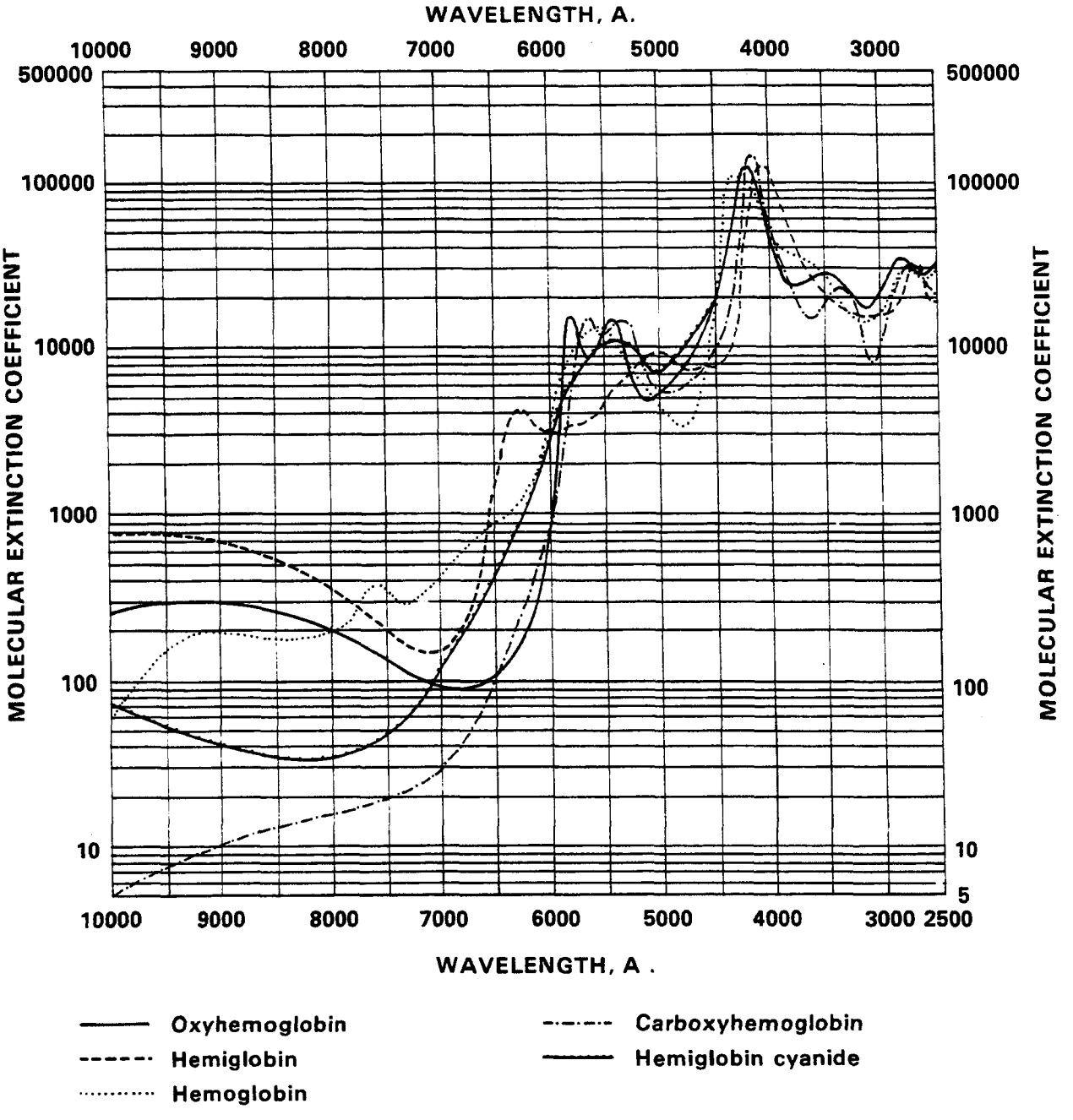


Figure 5.1 Absorption Spectra of Some Hemoglobin Derivatives (after Lemberg and Legge, 1949) (Reprinted by permission of Interscience Publishers, Inc.)

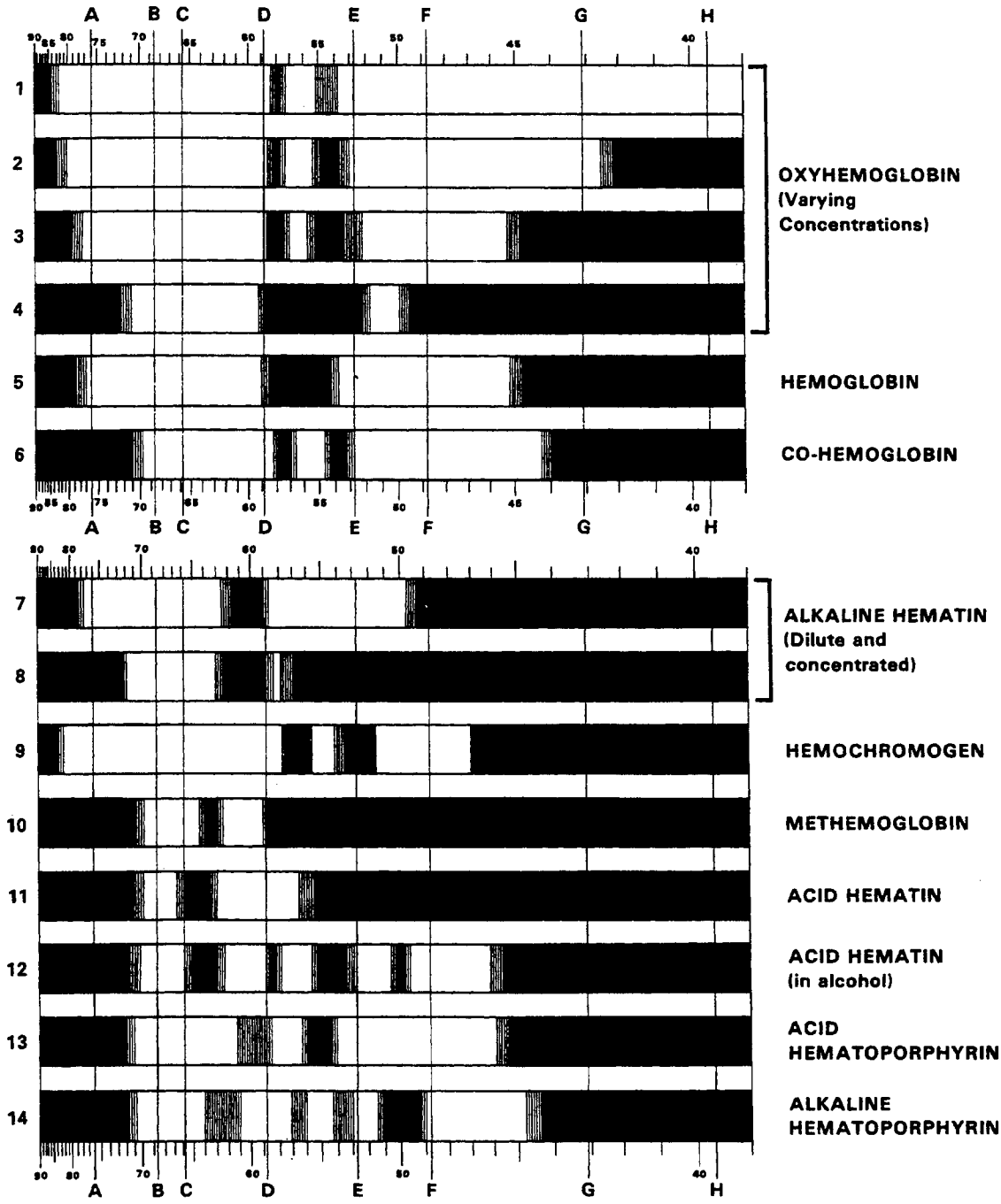


Figure 5.2 Absorption bands for hemoglobin and some derivatives. Wavelengths in nm for Fraunhofer lines: B 686.7, C 656.3, D 589.3, E 527.0, F 486.1, G 430.8. From: L. Hektoen and W. D. McNally *Medicolegal Examination of Blood and Bloodstains* in: F. Peterson, W. S. Haines & R. W. Webster (eds.) *Legal Medicine and Toxicology*, 2nd ed., V. II, 1923. Reprinted by permission of W. B. Saunders Co.

recommended this technique for forensic examinations. Dr. Taylor recommended Sorby's technique for medicolegal cases, and wrote a sort of introduction to the 1870 paper (Taylor, 1870).

The spectral methods were incorporated into the repertoire of techniques for examining bloodstains fairly quickly. The earlier texts of Chapman (1892), Ewell (1887) and Reese (1891) carry discussions of them. Relatively fresh, reasonably well-preserved stains may still show hemoglobin spectra. As aging proceeds, the hemoglobin is converted into methemoglobin and later into hematin. The last-mentioned is exceedingly less water-soluble than hemoglobin, it being necessary with old stains to extract with acid, base or solvents. The extracts may then be examined for the appropriate hemoglobin derivative. Reese (1891) was apparently recommending the preparation of alkaline methemoglobin in cases of stains, though present-day terminology for the derivatives was not then used. In the early English literature, as noted by Gamgee (1868), hemoglobin was called *cruorine,* after Stokes' suggestion (1864). HbO_2 was called "scarlet" cruorine, while Hb was "purple" cruorine. The early workers regarded the $Hb + O_2 \rightleftharpoons HbO_2$ reaction as one of oxidation-reduction, and they seem to have had difficulty squaring this concept with their results using oxidizing or reducing agents which were affecting the valence of iron. Sorby (1865) apparently suggested that the term "brown cruorine" be applied to methemoglobin. It was Hoppe-Seyler (1864) who introduced the present-day name "hemoglobin". "Um Verwechselungen zu vermeiden, nenne ich den Blutfarbstoff Hämoglobulin oder Hämoglobin," he wrote. [In order to avoid confusion, I call the blood pigment hemoglobulin or hemoglobin].

All hematin compounds can be transformed into hemochromogens by additon of base, reducing agent and a nitrogenous compound (Lemberg and Legge, 1949). The derivative, characterized spectrally by a sharp α-band in the 550–560 rm region, is therefore one of the most useful in diagnosing bloodstains. As discussed previously in section 4.2.4, heme will combine with a variety of nitrogenous bases to form hemochromogens. Bloodstain extract may be treated with strong alkali and ammonium sulfide to form hemochromogen (Sutherland, 1907). Simonin (1935) suggested extracting the bloodstain with 10 mN HCl, and treatment of the extract with KOH and hydrosulfite. The characteristic band at 560 nm is detectable by this method at 1:500 to 1:1000 dilutions of whole blood. Smith and Simpson (1956) and Glaister and Rentoul (1957) recommended a reagent made up by shaking 2 g $Na_2S_2O_4$ in 5 mℓ 10% NaOH or KOH and adding 1 mℓ alcohol. This solution must be freshly prepared before each use. Pyridine hemochromogen may be obtained using Takayama's solution (Smith and Simpson, 1956; Fiori, 1962), as discussed in Section 4.2.4. Riegler (1904) used a solution of hydrazine sulfate in alcoholic NaOH. A drop of hydrazine sulfate in 20% KOH, introduced under the cover slip on a slide containing the sample, was recommended by Hesselink (1931). Olbrycht (1950) dissolved the stain directly in pyridine, then added hydrazine sulfate as reducing agent. Meixner (1927) recommended KOH in glycerol as a good medium for extracting older stains. Hankin (1906), who was concerned with stains that underwent rapid putrefaction when damp, because of the tropical climate, recommended treatment with boiling water, followed by addition of ammonium sulfide on a slide. Cyanide hemochromogen may be prepared as well (Hankin, 1906; Fiori, 1962) if KCN solution is used to dissolve the bloodstain.

The use of the hematoporphyrin spectrum for bloodstain identification was first suggested by Struve in 1880, but not until Kratter published the results of his experiments in 1892 did the method begin to enjoy more general use. Hematoporphyrin is formed from bloodstains upon treatment with concentrated H_2SO_4. The iron atom is removed by this treatment and the vinyl residues of the porphyrin nucleus are oxidized to hydroxyethyl groups (see Table 4.1). This material was originally called "iron-free hematin". The treatment with sulfuric acid can cause charring of the fabric, and carbonized fabric fragments interfere with spectral examination. Ipsen (1899, 1900) thought that the treated material could be washed in order to get rid of such particles, and more acid added. But Ziemke (1901) found that this procedure did not work well. He advocated 24 hr sulfuric acid treatment of the stain, glass wool filtration and neutralization to form a precipitate. The precipitate is then washed, dried and an ammonia-alcohol extract of it examined spectrally. Takayama (1905) allowed the acid to act on the stain for 5–7 days, after which the material was heated, diluted to about three times its volume with water, filtered through glass wool, and examined.

The hematoporphyrin procedure may well be the least desirable of all spectral tests, if proof of the presence of blood is wanted. There are probably more naturally occurring substances with spectra similar to hematoporphyrin than to the other derivatives. When bloodstains are very old, however, or when they have been exposed to high temperatures for extended periods, they become very difficult to dissolve, and often fail to give hematin or hemochromogen. In these cases, hematoporphyrin preparation may be the only recourse. Fiori (1962) notes that strong acids should be avoided for spectral determination, and that hematoporphyrin is, therefore, best prepared by the milder method of Dotzauer and Keding (1955). The stain is dissolved in 0.1N HCl, with heating if necessary. After 15 minutes, concentrated thioglycollic acid is added and the material heated to boiling for 1 minute. Hematoporphyrin formation is complete in about a half hour. Olbrycht (1950) has pointed out that bloodstain extracts can easily be contaminated with substances from the substratum which may then interfere with the identification tests. The use of concentrated sulfuric acid would only tend to aggravate such contamination problems because the acid is such a strong solvent. This consideration strengthens Fiori's contention that strong acids should be avoided.

Many conditions to which bloodstained material can be exposed affect the spectral tests (as, of course, they affect any of the identification or grouping tests). Weathering,

submersion in water, exposure to sunlight, heat, washing, or rust can all cause great difficulty. Rust formation is accelerated on bloodstained ferrous metal surfaces (Buhtz, 1933), and rust contamination often precludes the identification of blood. Scheller (1937) studied the spectral identification of bloodstains on various substrates after exposure to air and weathering in different humidities. Damp environments cause more rapid deterioration of the stain than moderate ones, and accelerate the formation of rust on bloodstained ferrous metal surfaces and of $ZnCO_3$ on bloodstained galvanized sheet metal surfaces. Green plants are particularly difficult contaiminants of samples to be subjected to spectral tests because of the chlorophyll, which, being itself a magnesium porphyrin derivative, shares spectral characteristics with hematin compounds and which may be convertible under some circumstances to similar derivatives (Mayer, 1933). A bloodstain on a green leaf may therefore present special problems in this regard. Hirose (1966a, 1966b) studied the effects of soil on bloodstained fabrics buried in earth. Hemoglobin leeches out of such fabrics, the rate being directly proportional to soil moisture content, but independent of soil temperature. Hemoglobin solubility decreases with time in such samples as well. In the case of a bloodstained cotton cloth buried in soil of pH 5.3, Hirose found the order of efficiency of extraction of hemoglobin (from most efficient to least efficient) to be as follows: 20% pyridine 1% NaOH > Veronal buffer, pH 9.2 > Veronal buffer, pH 7 > Veronal buffer, pH 5.3 > water. The hemochromogen spectrum could be obtained in some samples after 40 days in the soil. Olbrycht (1950) carried out extensive studies on spectrophotometric and spectrographic identification of stains at various dilutions of blood. Microspectrometric determination can fail if stains on linen were made from blood diluted more than 1:150; spectrographic tests in the UV region of the spectrum, however, can detect stains made from up to 1:400 dilute non-hemolyzed blood and 1:750 dilute hemolyzed blood, these being too dilute to give any evidence to the naked eye of there being a bloodstain on the cloth. Sunlight, metal oxides (especially rust) and admixture with fine, aluminum and iron containing soils were found to be the most deleterious conditions in Olbrycht's studies. Haseeb (1972) reported spectral identification of Hb and metHb in a 12 year old bloodstain kept on the laboratory bench in the Sudan. Hirose (1976) mixed blood with iron powder and allowed the mixture to age in order to mimic the aging of a bloodstain on an iron surface.

The pyridine-hemochromogen spectrum test was carried out at 1, 11 and 51 days, with the results shown in Table 5.2. The 20% pyridine-1% NaOH extraction medium was found to be best one under these circumstances, and the higher pH veronal buffer more efficient than the lower pH one, just as he had found in the case of the bloodstained cloth buried in soil (Hirose, 1966b).

5.2 Spectrofluorimetric Methods

In 1916, Heller proposed the use of hematoporphyrin fluorescence, resulting from excitation by ultraviolet light, for the identification of bloodstains. Methods of preparing hematoporphyrin have been discussed in the previous section. Hematoporphyrin gives a red fluorescence when illuminated with 366 nm light (Fiori, 1962). Dotzauer and Keding (1955) studied this method in some detail, and their method for preparing hematoporphyrin is given above, in section 5.1. The fluorescence is dependent on the solution used, the substrate concentration, the pH and the temperature. Scheller (1973) suggested this method for identifying bloodstains on oxidized metals. Dotzauer and Kedding (1955) showed that bloodstains up to 10 years old, exposed to heat, sunlight or humidity can give positive results. Further, chlorophyll does not interfere under their conditions because the acid concentration is too low to bring about its conversion to hematoporphyrin. Ju-Hwa and Chu (1953) obtained hematoporphyrin and its characteristic fluorescence by adding a solution of 50 ml glacial acetic acid and 50 ml 0.1N HCl, containing 1 g hydrazine di-HCl, to very dilute blood solutions (1:5000 - 1:20,000).

Direct application of the reagent to stains on fabrics or paper was unsuccessful, but treatment of saline extracts gave good results. Chlorophyll, however, gave positive results in this system as well.

Schwerd (1977) recommended hematoporphyrin fluorescence as a certain test for the presence of blood in stains. He preferred the sulfuric acid method of preparation to the HCl/thioglycollic method of Dotzauer and Keding (1955). Amounts of blood in stains corresponding to 0.6 μg Hb could be detected, and Schwerd indicated that he thought the possibility of false positive results with this test, when properly carried out, had been overstressed.

The same constraints concerning interpretation must, of course, apply to the spectrofluorimetric test for hematoporphyrin as do to the spectrophotometric test for it. The ubiquitous biological occurrence of porphyrin compounds must be kept in mind. Bile, feces and meconium may give positive results in these tests because of porphyrin compounds contained in them (Fiori, 1962).

5.3 Microscopical Methods

5.3.1 Blood identification by microscopical techniques

Microscopical tests have been applied to bloodstains for a number of different reasons, including:

- Identification of blood in stains
- Determination of species by noting whether red cells have nuclei or not and/or structure of white cells
- Examination of leucocytes for chromatin bodies for cytological determination of sex of origin of the stain
- Examination for epithelial and other cells which might indicate the origin of the bloodstain from a particular organ or tissue
- Examination for the presence of pathological conditions, such as hematological diseases, presence of parasites, etc.

The discussion presented in this section has primarily to do with microscopical methods as means of identifying blood.

Table 5.2 Pyridine Hemochromogen Spectrum After Aging of Blood-Iron Powder Mixtures As Percent of Zero Time Control (after Hirose, 1976) Reprinted by permission of *Forensic Science* and Prof. H. Hirose.

Extraction Medium	Age in Days		
	1	11	51
20% pyridine - 1% NaOH	67	36	33
1.25% NaOH	41	14	11
25% pyridine	30	5	4
Veronal buffer, pH 9.4	22	4.2	3.1
Veronal buffer, pH 7.8	14	0.6	0.4
Saline	18	0	0

Microscopical measurement of red cells as a means of identifying the species of origin of blood will be discussed in a later section (Section 15) as will cytological methods for determination of sex of origin (Section 48).

In one of the earliest systematic investigations of the use of microscopy in medico-legal inquiries, Mandl (1842) discussed the identification of blood in stains through the recognition of blood cells. Although he appeared to think that microscopical examination should supplement chemical tests where possible, he clearly thought that the finding of blood cells provided an unequivocal identification criterion if the chemical tests failed to give results. Robin and Salmon (1857) strongly advocated microscopy for the identification of blood in stains. They said that identification of white and red blood cells in the stain along with fibrin threads yielded unequivocal proof of the presence of blood. In the paper, they were reporting on a case in which a suspect named Doiteau had been accused of killing an old woman. The defendant claimed that the stains on his clothing had come from a duck, but the experts said in their report that, based upon the microscopical examination, the stains were of human origin. Roussin (1865) said that microscopical examination of suspected stains was the only sure method of arriving at an identification of blood, and that chemical tests were unreliable. A positive Teichmann crystal test (Section 4.2.2) gave at least strong presumptive evidence of the presence of blood in his opinion, but he regarded the crystal test as unpredictable, and said that in many cases it did not work.

Over the years, a large number of different solutions have been proposed for treatment of suspected stains. Various claims were made for their properties, these mostly having to do with the ability of the solutions to bring about the extraction of "intact" red cells, and protect or restore their shape and size to the *in vivo* condition. A number of these solutions have been reviewed by Formad (1888), Sutherland (1907) and Ziemke (1938). They are very common in the old literature, mostly being named for their proposers, e.g. Hayem's fluid, Roussin's liquid, etc. A few examples of these are given below.

1. 4 g NaCl, 26 g glycerine, 2 g $HgCl_2$ and 226 mℓ water; diluted 2-3X with water before use. (Pacini, 1972)
2. 1 vol conc. H_2SO_4, 2 vol glycerine, and water to yield S.G. = 1.028 at 15° (Roussin, 1865)
3. 32% aqueous KOH (w/v) (Donders, 1847-48). This solution was recommended by Virchow (1857) and by Malinin (1875) and is sometimes called Virchow's fluid
4. 5 g Na_2SO_4, 1 g NaCl, 0.5 g $HgCl_2$ in 100 mℓ water (Hayem, 1889)
5. 2 g NaCl, 0.5 g $HgCl_2$ in 100 mℓ water (Vibert, 1911)
6. Artificial serum of Malassez and Potain: equal parts of aqueous solutions of gum arabic, NaCl and Na_2SO_4, all of S.G. = 1.020 (Sutherland, 1907, Formad, 1888)

These solutions are now very seldom used or encountered, though as recently as 1954, Kerr was still recommending Roussin's solution in the 5th edition of his text, *Forensic Medicine*. He stated that the microscopical finding of red cells in stain extracts constituted unequivocal proof of the

presence of blood. There can be no doubt that the statement is true. The difficulty lies in being sure that one is, in fact, looking at red blood cells. Even under the most ideal circumstances, the red cells of most mammals do not exhibit many characteristic structural features, being enucleate and quite small. Their shape is exceedingly sensitive to changes in the osmotic strength of their medium; within a certain range of osmolarity they behave as almost perfect osmometers. Red cells are readily crenated in hypertonic media, and swollen and lysed by hypotonic ones. Membranes can "re-heal" after lysis or disruption, forming membrane-bound vesicles which may or may not bear any resemblance to intact erythrocytes.

The changes that occur in the structure of red cells upon dessication, as in the formation of dried bloodstains, and subsequent "reconstitution" with one or another solution, are probably extreme, and little documented. Most of the available information is now quite old, and consists of "before-and-after" studies and observations. Lucas (1945) mentioned Vibert's solution for the reconstitution of red cells from bloodstains, but noted that the results were often highly unsatisfactory. These difficulties seem to have been recognized long ago, at least by some workers; Sutherland (1907) quotes Tourdes as having written in 1878 that it is of little use to look for red cells in bloodstain extracts that resemble those found in blood, because what will more likely be seen is ". . . . rounded bodies, more or less spherical or flattened, and of a more or less deep tint, mixed with amorphous debris, the result of the destruction of other erythrocytes." The difficulties accompanying the method were long ago underscored by Liman as well. In 1863, he wrote:

> "It is exceedingly difficult and risky to try to find blood cells with the microscope in old, dried and contaminated stains. The method is of no use if the blood cells have been destroyed by being washed or pulverized."

In spite of all the problems, the experienced observer can sometimes locate red cells or white cells in stain extracts leaving no doubt as to the origin of the stain.

In more recent times, investigators who have advocated microscopical examinations of stains have not restricted their attention to red cells, but have suggested techniques which would enable the visualization of leucocytes as well. A number of suggested methods have thus involved the use of various histological stains. Rojas and Daniel (1927) and Nicoletti (1933) suggested the staining of the cells with hematoxylin and eosin *in situ*. Fiori (1962) suggested that this sort of histological staining be avoided if material is precious. Such as is available in these cases should be preserved for more reliable methods of identification, he said, noting also that interpretation of results requires great care since various structures might be mistaken for red cells. Rojas and Daniel (1927) called for fixation of the stained material with equal parts by volume of alcohol and ether, followed by hematoxylin-eosin staining. Cevidalli and Dalla Volta (1923) followed a similar procedure except that fixation was effected by treatment with a series of aqueous alcohol solutions of decreasing alcohol concentration. They also employed Giemsa and May-Grünwald stains. These methods were especially recommended for bloodstains on relatively transparent and non-colored material. Otherwise the fibers had to be teased apart carefully. In cases of bloodstains on smooth surfaces (e.g. knife, floor tile, etc.) or on nontransparent textiles, transfer methods must be used. Fiori (1962) classifies the transfer methods as "fast" and "slow". The former consist essentially of scraping or chipping a bit of bloodstain onto a slide, crushing it up to give fine fragments or powder, reconstituting with a solution such as albumin-glycerol (Cevidalli and Dalla Volta, 1923) or human serum (Undritz and Hegg, 1959), and fixation followed by staining. The "slow transfer" method (De Dominicis, 1917) consists of covering the suspected stain with a thin layer of celloidin in alcohol-ether solution and allowing it to dry. The resulting film can be removed and will have blood cells attached to it. Celloidin, incidentally, is a form of cellulose nitrate contained in a solvent and used extensively in histology for embedding microscopic sections. Cevidalli and Dalla Volta (1923) discussed the celloidin method in detail, and it was recommended by Romanese and Pinolini (1922).

As suggested earlier, there are different reasons for making microscopic preparations and the selection of histological stains is determined by what is wanted. Staining of the red cells and white cells can serve to identify the stain as blood. The structure of certain of the leucocytes can help determine species of origin, while the polymorphonuclear leucocyte nuclei must be stained to look for Barr bodies to determine sex of origin. Leucocyte granules also possess a peroxidase activity that may be of limited value in assessing bloodstain age.

Stain preparations like many other things in biology, have tended to acquire the names of their proposers or early users. Hematoxylin is a very common histological stain. As discussed above, it is not itself a dye, and must be converted to hematein. Many preparations have been proposed over the years. Hematein itself, while colored, has little affinity for tissues, except in the presence of various metals, with which it forms variously colored so-called "lakes". Thus, for example, Hansen's hematoxylin is prepared by mixing 45 mℓ 10% iron alum, 7.5 mℓ 10% hematoxylin in absolute alcohol and 47.5 mℓ water, boiling, filtering, and making the filtrate up to 100 mℓ with water (Gurr, 1960). Eosin is a very common counterstain for hematoxylin. Hematoxylin-eosin preparations will stain leucocyte nuclei. Leucocytes may be structurally distinguished using May-Grünwald or Giemsa stains as well. Biebrich scarlet (Fig. 5.3) and the Feulgen method have been used to stain the red cell nuclei of invertebrate, avian, reptile, etc. bloods, and leucocyte nuclei. Giemsa stain is recommended in thicker blood smears for visualization of parasites (Clark, 1973). Neutrophils, basophils and eosinophils have granules possessing peroxidase activity which may be stained with preparations such as that of Graham (1916): blood smears are treated successively with solutions of α-naphthol in alcohol and peroxide, pyronin Y in alcohol

and aniline, and methylene blue. Neutrophil granules are stained purple-red, eosinophil granules, lighter red, and basophil granules, a deep purple. The nuclei are stained dark blue and the cytoplasm light blue. It is said that the monocyte granules become peroxidase negative in bloodstains in about a month, about 8–12 months being required for neutrophil granules to be negative, while eosinophil granules may be positive in up to 5-year old stains (Undritz and Hegg, 1959). Environmental influences can greatly influence these values. Undritz and Hegg also noted, as did Ziemke (1938), that tissue and organ cells could be histologically differentiated in blood stains, such as in cases on blood crusts on a knife which had been used in a stabbing, or where stains may contain nasal or epithelial cells. Le Breton did not share the same level of confidence in or enthusiasm for the microscopical methods (Fiori, 1962).

De Bernardi (1959) suggested paraffin embedding, thin sectioning and staining with hematoxylin preparations containing alum for bloodstains in deeply absorbed in wood. Däubler (1899) had employed a similar procedure much earlier. If blood crusts are detached from rusty surfaces by the celloidin technique, the film may be fixed and soaked in a saturated solution of oxalic acid containing a bit of uranium nitrate, then exposed to light to dissolved the rust, prior to histological staining (Romanese, 1930).

Some of the more recent texts in forensic medicine do not mention microscopical methods for identification of blood at all, e.g. *Glaister's Medical Jurisprudence and Toxicology*, 13th ed. (Rentoul and Smith, 1973). It should be kept in mind that these techniques require a comparatively large amount of material, and that a certain degree of skill with histological technique is doubtless required if good results are to be expected. In some instances, however, some workers prefer these methods. If whole (liquid) blood is encountered, microscopical identification might well be the method of choice, since a smear could be quickly and easily prepared. Fiori (1962) said emphatically, however, that microscopical methods should never be employed *in place of* other, more reliable procedures, such as spectral, chromatographic and immunological ones. Discussions of microscopical methods are, in any case, of more than purely historical interest, because of the newer methods for the cytological determination of sex of bloodstains (Section 48).

5.3.2. Biological stains and dyes

The histological stains employed in this work have known, and often differential, staining affinity for blood cells. Most of the stains commonly employed in histological work have been in use since the last century. Considerable information about the structure, properties and mode of action of biological stains is available (Clark, 1973; Conn, 1933; Gurr, 1960; Gurr, 1962; Lillie, 1977). In 1922 a Commission on Standardization of Biological Stains was set up in this country to devise standards of uniformity for commercially available products and techniques, and to disseminate information. The handbook of biological stains, published under the Commission's auspices, has gone through nine editions (Lillie, 1977). Conn's *History of Staining* (1933) was authorized by the Commission, and a guide to recommended staining procedures, now in its 3rd edition, is published as well (Clark, 1973). A brief discussion of the more commonly used stains is given here. Readers interested in histological stains should consult the above-cited specialized works on the subject.

Hematoxylin was first employed as a stain in 1863, apparently without success. It was successfully employed two years later by Böhmer (Clark, 1933). Hematoxylin itself is a naturally occurring glycoside from logwood, and is not a dye. It is easily oxidized to the dye form, however, by oxidizing agents or exposure to air. The oxidation product, which is the dye, has the unfortunate name *hematein*, and should *not* be confused with the hemoglobin derivative *hematin*. Fig. 5.4 (a) and (b) shows the structures of hematoxylin and hematein. Eosin, also called eosin Y, is a tetrabromofluorescein (Fig. 5.5), and was discovered in 1871. Giemsa stain, which came into use in 1902, is a mixture of methylene blue and its oxidation products, the azurs, in combination with eosin Y. May-Grünwald stain (1902) is a mixture of eosin Y and unoxidized methylene blue (Fig. 5.6), and is equivalent to Jenner stain (1899), after the latter of whom it should no doubt have been named. Wright's stain is the result of heating methylene blue in the presence of $NaHCO_3$, adding eosin, collecting the precipitate which forms and dissolving it in methanol. The so-called Feulgen reaction for staining of nuclei relies on the acid hydrolysis of the purine residues from DNA, and reaction of the liberated aldehyde groups with Schiff's fuchsin-sulfurous acid reagent to form red-purple complexes. Basic fuchsin is a mixture of pararosanilin and related compounds.

Biological stains and dyes will come up again in this book in other contexts, for example in the visualization of spermatozoa under the microscope (Section 10.2.1) and as coupling reagents for the visualization of the naphthols liberated when naphthyl phosphates are employed as substrates for acid phosphatase (Section 10.3.2). These dyes and stains have frequently had a number of names over the years. It is, thus, not always apparent to the uninitiated that two very different sounding names may refer to the same material. This problem has existed for a long time. There have been standardized lists of dyes and stains prepared over the years, but different lists have not been consistent with one another, nor has there always been consistency in the same list as it was revised and re-revised over time. Dye manufacturers and trade associations have usually designated products by number. There is a lengthy history to the various dye indexes (see Lillie, 1977), but for purposes of this book, suffice it to say that there is now a fairly widely accepted numbering system which is derived from the *Colour Index* in its most recent edition. Most stains and dyes are assigned a five digit "CI number".

In Table 5.3 are listed a number of stains and dyes which come up in medico-legal biology. The preferred name is given, along with synonyms and CI number.

Table 5.3 Biological Stains and Dyes

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Amido Black 10B	Naphthol Blue Black; Naphthalene Black 10B	20470	Acid Diazo Dye
Aniline Blue WS	Water Blue I; Cotton Blue; China Blue	42755	Acid Triphenylmethane Dye
Biebrich Scarlet	Scarlet 3B of B of EC; Croceine Scarlet	26905	Diazo Dye
Brilliant Indocyanin G	Coomassie Brilliant Blue G-250; Supranolcyanin G;	42655	Arylmethane Dye
Carmine; Carminic Acid	Cochineal	75470	Derivative of Anthraquinone Glycoside
Crystal Violet	Methyl Violet 10B; Gentian Violet	42555	Hexamethylpararosanilin
Eosin B, BMX	Eosin BN, BA, BS, BW or DHV; Saffrosin; Eosin Scarlet	45400	Dinitro derivative of dibromofluorescein
Eosin	Eosin Y or G;	45380	Tetrabromofluorescein
Erythrosin	Erythrosin R or G; Pyrosin J	45425	Diiodofluorescein
	Erythrosin B; N or JN; Pyrosin B	45430	Tetraiodofluorescein
Fast Blue B	Diazo Blue B; Dianisidine Blue; Fast Blue Salt BN; Naphthanil Blue B; Brentamine Fast Blue B	37235	See Fig. 10.3
Fast Blue RR	Blue RR; NRR; Diazo Blue RR	37155	Diazonium Dye
Fast Red AL	Naphthanil Diazo Red AL; Red AL; ALS	37275	See Fig. 10.2
Fast Red RC	Red RC; RCS; Red Salt I; Diazo Red RC; RS: Fast Red 4CA	37120	See Fig. 10.4
Hematoxylin; Hematein	Logwood	75290	See Fig. 5.4

Table 5.3 (cont'd)

Preferred Name	Synonyms	C.I. Number	Chemical Name or Nature
Kernechtrot	Nuclear Fast Red; Calcium Red	60760	Anthraquinone Derivative
Malachite Green	Solid Green, O; Victoria Green, B; Malachite Green BXN	42000	See Fig. 6.6
Methyl Blue	Helvetia Blue; Cotton Blue; Soluble Blue; Ink Blue; Sky Blue	42780	Aminotriarylmethane Dye
Methylene Blue	Methylene Blue Chloride	52015	A Thiazin Dye
Methylthiazolyldiphenyl Tetrazolium (BS8)	MTT; Chelating Tetrazole MTT	____	3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyl tetrazolium bromide
α-Naphthylamine	Fast Garnet B	37265	See Fig. 10.1
Nitro Blue Tetrazolium (BS8)	NBT; Nitro BT; Ditetrazolium Chloride	____	3,3'(4,4'-di-o-anisylene)-2,2'-di(p-nitrophenyl)-bis(5-phenyl)
Pararosanilin	Magenta O; Basic Fuchsin	42500	Triaminotriphenylmethane chloride
Ponceau S	Fast Ponceau 2B	27195	Polyazo Dye
Procion Blue HB	Cibachron Blue F.3GA	61211	Aminoanthraquinone Derivative
Procion Brilliant Red M-2B	Mikacion Brilliant Red 2BS	18158	A Monoazo Dye
Pyronin Y	Pyronin G	45005	Xanthene Derivative
Remazol Brilliant Blue R	Remalan Brilliant Blue R; Ostazin Brilliant Blue VB; Primazin Brilliant Blue RL	61200	Aminoanthraquinone Derivative
Rhodamine B	Brilliant Pink B; Rhodamine O	45170	Xanthene Derivative

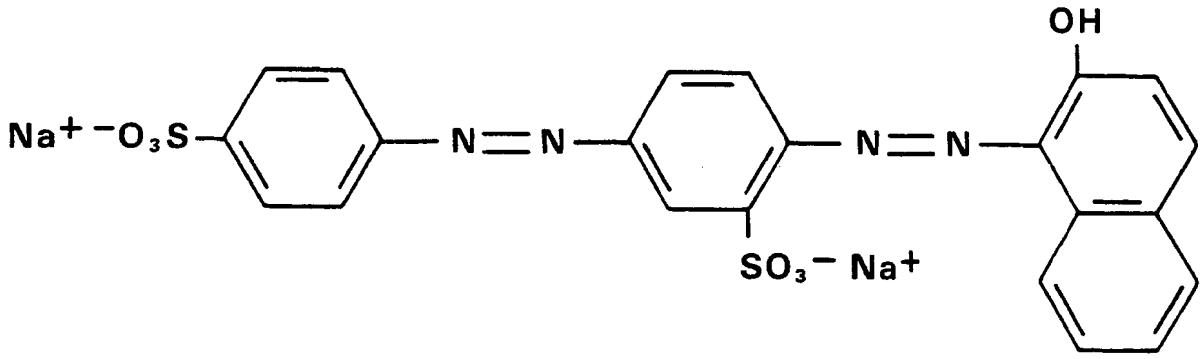
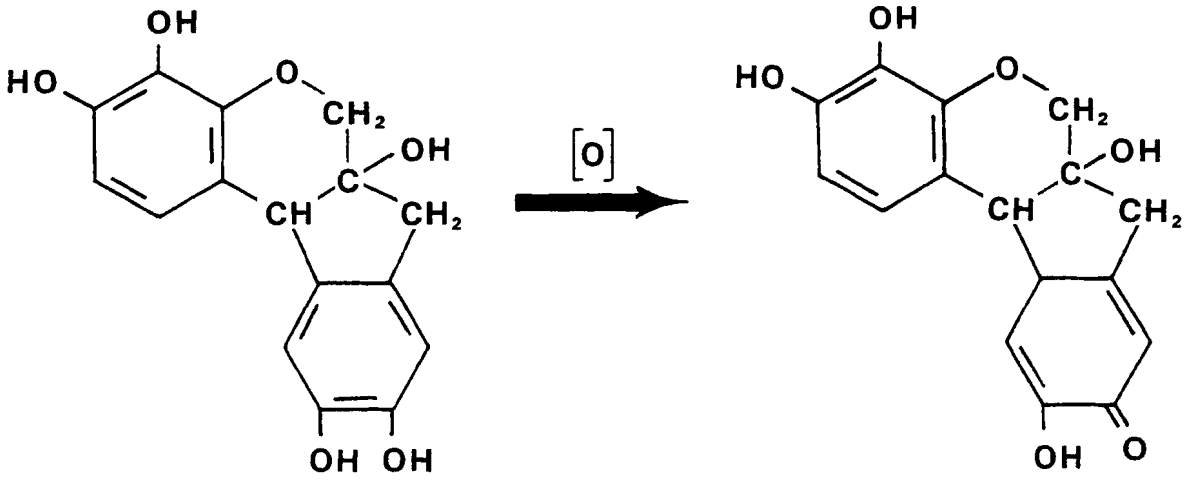


Figure 5.3 Biebrich Scarlet



(a) hematoxylin

(b) hematein
(colored dye)

Figure 5.4 Hematoxylin and Hematein

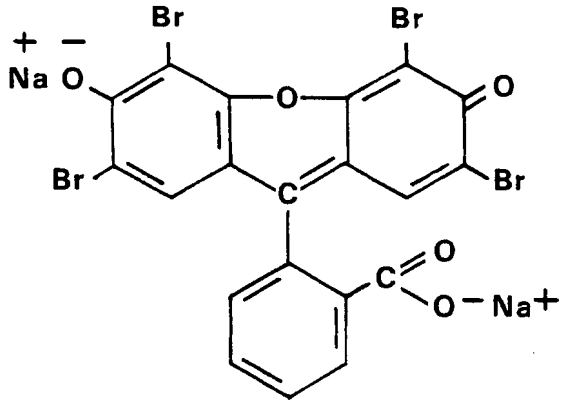


Figure 5.5 Eosin or Eosin Y

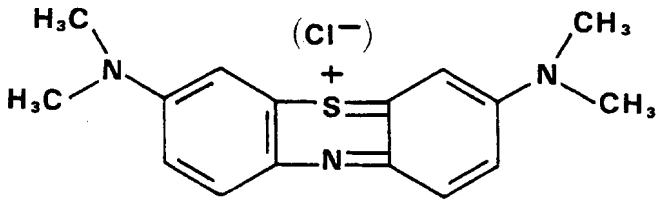


Figure 5.6 Methylene Blue

SECTION 6. CATALYTIC TESTS

Catalytic tests for blood are all based on the fact that hemoglobin and a number of its derivatives exhibit a peroxidase activity. If enzymes are defined as proteins which have *in vivo* catalytic functions, then hemoglobin is not an enzyme. It behaves as an enzyme in these tests, however, as do some of its derivatives, in that they catalyze the oxidation by peroxide of a number of organic compounds to yield colored products. Enzymes which catalyze the peroxide-mediated oxidation of organic compounds *in vivo* are called peroxidases; hemoglobin and the other derivatives which show this catalytic property are thus said to have "peroxidase activity". The "tests" based on the property are generally named after the compound undergoing oxidation (e.g. the guaiacum test, the benzidine test, the phenolphthalin test), or after the discoverer(s) (e.g. van Deen's test, Adler's test, the Kastle-Meyer test). The majority of tests which have been devised for the medico-legal identification of blood are based on the peroxide mediated oxidation of guaiacum, aloin, phenolphthalin, benzidine, the leuco base of malachite green, p-phenylenediamine, eosin hydrate, rhodamine, o-tolidine, o-toluidine, o-dianisidine or tetramethylbenzidine. Some of the tests have enjoyed wide use and great popularity, while others have been used by only one or a few workers. Likewise, some are very much in use today, while others have become a part of the historical archives of this field.

The generalized peroxidase reaction may be written

$$AH_2 + ROOH \rightarrow A + ROH + H_2O$$

where AH_2 is the donor and ROOH is the peroxide. Hydrogen peroxide is frequently used, in which case R = H, and the products would be $A + 2H_2O$. The AH_2 donor can be any oxidizable substrate yielding a detectable (usually colored) product A. In the special case of the catalase reaction, $AH_2 = H_2O_2$ and $2H_2O_2 \rightarrow O_2 + 2H_2O$. Additional information on the peroxidase and catalase reactions may be found in Mahler and Cordes (1971).

6.1 The Guaiacum Test

The subject of the peroxidase activity of blood was opened by Schönbein in 1857. He had shown that ozone and certain inorganic peroxides would cause guaiacum to turn blue, but that H_2O_2, "ozonized turpentine", and "ozonized ether" would not do so by themselves (Schönbein, 1848a, 1848b). The last three mentioned substances were called "antozones", this terminology being based on a theory of oxidation he held which said that "ozone" represented oxygen in a "positive polar state" while "antozone" represented it in a "negative polar state". Substances which behaved like ozone itself were called "ozonids" or "ozonides" while those behaving like "antozone" were called "antozonid(e)s". It was thought that the two forms of "polarized" oxygen reacted in some way to give oxygen. Ozone and "ozonides" would cause guaiacum to turn blue by themselves, while the so-called "antozonides" would not. The addition of a catalytic quantity of platinum black, however, would bring about the bluing reaction with H_2O_2, and the "antozones", and it was soon found that this same catalytic activity was possessed by blood and wheat gluten, neither of which worked by themselves (Schönbein, 1857). The catalytic principle in blood was demonstrable in red cell lysates, in the absence of fibrin and serum, and was not destroyed by boiling or drying.

Guaiacum is a resin isolated from *Guaiacum officinale* and *Guaiacum santum*, trees indigenous to Mexico, South America and the West Indies. It consists of a mixture of substances, one source saying that "guaiacum" is 70% guaiaconic acid, 10% guaiaretic acid and 15% resin (Snell and Snell, 1962). Guaiaconic acid has the formula $C_{20}H_{24}O_5$ according to Grant (1969) and Kastle and Loevenhart (1901), and can be substituted for guaiacum in the reaction (Mitchell, 1933; Buckmaster, 1907). Guaiacum is also sometimes called "guaiac".

In 1858, Schönbein showed that $FeSO_4$ catalyzed the guaiacum reaction. His colleague, Prof. Hiss, had shown that the catalytic activity of the red cells was proportional to their iron content, and he believed, therefore, that the catalytic principle in blood depended more on its iron content for activity than on any specific structural or organizational feature. In 1863, Schönbein suggested that the power of blood to blue guaiacum might serve as the basis of a medico-legal identification test. Van Deen (1862) extended the studies on the guaiacum reaction with blood, and was the first to suggest it as the basis of a medico-legal test. He showed that it occurred with minimal amounts of old, putrefied samples, as well as with blood which had been dried or boiled. Dilutions of whole blood of up to 1:40,000 in water still gave positive reactions. A number of inorganic salts gave positive reactions too, but could be eliminated using other methods. Van Deen used oil of turpentine as oxidizing agent. This so-called "ozonized turpentine", as well as "ozonized ether" could serve as oxidants because of the presence of organic peroxides which form upon exposure to air. According to Taylor (1868), not much notice was taken of Van Deen's work until Liman's extensive experiments on the subject were published in 1863. Liman thought that a negative reaction could be taken as an indication of the absence of blood without further testing. A positive result, however, did not constitute proof that blood was present, since various vegetable gums, milk casein, tanned sheep leather and some inorganic salts gave "false positive" reactions. Dr. Day in

Geelong, Australia, confirmed most of the results of earlier workers in 1867. He employed "ozonized ether" for the test, and correctly surmised that it worked because of the peroxides which formed when it was exposed to the air. In what may well have been the first reported use of the test in a medico-legal case, Dr. Day reported that he had detected blood on the trousers of a Chinese man suspected of a murder in a place called Scarsdale, Australia, on October 19, 1866. The trousers had been washed by the time they were taken as evidence, and the Government's forensic chemist could find no traces of blood on them by microscopical examination. Day wrote of this case to Dr. Taylor in London, and even enclosed a portion of the trousers on which he said he had detected blood. Dr. Taylor re-examined the cloth, some months old by then, by means of the guaiacum test, and confirmed Day's findings (Taylor, 1868). He reported this result in a rather lengthy study of the guaiacum test, prompted apparently by Day's communication. Taylor also noted that he gave the bloodstained cloth from the trousers in Day's case to Mr. Sorby, who was unable to confirm the presence of blood using his microspectroscopic method (see section 5.1). Taylor regarded the test as a useful one, to be used in conjunction with microscopical and microspectroscopic methods. Negative results were conclusive in his view, while a positive result ". . . enables a chemist to speak with reasonable certainty to the presence of blood . . .". The "false positive" results caused by oxidants which blued guaiacum in the absence of peroxide were easily eliminated by applying the guaiacum tincture first, and the peroxide after a short time if no reaction had taken place.

The guaiacum test was the first catalytic test devised for forensic blood identification, and, except for the aloin test which enjoyed very little popularity, was the only one in use for about 40 years. It is often referred to in the literature as Van Deen's test. Some English authors have called it Day's test. The old literature on this test was extensively and excellently reviewed by Kastle (1909). Kastle and Loevenhart (1901) stated that the product of the reaction, the so-called "guaiacum blue", had the formula $C_{20}H_{20}O_6$ and that guaiaconic acid, $C_{20}H_{24}O_5$, was the component of guaiacum resin which underwent oxidation. Guaiaconic acid can be separated, as it turns out, into α and β compounds (Richter, 1906), having the formulas $C_{22}H_{24}O_6$, and $C_{21}H_{45}O_5$, respectively. Guaiacum blue was said to be the oxidation product of the α compound, and to have the formula $C_{22}H_{24}O_9$. The guaiacum test has been applied to the detection of blood in urine (Schumm, 1909) and feces (Messerschmidt, 1909) for clinical purposes.

Medico-legal investigators were divided as to the relative value of the test as proof of the presence of blood. Buckmaster (1907) believed that, if the test were carried out on boiled samples (to eliminate vegetable peroxidases), a positive result was meaningful. Negative results indicated the absence of blood with certainty. Jenne (1896) thought that a positive result in a carefully performed test, using proper controls, warranted the conclusion that the stain was "surely blood". Siefert (1898) stated that a positive guaiacum test indicated a "high probability" of the stain being blood, while a negative result insured that it was not. Hemphill (1875) thought the test was a good and useful one, but did not clearly state that a positive result constituted conclusive evidence of the presence of blood. Schumm (1907) thought the test was trustworthy with certain precautions.

A larger number of authorities did not believe that a positive result was to be taken as proof of blood, but they did think the test had value as a preliminary or sorting technique (Chapman, 1892; Delearde and Benoit, 1908; Dérobert and Hausser, 1938; Ewell, 1887; Macnamara, 1873; Marx, 1905; Sutherland, 1914; Wood, 1901). Other workers believed that the primary value of the test was in eliminating stains that were not blood. They stressed the importance of negative results, which warranted the conclusion that blood was absent (Liman, 1863; Mialhe *et al.*, 1873; Mecke and Wimmer, 1895; Palleske, 1905a; Siefert, 1898; Whitney, 1909). A few investigators believed that the test was virtually worthless (Alsberg, 1908; Dervieux, 1910). Breteau (1898) said that very great caution should be used in interpreting results because of the large number of substances that would give a positive test.

The objections to the value of the test were based on the relatively large number of substances other than blood which had been reported to give positive results (Kastle, 1909). A number of inorganic elements and compounds, vegetable extracts, milk, gelatin, bile, gastric secretions, nasal mucus, saliva, pus, leather, soap, and certain types of papers have all been reported to give false positive reactions. It must be said that the way the test is carried out, the nature of the guaiacum, and of the oxidant used all make a difference in this regard. Carrying out the test on boiled samples (to eliminate vegetable peroxidases), addition of the guaiacum and peroxide in two steps (to eliminate inorganic oxidants), and the use of guaiaconic acid and H_2O_2 (to eliminate variability in the resin, ether and/or turpentine preparations) excludes the possibility of most, but not all, of the interfering substances. Workers who believed in the value of positive results recommended all these precautions, in addition, of course, to substratum controls.

Various claims have been made for the sensitivity of the test, it being difficult to compare the values in some cases because of the different ways in which they were expressed. Van Deen (1862) reported a positive test with a 1:40,000 dilution of whole blood in water. Mitchell (1933) and Kastle (1909) both say that Liman (1863) confirmed this result, but I have quite the opposite impression from Liman's paper. I understand him to say that he got a positive result with a 1:6,000 dilution of fresh blood in water, but that the reaction failed at dilutions of 1:40,000. However that may be, Schumm (1909) reported 1:40,000 to 1:100,000 dilutions of blood in water and 1:20,000 to 1:40,000 dilutions of blood in urine as the limits of sensitivity. Nicolesco (1934) gave 1:20,000 as the value. The most extravagant claim is that of Vitali (1903) who said that a $1:10^{11}$ dilution of dessicated blood in water still gave a positive result; this value is greater by six orders of magnitude than any other published figure.

Expressed as a dilution of whole blood in water, the range most often quoted for sensitivity is 1:20,000 to 1:100,000. The measurements are obviously affected by the reagents used.

The guaiacum test is now no longer employed as a catalytic test for the presence of blood in the forensic practice, having been supplanted by benzidine, phenolphthalin, etc. Schwarz (1936) used guaiacum as a substrate for testing the peroxidase activity of bloodstains in a series of experiments designed to correlate the color intensity with the age of the stain. In more recent times, guaiacol, which is o-methoxyphenol, and component of guaicum resing, has been employed as a staining substrate for haptoglobin (as the hemoglobin complex) in gels following electrophoresis (Reich, 1956; Queen and Peacock, 1966).

6.2 The Aloin Test

The aloin test for identification of blood in stains (and in urine and feces) is, like the guaiacum test, of primarily historical interest. In many ways, it is quite similar to van Deen's test. Aloin is a mixture of pentosides in the extracts of aloes, a genus of plants of the Family Lilaceae. Barbaloin, the major ingredient, is a hydroxyanthroquinone derivative of glucose. The structure is shown in Fig. 6.1 (Merck Index, 1968). Hemoglobin and some of its derivatives catalyze the oxidation of this material by H_2O_2 to yield a bright red product. Klunge (1882 and 1883) first noted that this test could be employed as a test for blood. A number of studies concentrated on chemical reactions of aloin, and the analogy between these and comparable guaiacum reactions (Neuberger, 1899; Schaer, 1900). Rossel (1901) noted that the test could be used for the detection of blood in urine. Buckmaster (1907) found that the test was less sensitive than the guaiacum test, but that the oxidation product (i.e. the color) was quite a bit more stable. The test was never as widely used as van Deen's, and not as much was written about it. Sutherland (1907) regarded it as a good confirmatory negative test.

6.3 The Phenolphthalin Test

In 1901, Kastle and Shedd showed that preparations of cellular "oxidases" would catalyze the oxidation of phenolphthalin to phenolphthalein in slightly alkaline solutions. At the time, the cellular enzymes responsible for the catalysis had not been purified to any great extent, nor had there been any attempt to systematize the enzyme nomenclature. The crude enzymatic preparations were often referred to as "oxidizing ferments". Phenolphthalein is, of course, pink to red in alkaline solution, while phenolphthalin is colorless. Thus, the latter was an excellent artificial substrate for assaying the "ferments" because the colored oxidation product was soluble and readily quantitatable colorimetrically. Meyer (1903) utilized phenolphthalin to detect the "oxidases" in leucocytes. In particular, he found differences in this activity between normal and leukemic samples. He noted further that this test could be used for the qualitative and quantitative determination of blood in urine. The first unequivocal suggestion that the test be applied to medico-legal blood identification was made later in 1903 by Utz. He reported that the test served well on bloodstains up to 1½ years old, gave a negative reaction with rust, but, not surprisingly, gave "false positive" reactions with pus and other leucocyte-containing secretions. The test became known as the "Kastle test", the "Meyer test" or the "Kastle-Meyer test."

It was soon quite clear that the test relied on the peroxidase activity of hemoglobin. Kastle and Amoss (1906) showed that the catalytic activity of blood toward the peroxide oxidation of phenolphthalin in alkaline solution was directly proportional to the hemoglobin content. The reagent, phenolphthalin, was prepared from phenolphthalein by reduction in the presence of Zn and strong NaOH or KOH. Kastle (1909) recommended the precipitation of the phenolphthalin by acidifying the reaction mixture, and collection of the precipitate. This material was recrystallized several times from minimal alcohol by cold water, and stored as a solid. Liquid solutions of the compound were stable for a matter of weeks if kept dark, and stability was greatly increased by the presence of a small quantity of zinc dust. One of the advantages of this test, in comparison to guaiac and aloin, was that the reagent was a pure compound. Kastle, who was very partial to this test for blood, discussed its many aspects in great detail in his review in 1909.

Deléarde and Benoit (1908a) studied the phenolphthalin test and showed that it was positive with hemoglobin, methemoglobin, hematin chlorhydrate, reduced hemoglobin, and old, putrefied blood. They got a positive test on a control bloodstain 26 years old, and believed that the test, properly controlled, was both sensitive and specific (1908b), in addition to its usefulness in detecting blood in urine, feces and gastric juice. Boas (1911) indicated that the test was useful for occult blood. Pozzi-Escot (1908), however, thought that no value should be attached to the test for blood because saliva, pus, malt extract, vegetable extracts and the salts of heavy metals such as Co, Mn, Pb and Fe could give false positive reactions. Dervieux (1910) agreed with this view, suggesting that the test had no value at all, positive or negative.

The phenolphthalin to phenolphthalein oxidation reaction, and the structures of the latter compound in both acidic and basic solution, are shown in Fig. 6.2. Because phenolphthalein is colorless in acidic solution but pink to red in basic solution, it has been widely employed as a pH indicator. Many have noted that the phenolphthalin test is more sensitive than either the guaiac or aloin tests. Deléarde and Benoit (1908a) and Nicolesco (1934) have indicated positive reactions with blood diluted $1:10^6$, as has Girdwood (1926). Gettler and Kaye (1943) reported a sensitivity of $1:10^7$ dilution of whole blood, but of $1:10^6$ dilution for old, decomposed blood. Glaister (1926a) noted that saline extracts of 1 year old stains reacted at 1:212,000 dilutions but that a 1:800,000 dilution of a water extract of the stain gave a positive result. Kastle (1909) did sensitivity experiments by dissolving

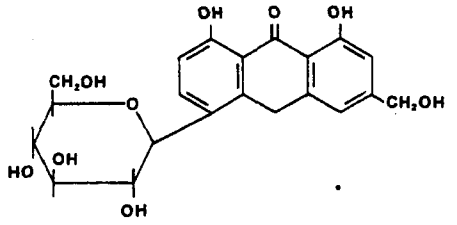


Figure 6.1 Barbaloin (1,8 dihydroxy - 3 - hydroxymethyl - 10 - (6 - hydroxymethyl - 3,4,5 - trihydroxy - 2 - pyranyl) - anthrone)

3.8 mg of blood in 100 mℓ water as a first dilution and making three serial ½ dilutions in addition. These, he denoted solutions (1), (2), (3) and (4), and they contained 38μg, 3.8 μg, 0.38 μg and 0.038 μg of blood per mℓ, respectively. One mℓ of each solution was tested by the addition of 2 mℓ reagent. Solution (3) which contained 0.38 μg/mℓ could be readily distinguished from the control colorimetrically. Solution (4), the weakest, could not be, but Kastle noted that two independent observers (he, presumably, being one of them) were able to distinguish the difference between solution (4) and the control by eye. In terms of dilutions of whole blood, therefore, since the assays were carried out in a total volume of 3 mℓ, solutions (3) and (4) correspond to about $1:8 \times 10^6$ and $1:80 \times 10^6$ parts blood to parts water. Kirk (1953) noted that $1:10^5$ dilutions of blood gave a positive reaction within 3 sec while $1:5 \times 10^6$ dilutions required 20 sec to do so.

Glaister (1926a) tested a variety of substances and body fluids, including rust, urine, saliva, semen, perspiration and milk, and got negative results with the phenolphthalin test. Kerr (1926b) took exception to Glaister's confidence in the method, noting that feces from patients taking aspirin gave a false positive test. Glaister (1926b) replied that, while he did not question the need for corroboration of the presence of blood, his own experience with the test had convinced him of its value in medico-legal cases. Girdwood (1926) noted that he did not think the test should be relied upon by itself as an indication of blood in stains, nor of occult blood in stool samples. Gettler and Kaye (1943) thought the test was more specific than guaiacum, benzidine or o-tolidine, and Gradwohl (1956) said that he preferred this test to benzidine. More recently, Higaki and Philp (1976) re-evaluated the test for blood in terms of sensitivity, reagent stability and specificity. The reagent was prepared essentially as recommended by Camps (1968), which follows almost exactly the original method of preparation used by Kastle (1909) except that the product is not isolated and recrystallized. Phenolphthalein (2 g) is dissolved in 100 mℓ water containing 20 g KOH and boiled with a reflux condenser in the presence of 20 g zinc powder until colorless. The resulting solution is kept in a brown bottle with some Zn dust present. The test was carried out in a number of different ways, with and without ethanol or methanol, and with peroxide or perborate. A so called one-stage test amounted to the addition of the combined reagents to the sample; a two-stage test consisted of the successive addition of reagent and either peroxide or perborate; a three-stage test involved the addition of the alcohol, the reagent, and the peroxide or perborate successively. Benzidine was employed as a control, because the experiments were designed to evaluate phenolphthalin as a substitute for benzidine in routine practice. It may be noted here that Camps (1976) recommended that the phenolphthalin test be substituted for the benzidine test. Dilutions of whole blood as well as stains prepared from them were tested. Stains were tested by applying reagents to a stained thread on filter paper. All results were recorded after 5 sec of observation. With liquid dilutions, ethanol or methanol, reagent and perborate, used in a one-stage test, proved to be most sensitive (in excess of $1:10^6$). A one-stage phenolphthalin-perborate test was sensitive to about $1:10^5$–$1:10^6$ dilutions, there being no advantage to a two-stage test with these reagents, nor to a three-stage test involving either alcohol. Reagent-peroxide combination, with or without alcohol, regardless of the number of stages, gave

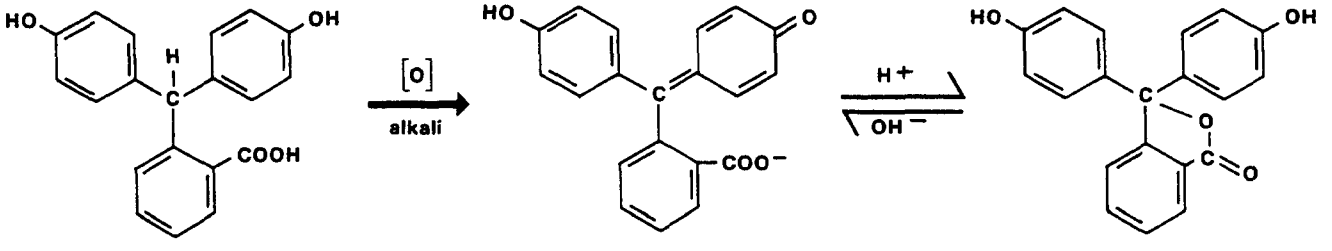


Figure 6.2 Phenolphthalin Oxidation and Phenolphthalein

sensitivities of about $1:10^4$-$1:10^5$ dilutions, fairly comparable to the benzidine control. With bloodstains, the one-stage methanol-reagent-perborate test was most sensitive, to about $1:10^3$ with the benzidine control being about $1:10^4$. Reagent, with peroxide or perborate, was considerably less sensitive in a one-stage test (neat-$1:10^2$ dilutions), and was unsatisfactory in a two-stage test, giving weak reactions at all dilutions to $1:10^2$. From the standpoint of stability, all perborate and one-stage reagents were relatively unstable. A three-stage test with peroxide, reagent and either alcohol, stored separately, was most desirable from the point of view of reagent stability. This test was recommended for screening, in spite of the fact that its sensitivity was of the order of $1:10^3$, because if greater sensitivity were required, the stain could be extracted, and the extract tested. Under these conditions, the sensitivity is increased by one to two orders of magnitude. The tests were performed on a number of vegetable extracts as well, the phenolphthalin test being found to be more specific than benzidine, particularly if the three-stage test with ethanol and peroxide was employed. Hunt *et al.* (1960) found that phenolphthalin gave positive reactions at blood dilutions of $1:10^7$, but that the reagent was useful for the examination of bloodstains only if extracts were made. The results obtained directly on stains, or on filter paper rubbings, were found to be less than satisfactory. These investigators noted further that the test appeared to be more specific for blood than some of the other catalytic tests, false positives having been noted only with copper salts, as had been noted much earlier by Glaister (1926a).

6.4 Benzidine Test

This test may well be the most familiar and widely used of the catalytic tests for blood. Its employment for that purpose began with a quite extensive series of experiments by Rudolf and Oscar Adler, published in 1904. A large number of oxidizable organic compounds were tested for their ability to form colored products in the presence of H_2O_2 and a $1:10^5$ dilution of rabbit blood in water. Among the substances tested were: aniline, its monomethyl-, dimethyl- and diphenyl- derivatives, p-toluidine, xylidine, the o-, m- and p-isomers of phenylenediamine, dimethyl-p-phenylenediamine, tetramethyl-p-phenylenediamine, phenol, p-amidophenol, the cresols, thymol, catechol, resorcinol, guaiacol, hydroquinone, pyrogallol, benzoic and salicylic acids, benzidine, tolidine, α- and β-naphthols, α-naphthylamine, the leuco bases of malachite green, brilliant green and acid green, methyl violet, crystal violet, a number of triphenylmethane dyes, eosins and rhodamines. The leuco base of malachite green (section 6.5) was used to test bloodstains, and this reagent, as well as benzidine and the leuco base of crystal violet, were recommended for the identificaiton of dilute blood in aqueous solutions. Benzidine and leucomalachite green were also recommended for testing for the presence of blood in urine and feces.

Benzidine is p-diaminodiphenyl (Fig. 6.3). The blue oxidation product obtained with peroxide in the presence of blood is often called "benzidine blue". The course of benzidine oxidation by peroxide is generally carried out at acid pH; a possible mechanism for the reaction is indicated in Fig. 6.4. The benzidine blue is an intermediate in the reaction. Eventually, the diimine compound forms, and it is brown. This latter may, in turn, polymerize. For histochemical work, the blue intermediate is more desirable than the brown end product and there have been efforts to find conditions which render it more stable. It is known that the stability is maximized at pH 4.5, and the blood test is usually carried out in acetic acid to achieve approximately this condition. The structure of benzidine blue is given by Feigl (1966) as consisting of one mole of amine, one mole of imine and one mole of whatever acid is present. Van Duijn (1955) looked into this question. He found that if milk peroxidase is used to form benzidine blue from benzidine and H_2O_2 in the presence of 5% NH_4Cl, the product had the composition $C_{24}H_{24}N_4Cl{\cdot}2H_2O$. The ammonium chloride had been found to stabilize the benzidine blue intermediate, a desirable goal in histochemical staining work. The amounts of time involved in the transition from benzidine blue to the brown diimine, which are of the order of minutes at acid pH, are too long to be of any practical concern for the medico-legal blood test, which occurs instantaneously or within a few sec.

This test quickly gained favor, and has become widely used. Messerschmidt (1909) preferred the test for detecting occult blood in feces, and recommended the method of Schlesinger and Holst (1906). Lyle *et al.* (1914) conducted experiments to determine the optimal concentrations of reagent, acetic acid and H_2O_2, primarily for the clinical test. They could achieve positive results with blood diluted $1:5 \times 10^6$ under optimal conditions, but were willing to allow up to 5 min for color development. In testing for the presence of blood in medico-legal exhibits, where little is known of the history of the article, the benzidine test has suffered the same criticisms levelled against other catalytic tests concerning specificity. Michel (1911) addressed the specificity problem in regard to metals. He reported that iron, copper and certain of their oxides, will oxidize guaiac, benzidine and leucomalachite green in the presence of H_2O_2. He noted, however, that treatment of the colored product obtained in the test with 2,4-diaminophenol would cause formation of a red-colored product if the original oxidation had been catalyzed by metals, but no such change would result if the original catalyst had been blood or pus. He added, however, that chloride or permanganate can oxidize the testing reagents, but are not subsequently affected by the 2,4-diaminophenol. Glemser (1939) noted that a variety of iron oxides cause the oxidation of benzidine, and concluded that the test could not be regarded as specific in the presence of rust. In 1973, Eisele tested approximately 50 substances for false positive benzidine reactions, and confirmed many of the observations of earlier workers. In addition, he confirmed the fact that fruit and vegetable extracts which contain peroxidases can give the reaction. Hunt *et al.* (1960) in their extensive studies noted that feces often gave a false positive,

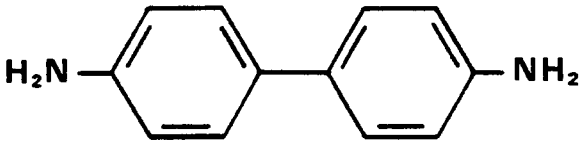


Figure 6.3 Benzidine

as did green leaf material smeared on filter paper. Smears of shoe polish (or leather) from used shoes gave a false positive reaction in one case of 30 tested. The fingernail scrapings from a grocer who handled fruits and vegetables gave the test, as did axillary wipings from normal men. These workers decidedly regarded the test as presumptive, and unequivocally stated so:

> . . . occasions do occur when a garment which is expected to be contaminated with blood gives a positive presumptive test for blood, although the deposit is insufficient or unsuitable for more specific tests. However tempting it may be to use this as evidence, it is scientifically and morally incorrect to do so, for it is clearly recognized that such tests are not specific and their introduction into evidence may well mislead. It is no argument that the evidence can be challenged, for to do so implies scientific advice which is not always available.

This strong remark was prompted in part by a case, which the authors review in the paper, in which a suspect willingly submitted to scientific investigation. His clothes and a leather container, alleged to have been used to transport the murder weapon (which was not recovered), were examined using the benzidine test. Material was insufficient for further tests. The Crown's expert testified that the benzidine test was presumptive, but upon being questioned, said that in skilled hands, it could be accepted as proof of blood's presence. That judgment, he said, would be based in large part on the time taken for the test to come up, but would not commit himself to a specific time period. In the estimation of Hunt *et al.*, the impression was left with the jury that blood had indeed been found, and there was an unstated implication that it was human. An opposite point of view on this question is discussed below.

In 1951, Grodsky *et al.* did a study of the catalytic tests and the luminol test (see Section 6.7). In addition to a number of important points discussed below, they noted that the use of sodium perborate instead of H_2O_2 for the benzidine, phenolphthalin and leucomalachite green tests represented a great improvement for the simple reason that the concentration of the perborate could be accurately determined, once reagent concentrations had been optimized. It should be noted that sodium perborate had been recommended previously as a substitute for H_2O_2 in the benzidine test (Lucas, 1935), but that Grodsky *et al.* (1951) were the first to suggest its use with phenolphthalin (Cf Higaki and Philp, 1976) and leucomalachite green. Grodsky *et al.* did not deny that a positive benzidine test alone should not be interpreted as positive evidence of blood without corroboration, but they did suggest that the use of several catalytic tests along with a luminol test provided far more convincing evidence than any one test alone (see Section 6.8).

There is no doubt that the majority of authorities have regarded the benzidine test as a sorting, or presumptive, test (Camps, 1968; Dérobert, 1974; Fiori, 1962; Gonzales *et al.*,

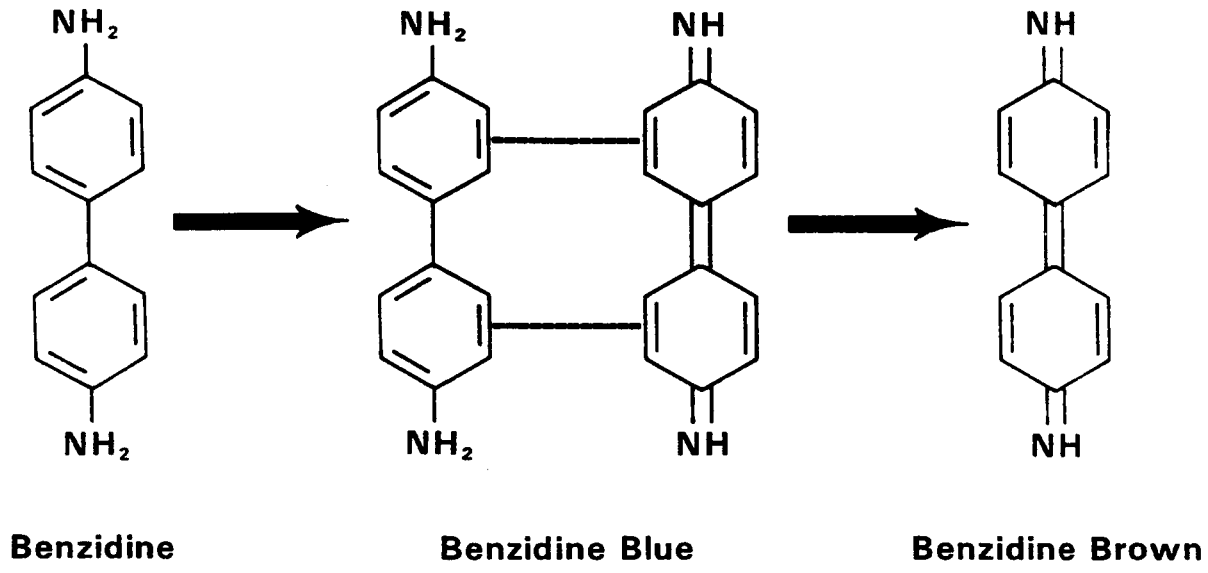


Figure 6.4 Course of Benzidine Oxidation by Peroxide

1954; Hunt *et al.*, 1960; Kerr, 1954; Lucas, 1935 & 1945; Mikami *et al.*, 1966; Prokop, 1966; Simpson, 1965; Thomas, 1937). In 1964, the benzidine test was critically examined by Culliford and Nickolls. They noted that by 1931, the test had fallen into disfavor, at least in some quarters, as a certain test for blood, Glaister having written in that year in the 5th edition of *Medical Jurisprudence and Toxicology:*

> While some employ this test, it has the disadvantage that, like the Guaiacum test, it can only be of value as a negative test, in that if no colour reaction occurs—blue or green—on applying it to a stain, it indicates the absence of blood. Should the colour reaction take place, it only suggests the presence of blood, since gluten, many plant juices as horseradish, and hypochlorites will give the blue colour reaction, although these may give the reaction either before or without the addition of ozonised ether. We do not put our trust in this test.
>
> We have abandoned completely the Guaiacum and Benzidine tests for the reason chiefly that the reaction obtained in the presence of minute amounts of known blood is uncertain and doubtful, and also because a reaction may be produced by it by substances other than blood. These objections do not apply to the Kastle-Meyer test.

On the other hand, Gradwohl (1954) wrote in his book, *Legal Medicine,* that positive reactions with benzidine (or phenolphthalin), assuming properly negative controls, do indicate "the presence of blood." This statement suggests that many laboratories regarded the test as considerably more valuable than did Dr. Glaister. Culliford and Nickolls point out, as did Grodsky *et al.*, (1951), that the judgment made about the test must be evaluated in the context of a number of variable parameters, such as the precise way in which the test was done (i.e., one-stage, two-stage, what controls were used, testing stains directly, testing extracts, etc.), the concentration and purity of the reagents used, whether peroxide or perborate was used, and the experience and judgment of the person carrying out the procedure. The major sources of "false positives" were categorized by these workers as: (1) blood contamination; (2) chemical oxidants or catalysts; and (3) vegetable or fruit peroxidases. Contamination should not be a problem in practice if the reagents are pure, the glassware clean, and the examination area kept scrupulously uncontaminated. The test is exceedingly sensitive, and contamination does not have to be very great to be a serious problem. Chemical oxidant interference is readily dispensed with by adding the reagent and the peroxide in successive steps; if color develops upon the addition of reagent alone, the presence of a chemical oxidant is indicated. Chemical catalysts, which work only in the presence of the peroxide, are not eliminated by the two-stage procedure, but Culliford and Nickolls argued that reactions caused by these materials appear quite different to the experienced eye than do those caused by blood. The plant peroxidases are heat-labile, and testing samples that have been heated to 100° for a few minutes serves to differentiate them from blood, which still reacts readily after the heating step. It was further shown that the majority of plant peroxidases which would give the reaction were quite labile in the dried state. These gave weak to negative benzidine reactions after three days in the dried state, whether the test was done directly on cloth, on a rubbing, or on an extract. Finally, these investigators described a simple electrophoretic procedure, carried out on 1% agar gels, for the differentiation of a great number of substances which could give misleading results in the benzidine test. The procedure is also described by Culliford (1971). It should be noted that these authors took strong exception to the statements of Hunt *et al.* (1960) above, to the effect that the reporting of a positive presumptive test in a case where there was no additional material available for testing would be scientifically and morally incorrect, because it could be misleading. Failure to report such a result, Culliford and Nickolls argued, would be to usurp the prerogatives of the Court, and in such a case as the one discussed by Hunt *et al.*, the result should be reported with a suitable explanation of its meaning.

Reports of the sensitivity of the benzidine test vary in the literature. Adler and Adler (1904) originally reported a sensitivity limit of $1:10^5$ dilution of whole blood in water. Nicolesco (1934), Dérobert and Hausser (1938) and Thomas (1937) all cite 1:200,000 dilutions as the limit. Grodsky *et al.* (1951), Hunt *et al.* (1960) and others have noted that the sensitivity quotations can be misleading, because the results depend on so many different parameters. Unless the technique and the reagents used are fully described, it is not at all certain that the results will be able to be duplicated exactly. Indeed, while it is easy enough to compare the sensitivities of reagents in terms of the maximal dilutions of whole blood which still give positive tests, it is not always clear how such values translate in their applicability to bloodstains. Hunt *et al.* (1960) noted great variability in different lots of commercially obtained benzidine. Expressed as the highest dilution of blood, dried onto filter paper, which gave the test, the sensitivity ranged from 1:20,000 to over 1:150,000, and was even more variable if the amount of time required for the color to develop were taken into account. Grodsky *et al.* (1951) noted that stains made from 1:300,000 dilutions of blood came up within 10 sec while those made from 1:100,000 dilutions came up within 1 sec. Akaishi (1965) reported a sensivity of only 1:12,800 for benzidine in stains made at that dilution, and noted that 20 sec was allowed for color development.

It may be noted here that Alavi and Tripathi (1969) recommended that blood testing in the field (e.g. at scenes etc.) could be done with benzidine-impregnated filter papers. The suspected material was moistened with water, the benzidine paper then pressed against it, and peroxide added to the paper from a sealed vial. The papers were said to be stable for up to 1 year and could be regenerated by soaking again in benzidine solution and drying.

The foregoing discussion of the benzidine test, without which the sourcebook would obviously be incomplete, may nevertheless be almost purely academic from a practical point of view. That benzidine was a chemical carcinogen has

apparently been known for some time. Hunt *et al.* (1960) mention that the manufacture of Analar Benzidine was discontinued in 1951 for that reason. Camps (1976) notes that phenolphthalin has replaced benzidine in routine practice. Rentoul and Smith (1973), in the 13th edition of *Glaister's Medical Jurisprudence and Toxicology,* suggest a saturated solution of amidopyrine in 95% ethanol as a benzidine replacement. (See Section 6.6.8). Apparently, therefore, the use of benzidine has been largely discontinued in England. Higaki and Philp (1976) carried out their study of the phenolphthalin test (see Section 6.3) primarily to check its applicability as a substitute for the benzidine test, suggesting that benzidine has been abandoned in Canada as well.

In this country, the use and manufacture of benzidine has become subject to extremely stringent restrictions and controls, according to regulations issued by the Occupational Safety and Health Administration of the Department of Labor (Code of Federal Regulations, 1976). While manufacture and use have not been ordered to cease, the regulations and restrictions are prohibitively involved for a laboratory doing routine work. Regardless of the merits of the test, or the qualifications that should or should not be place on interpretation of the results obtained with it in medico-legal cases, it is probable that the substance will shortly be unavailable. Supervisors will probably be increasingly unwilling to place their examiners at risk by continued use of the reagent, and the OSHA Regulations may also be sufficiently constraining that manufacturers will consider them prohibitive as well. It is likely, therefore, that the benzidine literature will shortly become a part of the archives of this field.

6.5 Leucomalachite Green and Leucocrystal Violet Tests

The use of the leuco base of malachite green as a blood testing reagent was first reported by Adler and Adler (1904), as noted above. The term "leuco compound", or in this case, "leuco base", comes from the literature of biological stains and dyes (Lillie, 1969). Compounds to be employed as stains or dyes obviously have to be colored. Although they differ greatly from one another chemically, all contain a chromophore group, a structure which renders them colored. They all share in common additionally the property of being reducible and reduction alters the chromophore group rendering the compound colorless. These colorless reduction products are referred to as "leuco compounds". Clearly, the leuco compounds are oxidizable to the dye forms. The "leuco bases" are particular types of leuco compounds, usually carbinols, and characteristic of the triphenylmethyl derivatives.

In the original work, the Adlers used leucomalachite green and leucocrystal violet, in addition to benzidine, for blood detection in aqueous solution. The structures of crystal violet (hexamethylpararosanilin) and malachite green are shown in Figs. 6.5 and 6.6, respectively. Only the leuco base of the latter has been widely used in forensic practice. The leucomalachite green test had a sensitivity limit, like

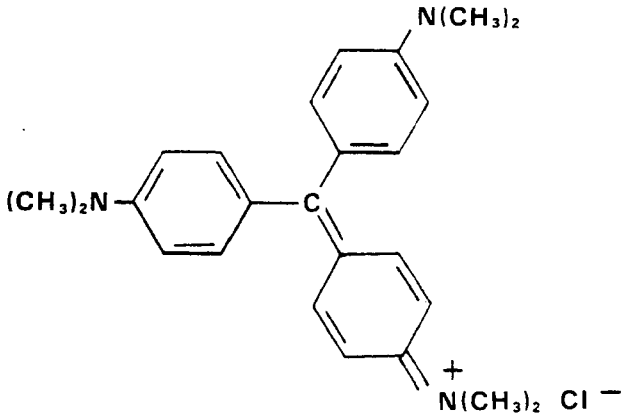


Figure 6.5 Crystal Violet

benzidine, of 1:10^5 dilution of blood. Michel (1911a) recommended this test and said that it was more sensitive then phenolphthalin. Von Fürth (1911) utilized the test on bloodstain extracts prepared by digesting the bloodstained material with 50% KOH in ethanol, and extracting that solution with pyridine. The test was then performed on a piece of filter paper, moistened with the pyridine extract. Medinger (1933) strongly recommended the reagent, and tested various physiological fluids, plant extracts and inorganic compounds, all with negative results, provided the peroxide was added in a second step after no color had developed in the presence of reagent alone.

White (1977) showed that leucocrystal violet was as sensitive in detecting iron (III) mesoporphyrin IX as a spot on filter paper as tetramethylbenzidine, guaiacum and aminodiphenylamine.

Alvarez de Toledo y Valero (1935) review the test rather extensively, and tested a large number of organic and inorganic compounds for false positive reactions. He found that there are many chemical oxidants that will give the reaction in the absence of peroxide, as well as some that catalyze the

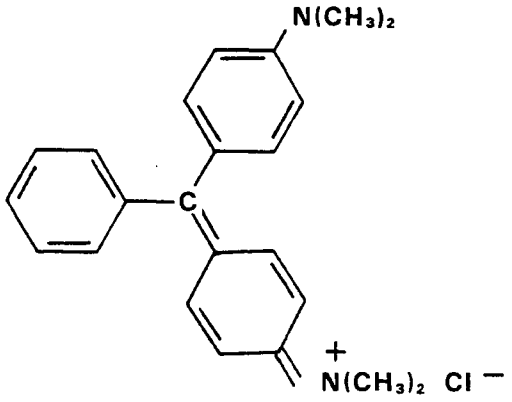


Figure 6.6 Malachite Green

reaction in its presence in much the same manner as does blood. The test is, therefore, not more specific for blood than most of the other catalytic tests.

The sensitivity of the test was originally reported to be $1:10^5$ dilution of blood by Adler and Adler (1904). Alvarez de Toledo y Valero (1935) reported the same value, but Nicolesco (1934) quoted the sensitivity as a 1:20,000 dilution of blood. Alvarez de Toledo y Valero noted that more dilute solutions of blood required longer times for color development, 1:1000 dilutions being instantaneous, 1:2,000 dilutions requiring 15–20 sec, and so on until 55 sec was necessary at $1:10^5$ dilutions and 25 min was needed for $1:2 \times 10^5$ dilutions. Grodsky *et al.* (1951) reported that stains made from $1:10^5$ dilutions of blood came up in 15 sec, using a reagent prepared by dissolving 0.1 g leucomalachite green and 0.32 g sodium perborate in 10 mℓ of 2:1 (v/v) glacial acetic acid in water. The benzidine test on the same sample, by contrast, came up in 1 sec. Hunt *et al.* (1960) similarly reported leucomalachite green to be less sensitive than benzidine, and apparently more specific. But the apparent increase in specificity was attributed to the lower sensitivity, and was thus not considered an advantage.

6.6 Other Catalytic Tests

Over the years a number of catalytic tests have been proposed for the detection of blood in stains, or of blood in feces or urine, which have enjoyed only limited use, or about which there is not a great deal of literature. All these tests are briefly discussed together in this section.

6.6.1 Peroxide

It should be mentioned that peroxide was sometimes used as a reagent for blood detection, especially in the older literature. That blood possessed a peroxidase activity, and acting as a catalase was thus capable of evolving oxygen from peroxide, has been known since the experiments of Schönbein (1863). All the catalytic tests are based on this principle, as discussed in the preceding sections; but all have relied upon the coupling of the peroxidase activity to the oxidation of a compound which formed a colored product.

Zahn (1871) specifically noted that peroxide could be used to detect blood in stains, though it is not clear that he was aware of Schönbein's work. If peroxide is brought into contact with a bloodstain the peroxidase reaction takes place after a minute or so, and is evidenced by the formation of large numbers of tiny bubbles. Gantter (1895) suggested that the test had substantial value if negative, i.e., was a good indication of the absence of blood. Sutherland (1907) referred to the test as the Zahn-Gantter Test, and noted that many of the substances now known to give false positive catalytic tests, such as vegetable extracts, gave this test as well. Cotton (1904) studied the evolution of oxygen in the presence of H_2O_2 from the blood of a number of different species. Palleske (1905b) also conducted studies on the test with different bloods. A positive test could be obtained with a drop of blood in 1500 mℓ of water, which, if we assumed that 20 of his drops would equal 1 mℓ, would represent a sensitivity of a 1:30,000 dilution. Sutherland (1907) thought the test was useful when negative, except where the stain had been heated above 120°. Leers (1910) presented the test as a presumptive one, noting that other substances than blood gave positive results. He called the test "Die Vorprobe mit Wasserstoffsuperoxyd" or, simply, "the preliminary test with hydrogen peroxide."

6.6.2 Eosin

In 1910, Ganassini proposed the use of an eosin reagent, prepared from crystalline eosin by heating in strong base, and collection and washing of the acid precipitate. This reagent in alcoholic solution in the presence of strong alkali and H_2O_2 gave a momentary yellow to red colored product. He believed the reagent to be specific for blood. Belussi (1911) disagreed, noting that other substances gave positive tests, and that the sensitivity of Ganassini's test was far lower than that of benzidine.

6.6.3 2,7-Diaminofluorene

There is a brief report in the literature by Schmidt and Eitel (1932) on blood identification using 2,7-diaminofluorene (also called 2,7-fluorenediamine). This report has mainly to do with a problem concerning the stability of the reagent, but the implication that the reagent was in use for the detection of blood is quite clear. The structure of 2,7-diamonofluorene is indicated in Fig. 6.7. The 7th edition of the *Merck Index* indicates that the reagent is used to determine halides, nitrate, persulfate and several metals. It seems likely, therefore, that these might be expected to give false positive reactions in the test for blood using this reagent.

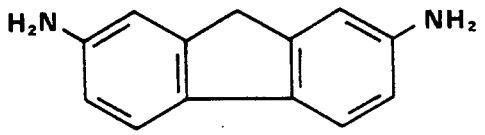


Figure 6.7 2,7 - Diaminofluorene

6.6.4 Rhodamine B

In 1917, Fuld recommended the use of a Rhodamine B reagent for blood detection. The reagent was prepared from Rhodamine B (Fig. 6.8) by reduction in base in the presence of zinc. This reagent detected blood at a dilution of $1:10^7$, according to Fuld. Alke (1922) studied the reaction in some detail, and reported that it is not given by semen, saliva, urine or a number of other biological substances. The usual inorganic oxidants will oxidize the reagents in the absence of H_2O_2. The reaction was negative with rust, and Alke reported a sensitivity limit of $1:10^5$ to $1:10^6$ dilution of blood. Ziemke (1938) notes that an investigator named Diels showed that chlorophyll gives the test. The reaction is apparently still positive with blood which is putrefied, or which has been heated above 200°.

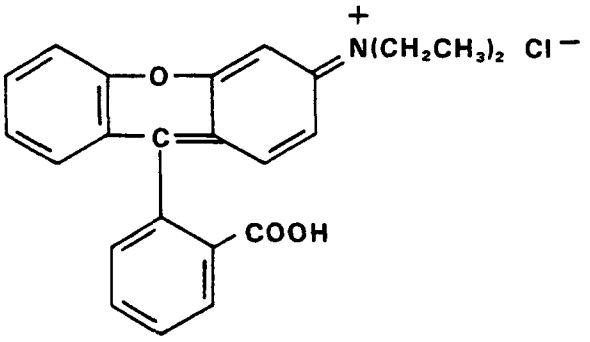


Figure 6.8 Rhodamine B

6.6.5 Para-Phenylenediamine

Boas, in 1906, recommended p-phenylenediamine (p-diaminobenzene) as a reagent for detecting occult blood in feces. Schumm and Remstedt (1906) evaluated the reaction, noting that under their conditions, the sensitivity could be as high as $1{:}10^5$ dilution of blood. The colors obtained varied, however, with different blood dilutions. Adler and Adler (1904) had tested this material, as well as the o- and m-isomers, in their experiments and obtained color reactions at sensitivities of 7:100,000 dilutions of blood, or about 1:14,285.

6.6.6 Ortho-Tolidine and ortho-toluidine

Ruttan and Hardisty (1912) recommended o-tolidine as a reagent for detecting occult blood. This reagent had a sensitivity limit of $1{:}7 \times 10^6$, an order of magnitude greater than that for benzidine, but somewhat less than that for phenolphthalin, which they reported as a $1{:}10^7$ dilution. The test was also recommended by Kohn and O'Kelly (1955) as a substitute for the benzidine test for occult blood, and Jacobs (1958) found the reagent to be a good substitute for benzidine for the staining of leucocyte peroxidases. In 1939, Gershenfeld wrote a paper noting that there had come to be some confusion in the literature as to the difference between o-tolidine and o-toluidine. The former is 3,3′-dimethylbenzidine (Fig. 6.9), while the latter is o-methylaniline (or 2-aminotoluene) (Fig. 6.10). Gershenfeld mentioned that some writers had erroneously credited the employment of o-toluidine for blood testing to Ruttan and Hardisty (1912), while these workers were responsible only for the employment of o-tolidine. Adler and Adler (1904) had tested p-toluidine but not o-toluidine in their studies. Either o-tolidine or o-toluidine may be used as reagents for blood testing, the former being more sensitive and giving a blue-colored oxidation product, while the latter gives a violet-colored one. The distinction is important, and should be kept in mind. Holland *et al.* (1974) confirmed Ruttan and Hardisty's earlier finding that o-tolidine is more sensitive than benzidine for blood testing, although the comparison in the more recent study was made on the basis of molar extinctions at maximum wavelength of visible absorption,

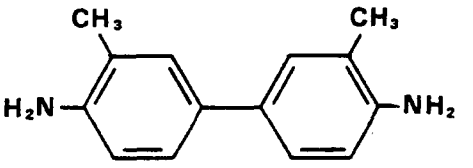


Figure 6.9 o-Tolidine

rather than in terms of maximal dilutions of blood which still gave positive reactions. Although o-tolidine has apparently not been placed on the OSHA regulations list (Cf:29 Code of Federal Regulations 1910.1000 *et seq.*), Holland *et al.* (1974) reported that it is carcinogenic in rats. Ferretti *et al.* (1977) reported that o-tolidine was mutagenic in the Salmonella/mammalian microsome test, indicating a high probability of carcinogenicity. Ortho-toluidine was not mutagenic in the test. Hunt *et al.* (1960) tested o-tolidine and found it to be quite similar to benzidine in most respects, and weak but slow positive reactions were obtained at $1{:}10^6$ dilutions of blood. Culliford (1971) mentioned that o-tolidine could be substituted for benzidine for routine identification testing.

6.6.7 Ortho-Dianisidine

Ortho-dianisidine is 3,3′-dimethoxybenzidine (Fig. 6.11). Owen *et al.* (1958) tested a number of compounds for the detection of the hemoglobin-haptoglobin complex in gels following zone electrophoresis. They found o-dianisidine to be the most sensitive, and to give the most stable color. The latter property led to its being adopted in clinical situations for the quantitative, colorimetric determination of heme compounds (Lupovitch and Zak, 1964; Ahlquist and Schwartz, 1975). Compton *et al.* (1976) used it to detect Hb-Hp complexes on cellulose acetate membranes following electrophoresis. Rye *et al.* (1970) said that there was no evidence that o-dianisidine was carcinogenic in humans, and that it was in fact metabolized by a different pathway than is benzidine, but Ferretti *et al.* (1977) reported that it behaved like benzidine and o-tolidine in the Salmonella/mammalian microsome test indicating that it might well be found to be carcinogenic. Culliford (1971) noted that this compound could be substituted for benzidine in identification tests for blood stains.

6.6.8 Amidopyrine

Amidopyrine, also known as aminopyrine, pyramidon, aminopyrazine, 4-dimethylaminoantipyrine, and a variety of other names, is 4-dimethylamino-2, 3-dimethyl-1-phenyl-3-pyrazolin-5-one (Fig. 6.12). Caplan and Discombe (1951) tested amidopyrine for the detection of blood in urine, and found it to be more sensitive than guaiacum, but less so than o-tolidine, benzidine or phenolphthalin. White (1977) found it to be among the least sensitive of a number of compounds he tested for the detection of Fe(III) mesoporphyrin spotted on filter paper. Owen *et al.* (1958) tested this material for

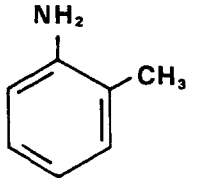


Figure 6.10 o-Toluidine

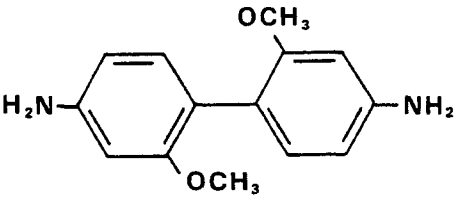


Figure 6.11 o-Dianisidine

the detection of Hb-Hp complexes following electrophoresis, but got poor results with it. As noted briefly above, however, Rentoul and Smith (1973) recommended amidopyrine as a benzidine substitute in blood identification tests. The suspect material is touched with a piece of filter paper, which is then first treated with a saturated solution of amidopyrine in 95% ethanol, and then with H_2O_2. A purple color appears in a few seconds if the test is positive, and the test was regarded as presumptive only. The test was said to work better with higher concentrations of peroxide, but these were not used because they were caustic to the skin.

6.6.9 Benzylidine dimethylaniline

In 1956, MacPhail reported that p,p′-benzylidine bis (N,N′-dimethylaniline) was an excellent reagent for the catalytic blood identification test. The compound is also known as 4,4′-tetramethyldiaminotriphenylmethane. Saline-moistened filter paper was placed in contact with the stain and then tested in a two-stage reaction. Reagent was made up 2 grains/oz 40% acetic acid. Sodium perborate (23 grains/oz 40% acetic acid) was used in the second stage. The oxidized product is momentarily green, passing quickly to a dark blue-green. Sensitivity was said to be in excess of $1:10^6$ dilution of blood, and the reaction only occurred with mammalian blood. False positives were not observed if the two-stage technique was employed. Williams (1974) recommended the reagent and said that it was superior to benzidine, there being less chance for false positive reactions.

6.6.10 3,3′,5,5′-Tetramethylbenzidine (TMB)

In an effort to find a safe substitute for benzidine, Holland *et al.* (1974) synthesized and tested 3,3′,5,5′-tetramethylbenzidine (TMB). The compound was prepared from 2,6-dimethylaniline, and its structure is shown in Fig. 6.13. There was some evidence, according to these authors, that carcinogenicity in these aromatic amines is related to the ability of the compound to undergo metabolic, or *in vivo*, o-hydroxylation. Methylation at all four o-positions renders this reaction impossible, and was expected, therefore, to decrease carcinogenicity. TMB was found to be much less carcinogenic in rats than either benzidine or o-tolidine, and more sensitive than either of them for blood testing. Garner *et al.* (1976) evaluated TMB as a reagent for the identification of blood in stains in comparison to benzidine. The sensitivity of the reagents, expressed as the weakest dilution of blood in saline that gave a positive test, was found to be identical at reagent concentrations of 0.05M, 0.1M and 0.2M, the last-mentioned being the saturating concentration of TMB in glacial acetic acid. At 0.2M strength, both reagents detected $1:10^6$ dilutions of blood. Studies on specificity were carried out by testing a number of plant extracts deposited on cotton cloth for false positive reactions. Testing was carried out on rubbings, extracts, and directly on the cloth. Positive reactions were obtained with both reagents on rubbings of horseradish stain, and with TMB but not with benzidine on rubbings of garlic stain. All other "false positive" reactions on rubbings or cloth were distinguishable from those of blood on the basis of the time of reaction or nature of the color developed. All saline extracts tested were negative with both reagents. Some types of papers gave positive reactions as well, but these too were qualitatively distinguishable from the blood reactions. Still, blood identification in stains on paper should be carried out with great care if TMB is employed. The reagents showed parallel decreases in sensitivity as a function of storage as 0.2M solutions in glacial acetic acid. The sensitivity had decreased to $1:10^5$ dilution of blood by the second day of storage, and to $1:10^4$ dilution by the eighth day. It made no difference to this decrease if the reagents were stored dark, or dark and refrigerated.

Nardelli (1976) has noted that parts of tangerine peels give positive but slow TMB reactions, a result which is not altogether surprising in view of the observation by Grodsky *et al.* (1951) that the white pulp, but not the juice, of the orange gave false positive reactions with benzidine. Nardelli also mentioned that saliva and asbestos glove material gave false positives. Garner *et al.* (1976) pointed out that as demand for and supply of TMB increases, and price decreases, the reagent may come to be used as commonly in forensic laboratories as was benzidine.

6.6.11 Chlorpromazine

Chlorpromazine is 2-chloro-10-(3-dimethylamino propyl)-phenothiazine. It can be oxidized by peroxide to a red free radical form, the reaction being catalyzed by peroxidases, including hemoglobin. The chromophore has an absorption maximum at 530 nm and Lee and Ling (1969) showed that the reagent could be used to quantitate Hb in serum. Collier (1974) confirmed these results and recommended chlorpromazine as a substitute for o-dianisidine and o-tolidine. White (1977) reported that chlorpromazine and

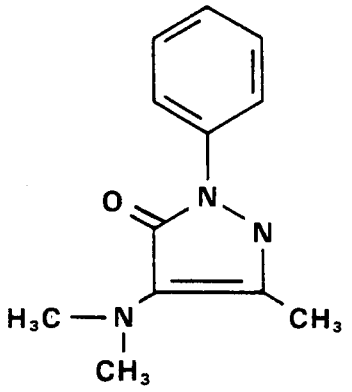


Figure 6.12 Amidopyrine

H_2O_2 detect Fe(III) mesoporphyrin IX as a microspot on filter paper.

6.6.12 Diphenylamine

In 1970, Woodman proposed the use of diphenylamine as a catalytic test reagent for blood in feces. The test was sensitive to about 1:4000 dilutions of blood in water and the oxidized chromophore is a green color. The reagent is also suitable for detecting hematin compounds on paper or cellulose acetate membranes following electrophoresis, and was recommended because it is not carcinogenic.

6.6.13 Fluorescin

Fluorescin is the reduced form of fluorescein, the latter being 9-(o-carboxyphenyl)-6-hydroxy-3H-xanthen-3-one. Fluorescein is soluble in alkali hydroxides and carbonates at room temperature, and exhibits an intense green fluorescence. In 1910, Fleig proposed using fluorescin to detect blood in the urine of patients. He prepared the reagent by reducing fluorescein in the presence of KOH, zinc dust and heat. He said that it was a more sensitive reagent than phenolphthalin, and suggested that it could be used to detect blood in dried stains as well.

We have conducted studies on the use of fluorescin as a presumptive test reagent, and have found that it is entirely satisfactory (Lee *et al.* 1979). We were unaware of Fleig's paper until quite recently. Fluorescin can be prepared from fluorescein in the same way that phenolphthalin is prepared from phenolphthalein. We find that the reagent is stable for months if kept over some zinc dust at 4°. We dilute it to about 1:60 with water for use, and the dilute solution is not as stable as the stock one. Water is preferable to ethanol as a diluent. A positive test can be obtained on blood dilutions up to $1:10^7$. It works on bloodstains on many different substrata.

6.7 Luminol Test

The luminol test is based on the fact that a number of hemoglobin derivatives greatly enhance the chemiluminescence exhibited by luminol upon its oxidation in alkaline solution. According to Proescher and Moody (1939), the compound was first synthesized by A. Schmitz in Heidelberg in 1902, under the direction of Prof. T. Curtius. Curtius and Semper (1913) then synthesized it in a different way, and referred to Schmitz's earlier work. But none of these workers noticed the chemiluminescence properties of the molecule. The intense blue chemiluminescence of the compound during its oxidation in alkaline solution was first observed by W. Lommel in Leverkusen (Germany) who brought it to the attention of H. Kautsky. Kautsky apparently interested H. O. Albrecht in looking into the properties of the phenomenon, the results of the investigation having appeared in 1928. Albrecht found that a number of oxidizing agents, which could be used in alkaline solution, brought about the luminescence, that H_2O_2 alone brought about only a feeble luminescence, and that luminescence was visible in a darkened room even at luminol concentrations of 10^{-8} M. Ferricyanide or hypochlorite greatly enhanced the luminescence obtained with H_2O_2, as did plant peroxidases and blood. Albrecht suggested a mechanism for the reaction as well, and this matter is discussed in more detail below.

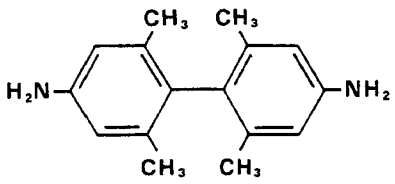


Figure 6.13 3, 3', 5, 5' - Tetramethylbenzidine

Luminol is 3-aminophthalhydrazide (Fig. 6.14). In 1934, Huntress *et al.* reported a method for the synthesis of the compound from 3-nitrophthalic acid and hydrazine sulfate, and named it "luminol". A year later, Harris and Parker, in the same laboratory, published studies on the quantum yield of the chemiluminescence. Gleu and Pfannstiel (1936) observed that crystalline hemin produced an especially intense luminescence, a fact soon confirmed by Tamamashi (1937). Specht (1937a, 1937b) did an extensive series of studies intended to design a useful medico-legal blood identification test based on luminol chemiluminescence. Old as well as recent bloodstains were examined, and able to be detected reliably using luminol reagent. Specht made two solutions, and noted that either one worked well: (1) 0.1 g luminol, 5 g $CaCO_3$, and 15 mℓ 30% H_2O_2 in 100 mℓ H_2O; (2) 0.1 g luminol in 100 mℓ 0.5% aqueous sodium peroxide. He tested a variety of substances for their ability to enhance peroxide-luminol luminescence in the same manner as did bloodstains. These included milk, coffee stains, semen, saliva, urine, feces, dyes, moldy bread, leather, fabrics, oils, varnish, wax, shoe polish, wood, grass, leaves and a number of metals, and all gave negative results. The reagent could be used in solution or sprayed onto suspected surfaces of all types using an atomizer. The spraying of luminol reagent onto bloodstain

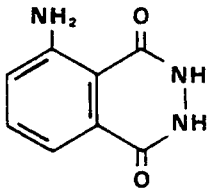


Figure 6.14 Luminol (3-aminophthalhydrazide)

material was said not to interfere with subsequent crystal or spectral tests, nor with serological tests for species or blood groups. The luminescence lasts for quite a while, at least a matter of minutes, and under proper conditions can be photographed to provide a record of the location of stains. Specht recommended the test strongly for medico-legal examinations, and believed that it was quite specific for blood. Proescher and Moody (1939) looked into the test fairly extensively, using commercial luminol at a concentration of 0.1 g in 100 mℓ 5% Na_2CO_3. This reagent solution was indefinitely stable. The test was performed by adding 15-20 mℓ 3% H_2O_2 or 1 g sodium peroxide to 100 mℓ luminol solution just prior to making the test. For the detection of fresh blood in solution, the hemoglobin was converted to hematin by the addition of concentrated HCl to the sample, which was then boiled briefly to destroy vegetable peroxidases. The test solution was then made alkaline again with sodium carbonate, and luminol reagent added. For bloodstains, the surfaces were first sprayed with 1-2% HCl, sprayed again after 10-15 min with sodium carbonate solution, and finally with luminol-peroxide reagent. Proescher and Moody regarded the test as extremely useful, but presumptive. The test could be given by hypochlorites, ferricyanide, and several other inorganic substances. Many of the substances with which Specht (1937a, 1937b) had obtained negative results were tested, and the negative results confirmed. Bloodstains were found to give more intense and longer lasting luminescence than fresh blood, and could be made luminescent many times by allowing the sprayed reagents to dry, and then re-spraying.

McGrath (1942) recommended the luminol test for use in forensic blood detection. He noted, as had others, that older stains gave more intense and longer-lived luminescence than fresher ones, because more met-Hb and hematin has formed in the older stains. He believed the test to be quite specific for blood, having obtained negative reactions with serum, bile, pus, semen, pleural fluid, earth, feces, fresh and spoiled vegetable material, various paints, metals, wood and shoe polish. He nevertheless cautioned that the test should not be used as a final, specific test for blood by itself. The main disadvantage of the test, in McGrath's mind, was that it had to be carried out in the dark. Kraul *et al.* (1941) noted that they regarded the test as a good presumptive one, but that it was not specific for blood. They also determined the wavelength of maximum chemiluminescence to be at 441 nm, with a shift to longer wavelength, 452 nm, in the presence of old blood. Schneider (1941) reported that a number of iron chlorophyll derivatives give luminescence with luminol-peroxide in sodium carbonate solution.

Grodsky *et al.* (1951) described a number of studies on the luminol test. They recommended a reagent consisting of 0.07 g sodium perborate in 10 mℓ water, to which is then added 0.01 g luminol and 0.5 g Na_2CO_3. The order of addition was important because the perborate is more soluble in water than in sodium carbonate, while the opposite is true of luminol. Reagents for the preparation of this reagent were incorporated into a field test kit recommended by the authors. The test was considered to be quite specific for blood, no false positives having been observed with a variety of materials that affected other catalytic tests. A noteworthy exception was copper salts. Grodsky *et al.* found that most brass, bronze and similar alloys which contain copper gave the reaction, a very important consideration if one is dealing with locks, door handles or other fixtures constructed of these materials. Indeed, Steigmann (1941) recommended the use of luminol for the detection of copper, as well as iron and cobalt, ions. He noted in 1942 that the reagent could be used for peroxide determinations as well.

Zweidinger *et al.* (1973) evaluated a number of types of film for the photography of bloodstains sprayed with luminol reagent. Various reagents, made with peroxide or perborate, were also tested. In aqueous solutions, the peroxide yielded a more intense, but shorter-lived chemiluminescence than the perborate. Similar results were obtained if the reagents were made up in 95% ethanol, it being necessary in this latter case to basicify the solution with 0.02 M KOH since sodium carbonate is quite insoluble in ethanol. A number of different photographic films were investigated, along with the effects of varying f/stop, exposure time and development conditions. It was possible to obtain good photographic records of bloodstains on items of evidence, and the procedure was recommended for adoption in routine practice.

One of the claims that has been made for the luminol test, particularly by those recommending sprayed reagents, is that its presence does not interfere with subsequent confirmatory blood tests or serological tests (Specht, 1937a, 1937b; Proescher and Moody, 1939; McGrath, 1942; Grodsky *et al.*, 1951). Srch (1971) reported, however, that the presence of luminol reagent on a sample may interfere with the Takayama test, the determination of ABH agglutinins by the method of Lattes, and the absorption-inhibition test for ABH agglutinogens. It did not interfere with the benzidine test, nor with species determination by the precipitin test. Schwerd and Birkenberger (1977) confirmed Srch's finding that luminol-peroxide spray can interfere with ABO grouping by inhibition technique, especially in small stains. Mixed agglutination could still be used, but did not work as well as on unsprayed controls. The precipitin test was not affected by the spray reagent.

An advantage of the luminol test is its great sensitivity. Albrecht (1928) stated that chemiluminescence was obtainable at luminol concentrations of 10^{-8} M, and Wegler (1937) reported luminescence at 10^{-10} g/mℓ luminol, or about

5.6×10^{-10} M (the MW of luminol is 177.16). The more usual way of expressing sensitivity, of course, is in terms of maximal dilutions of blood which still give positive reactions at some constant reagent concentration. Most authors have used 0.1% (w/v) luminol solutions, corresponding to about 0.056 M. Proescher and Moody (1939) said that the test was sensitive to $1{:}10^9$ dilutions of hematin. Grodsky *et al.* (1951) obtained luminescence lasting at least 15 minutes within 5 sec of spraying the reagent on stains made from $1{:}5 \times 10^6$ dilutions of blood. Weber (1966) quoted a sensitivity of $1{:}10^7$ dilution of blood using a photomultiplier tube detection system.

Bujan (1948) attempted to take advantage of the fact that luminol gives its intense luminescence with hematin, which is formed upon bloodstain aging. The luminescence intensity, measured photoelectrically, could be correlated with the age of the bloodstain, or with the amount of blood that was present in the stain.

The luminol reaction is somewhat more complex than those involved in the phenolphthalin, benzidine, leucomalachite green, and other catalytic tests. While it is probably not wrong to refer to the luminol test as a "catalytic test", it is not mechanistically a catalytic test in quite the same way as are the others. In dilute acid solution, luminol is relatively insoluble, and has the structure shown in Fig. 6.15(a). This compound gives a strong blue fluorescence with UV light. The tautomeric forms shown in Fig. 6.15(b) exist in alkaline solution, and it is these which produce chemiluminescence upon oxidation. Albrecht (1928) proposed a mechanism for chemiluminescence (Fig. 6.16), in which the phthalazine (I) was oxidized to form a diimide compound (II). This material would be hydrolyzed in the basic solution to yield the phthalic acid compound (III) and N_2H_2, which reacts with an additional mole of II to form nitrogen, IV and light. Compound IV, it will be noticed, is the form of luminol which exists in acid solutions (Fig. 6.15(a)), and thus presumably a partial regeneration of starting material. Tamamushi and Akiyama (1938) studied the reaction and their results were consistent with this mechanism, but Stross and Branch (1938) obtained results using fast-flow methods which could not be explained by it. Other studies were done by Sveshnikov (1938) whose results suggested a prior hydrolysis, with luminescence being due to the oxidation of a hydrolysis product. Kubal (1938) and Plotnikov and Kubal (1938) investigated the spectral changes associated with the reactions. In the presence of rhodamine or fluorescein, some of the chemiluminescent energy is apparently absorbed by the dyes, which then fluoresce at wavelengths longer than that of the luminescence. This phenomenon, they called chemifluorescence. Baur (1940) said that the decay of luminescence of luminol in the presence of hemin and peroxide followed a bimolecular rate law. Weber and co-workers carried out extensive studies on the luminescence reaction, and state, among other things, that substances which greatly increased the peroxide oxidation-dependent luminescence, such as chlorhemin, met-Hb and ferritin, do not, strictly

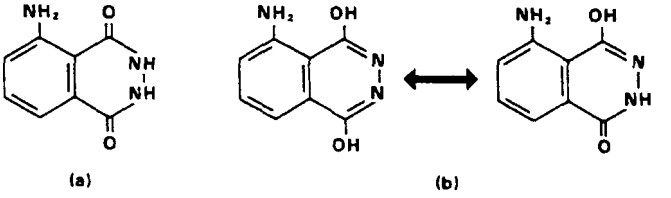


Figure 6.15 Luminol Structures in Solution

speaking, act as catalysts under all conditions (Weber, 1942; Weber *et al.*, 1942; Weber and Krajčinović, 1942). Apparently, these compounds are best thought of as "accelerators", which may act catalytically. Weber *et al.* (1942) suggested that the products of the initial oxidation reaction included O_2 and reduced accelerator. If the reduced accelerator could be reoxidized by O_2, the compound would be acting catalytically, while if the reoxidation were not possible, the accelerator would have acted as a reactant. The subject is complex, and it may be that the mechanism is not the same with every "accelerator" or catalyst. Shevlin and Neufeld (1970) studied the mechanism of the ferricyanide-catalyzed luminescence of luminol, and proposed the scheme shown in Fig. 6.17 to explain their data. It should be noted that the ferricyanide acts catalytically in this scheme. The exact role of the catalysts or accelerators is not clear from Albrecht's scheme (Fig. 6.16). White and Roswell (1970) reviewed the chemiluminescence phenomena characteristic of organic hydrazides generally, including luminol. Isaccson and Wettermark (1974) noted that the mechanism of luminol oxidation in aqueous solution has still not been satisfactorily elucidated in spite of many studies.

It may be mentioned, finally, that Weber (1966) proposed an improved reagent for blood testing. Stock solutions were: (A) 8 g NaOH in 500 mℓ H_2O, or 0.4N; (B) 10 mℓ 30% H_2O_2 in 490 mℓ H_2O, or 0.176M; (C) 0.354 g luminol in 62.5 mℓ 0.4N NaOH to a final volume of 500 mℓ with water, or 0.004M luminol. To make up testing reagent, 10 mℓ of each of these solutions is mixed with 70 mℓ H_2O. It will be noted that the Na_2CO_3 used by others is here replaced by NaOH, and that the luminol and peroxide concentrations are very much lower. This reagent works well with both fresh and dried blood, whereas the older reagents did not readily react with fresh blood because there was too little met-Hb or hematin present. The fact that this reagent serves with fresh stains is explained by the rapid conversion of Hb to met-Hb and/or hematin in the strong base. The reagent is more sensitive than the older ones because of the lowered H_2O_2 and luminol concentrations. Higher concentrations of these compounds tend to be inhibitory.

6.8 Catalytic Tests—General Considerations

There can be no doubt that most authorities have considered the catalytic tests as presumptive when used alone. Their value is ascribed to the ease and rapidity with which

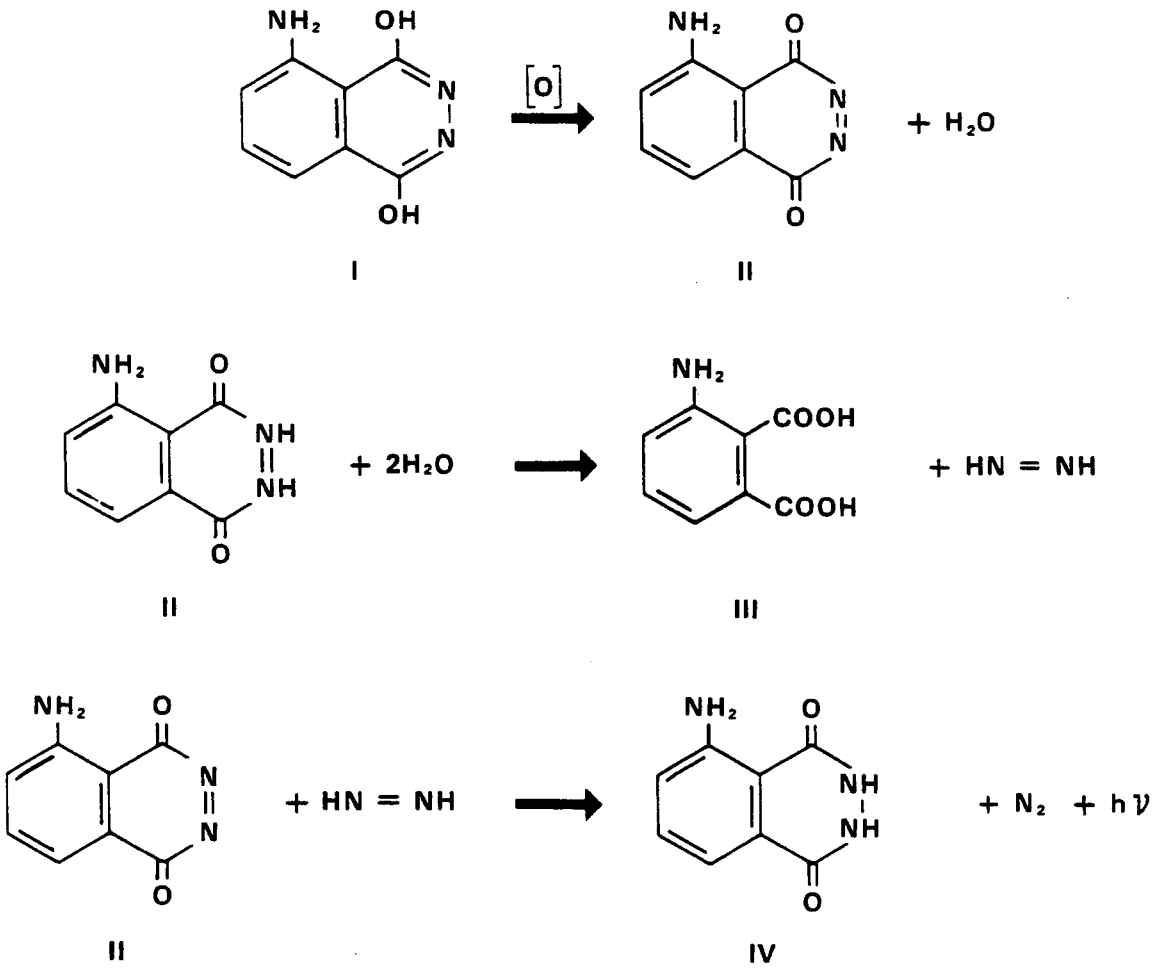


Figure 6.16 Albrecht Mechanism

they can be carried out in order to decide which exhibits should be subjected to further tests. It has occasionally been persuasively argued that in the hands of an experienced investigator, who is aware of the principles underlying the test and of the materials other than blood likely to give misleading reactions, the tests can be virtually specific. Grodsky *et al.* (1951) made something of a case for the combined use of several tests, namely benzidine, phenolphthalin, leucomalachite green and luminol, especially in cases where there was a limited amount of material to work with. These recommendations were based in part on their own studies, as well as on a set of experiments conducted by Pinker (1934). Pinker tested some 200 different biological substances and organic and inorganic chemicals for reactions with benzidine, phenophthalin and leucomalachite green reagents. A very small amount of the substance or stained material was treated directly with the reagent in a spotting plate. The reactions were carefully observed in terms of time, intensity, color produced, and other characteristic properties such as precipitate formation. Vegetable peroxidase interference could be eliminated by testing samples which had been heated, and chemical oxidants were detected by the fact that they react in the absence of peroxide. With these materials excluded, there was not found one interfering substance which would give a false positive reaction (indistinguishable from the blood reaction) with *all three* reagents. Pinker therefore argued that if positive reactions were given by all three reagents in carefully performed tests, the likelihood of error was exceedingly remote. Not all the same substances are likely to interfere with all the tests, especially with luminol as against the others. Grodsky *et al.* felt that there was no significant probability that any substance other than blood would be found which would give positive reactions in all four tests which would be indistinguishable from the blood reaction by an experienced worker. The argument was not put forth to suggest that these tests

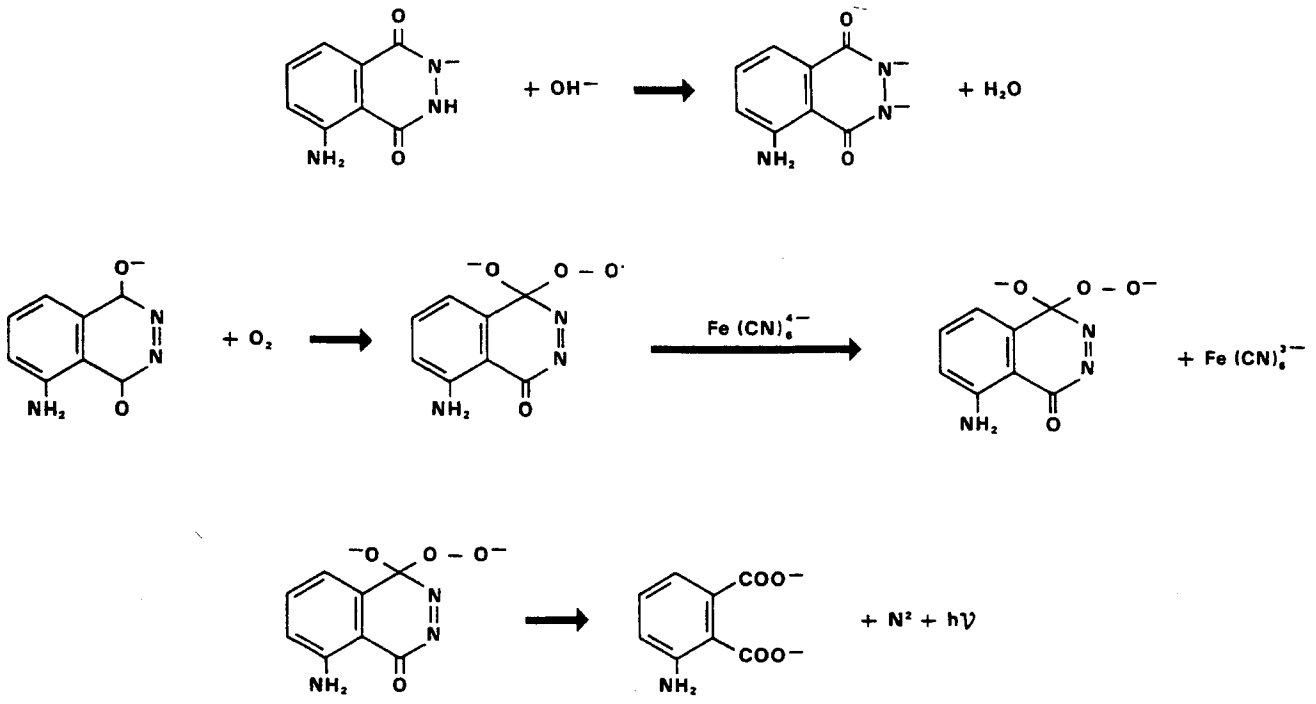


Figure 6.17 Shevlin and Neufeld Mechanism

replace crystal and spectroscopic tests; but cases do arise in which there is insufficient material for further testing. The results of Blake and Dillon (1973), discussed in Section 4.2.4, would suggest that for a number of examples of bacteria, whether in fresh culture or dried out on filter paper for 2 months, the use of several catalytic tests and/or a luminol test (Section 6.7) would not necessarily exclude false positive reactions. The ease with which suspensions of microorganisms gave the benzidine test was directly related to their catalase content, and the false positive reaction was not inhibited by exposure to high temperatures for periods of up to 30 min. Ultimately, of course, the decision concerning what proof value should be placed on the outcome of any test on a given exhibit rests with the individual expert. In the final analysis, the individual is giving his or her opinion as to what the findings mean.

A somewhat different issue, of no less concern, is raised by studies that have indicated the presence of blood on clothing and other samples that were randomly selected, and had nothing whatever to do with case material. Owen and Smalldon (1975) got positive benzidine reactions on 5 jackets of 100 tested, and on 16 pairs of trousers of 100 tested, randomly selected at a dry cleaning shop. One pair of used shoes of 100 pairs examined (50 men's and 50 women's) gave a positive reaction as well. Hunt *et al.* (1960) obtained strongly positive benzidine reactions (i.e. within 3 sec) on one of 30 used shoes randomly selected at a shoe repairer, and weakly positive reactions (i.e. within 5-10 sec) on six others. Terörde (1939) examined fingernail scrapings from 606 people in the general population for benzidine reactions. 195 samples were positive. 165 positive samples came from the 546 men in the sample, while the remaining 30 were from the 60 women. The individual's occupation or walk of life seemed to matter little, except in the case of butchers, whose scrapings were all positive. Additionally, Terörde got a positive precipitin reaction against anti-human serum with one of six of the benzidine-positive samples. The results strongly indicate that some consideration must be given to the actual evidentiary value of samples when positive results are obtained, especially in cases where little material is available for further testing.

Briggs (1978) discussed the matter of the probative value of bloodstains on clothing in connection with a case, which had required the examination of stains on a very large number of different articles. The relative occurrence of particular combinations of blood groups in the bloodstains enters into this discussion as well.

SECTION 7. OTHER TESTS

7.1 Immunological Tests With Anti-Human Hemoglobin

The first antiserum to a hemoglobin was reported by Leblanc (1901). He prepared anti-cow Hb sera in rabbits, with which a precipitin reaction could be obtained. Ide (1902) confirmed these results, although his antisera were hemolytic as well. His student, Demees (1907) was able to prepare a non-hemolytic anti-Hb precipitin antibody by using a more thoroughly purified Hb as immunizing antigen. Not all investigators obtained identical results in the early investigations, however. Gay and Robertson (1913) concluded that globin alone was not antigenic, but when combined with casein into what they referred to as "globin-caseinate", an antibody could be raised which fixed complement (see section 1.3.5.3) with the antigen as well as with casein alone. Browning and Wilson prepared antibodies to guinea pig globin in rabbits in 1909. In 1920, they enlarged these studies somewhat, noting that ox globin was antigenic as well. The anti-guinea pig serum was quite species specific, but the anti-ox globin was not. Ford and Halsey (1904) were unable to raise precipitin antisera to purified, crystalline dog or hen hemoglobin in either rabbits or guinea pigs. In 1919, Schmidt and Bennett got similar results. Unable to obtain any precipitin antisera against pure, crystalline dog hemoglobin, they concluded that hemoglobin was not antigenic. Klein (1904, 1905a) conducted extensive experiments on the immunization of rabbits with serum, whole red blood cells, and red cell extracts. Antibodies were obtained to the red cell extracts, which he called "erythropräzipitine"; these antibodies he believed to be different from the anti-serum antibodies (obtained by immunizing with serum). Klein was the first to suggest (1905b) that the anti-red cell extract serum should be employed in medico-legal investigations. He believed the antisera to be species-specific, and that their use would combine into one procedure the determination of the presence of blood and its species of origin. Leers (1910) agreed with this viewpoint. He prepared specific "erythroprecipitin" antisera of his own, and he discussed the use of these reagents in carrying out medico-legal tests for blood.

The issue of hemoglobin's antigenicity began to be settled in 1922, when Hektoen and Schulhof prepared precipitating antibodies against extracts of cow, dog, goat, guinea pig, horse, human, rat, sheep and pig red cells. Some of these were species-specific, while others showed cross reactions. The following year, they obtained many of the same antisera in species-specific form, and concluded that the red cell precipitinogen giving rise to these antibodies was in fact hemoglobin. They suggested that the antisera would be useful in solving medico-legal cases. Higashi (1923) independently arrived at the same conclusions, suggesting that the antisera be called "hämoglobinopräzipitin", and recommending that they be used in forensic practice. Heidelberger and Landsteiner confirmed these findings in 1923. They knew of Higashi's work, but had apparently not yet seen Hektoen and Schulhof's 1923 paper. Sera produced with crystalline hemoglobin, they said, reacted species-specifically with homologous antigen. The antisera worked equally well with homologous met-Hb, HbCN and HbCO. Fujiwara (1928) carried out studies on anti-hemoglobin precipitin sera in parallel with anti-serum precipitin sera. He obtained species-specific anti-Hb sera, which had titers as high as 1:40,000 against whole blood or hemoglobin.

Some years later, there was recurrent interest in antisera to hemoglobin, especially with the objective of developing immunological methods for differentiating the various human hemoglobin variants, which were coming to be known. These studies were also done as an immunological approach to discovering the differences in structure among the human hemoglobin variants. The subject of hemoglobin variants itself is involved, and will not be discussed here (see section 38).

Darrow *et al.* (1940) prepared antibodies to cord blood and to adult blood. The former, if absorbed with adult cells, could be rendered specific for cord blood hemoglobin (Hb-F; fetal hemoglobin). Antibodies to adult hemoglobin (Hb-A) reacted with both adult and fetal blood, and absorption of the antisera with either adult or fetal cells brought down all the antibodies. Aksoy (1955) prepared rabbit antibodies against cord blood, but found that in most of the preparations, absorption with adult cells precipitated all the antibody. In only one case was the serum rendered specific for Hb-F by absorption with adult cells.

In 1953, Chernoff published a pair of papers, the first of which (1953a) reported the preparation of anti-Hb-A, anti-Hb-F, anti-sickle cell hemoglobin (anti-Hb-S), and anti-guinea pig Hb, using Freund's adjuvant for the first time. The anti-Hb-F was specific for cord blood, but the antisera to Hb-A and Hb-S cross reacted. The second paper (1953b) reported the use of the specific anti-Hb-F to measure the amount of fetal hemoglobin in adult blood. Kohn and Payne (1972) described a radial immunodiffusion procedure for the determination of Hb-F, using a commercial antiserum. Goodman and Campbell (1953) showed that rabbit antisera to Hb-A and Hb-S did not differentiate them well, but that antisera prepared in chickens could be used to do so. Major

differences in reactivity between anti-Hb-A and anti-Hb-F were also confirmed. Ikin *et al.* (1953) prepared agglutinins to cord blood cells, which showed good titers only if used in the presence of 10% albumin and at 37°. Diacono and Castay (1955a) obtained antibodies from guinea pigs which could be used to distinguish between adult and fetal red cells, and they showed further (1955b) that the anti-Hb activity was not identical to the hemolysin activity. Boivin and his collaborators studied the reactions of anti-Hb-A and anti-Hb-F with a number of variant hemoglobins using Ouchterlony gel diffusion and immunoelectrophoretic techniques. They showed that hemoglobins A, F, S, C and E showed a common antigenic determinant (Boivin and Hartmann, 1958b; Boivin *et al.*, 1959). They also showed that anti-Hb-A reacted with hemoglobin-A in the hemoglobin-haptoglobin complex in gels (Boivin and Hartmann, 1958a). In 1961, Muller *et al.* obtained a rabbit immune anti-human Hb serum, which did not discriminate between Hb-A and Hb-F. But it did not react with human serum, nor with animal hemoglobins, and these workers suggested that it could be very useful in medico-legal work for confirmation of species of origin of bloodstains. Likewise, Fiori and Marigo (1962) prepared anti-Hb-A and anti-Hb-F which were species-specific for human blood and recommended their use in forensic bloodstain analysis. The method was also described by Fiori (1962) in his review.

Adult bloodstains react only with anti-Hb-A, while fetal, or newborn bloodstains react with both antisera. Stains of human body fluids other than blood may react with anti-human serum serum, but will not react with the anti-Hb sera. Hematin does not react with the anti-Hb sera, so that with old bloodstains other methods of identification must be used.

Mori (1967) prepared an anti-human Hb in goats and studied its reactivity and cross-reactivity with human and animal hemoglobins by immunodiffusion and immunoelectrophoresis. At least four precipitin lines could be observed in the reaction between crude human Hb and its antiserum. The antiserum cross-reacted with monkey, horse and dog hemoglobin as well. Absorption with monkey hemoglobin rendered the antiserum human-specific, but it was clear that human Hb seemed to share some antigenic determinants in common with monkey and several other animal hemoglobins. Ohya prepared antisera to human Hb (1970a) as well as to non-hemoglobin red cell proteins (1970b) in rabbits. The anti-Hb was found to cross react with monkey, dog and horse hemoglobin preparations. It could be shown that the cross reactivity was due not to hemoglobin, but to carbonic anhydrase in the non-hemoglobin red cell protein. The anti-human non-hemoglobin red cell protein sera readily cross reacted with many animal lysates, and it was shown that the cross-reacting protein was carbonic anhydrase. The carbonic anhydrases of humans and animals, therefore, do have antigenic determinants in common. If these are not rigorously excluded from the "crude hemoglobin" preparations used for immunization, the "anti-Hb" thus raised will contain non-species-specific anti-carbonic anhydrase antibodies as well. Ohya cautioned that great care should be exercised in using anti-Hb sera for confirmation of species of origin. Fukae *et al.* (1976) prepared antisera in rabbits against carefully purified human Hb-A and Hb-F. The antisera were then absorbed with human non-hemoglobin red cell proteins to render them species-specific. The anti-Hb-A was shown to contain approximately equal amounts of anti-α-chain and anti-β-chain antibodies, while the anti-Hb-F contained a preponderance of anti-γ-chain antibodies. This observation accounted for the superior antigenicity of Hb-F in comparison with Hb-A.

Baxter and Rees (1974a) tested a commercial anti-human Hb. They found that if the antiserum was used undiluted, it showed a titer of 1:8000 against human blood, 1:2000 against baboon blood, 1:64 against human serum and 1:16 against human semen and saliva. The antiserum could, therefore, be rendered specific for blood by moderate dilution, and further dilution would abolish the cross reaction with baboon blood (see section 16.8). These same investigators (1974b) evaluated commercial anti-human Hb-F serum in combination with anti-Hb-A for the discrimination of Hb-A and Hb-F in medico-legal cases. The anti-Hb-A reacted with cord blood, even at blood dilutions of 1:6400, but the anti-Hb-F did not cross-react with adult blood at blood dilutions as low as 1:100. Baxter and Rees recommended this method in appropriated cases (e.g. infanticide), but cautioned that there are adults whose cells may possess Hb-F in small, residual amounts, or in larger amounts because of pathological conditions. This fact must be taken into account in interpreting the results of precipitin tests using these antisera.

The use of anti-Hb sera in bloodstain detection and assessment is, of course, very closely related to the use of antiserum sera for species of origin determination. The latter is discussed in great detail in a later section (section 16.1). The advantage of anti-Hb sera is obviously that it may serve to combine the identification of blood with the determination of species of origin into a single test. This point was made emphatically by De Forest and Lee (1977). They prepared a high titer anti-human Hb and tested its applicability in medico-legal blood identification. The use of the reagent was highly recommended.

The cross-reactivity of anti-Hb antisera among different species is of theoretical interest, as well as being of considerable practical importance. Immunological relationships between the same protein(s) of different species can be used to draw conclusions about phylogeny, and various evolutionary relationships between species. This subject will not be discussed here with respect to hemoglobin. It will be discussed in somewhat more detail in the sections dealing with determination of species of origin (sections 16.8 and 16.9). The relationship between the structure and evolution of proteins and their immunogenicity forms the basis for the application of immunological techniques to identification and species determination.

7.2 Chromatographic Methods

In 1957, Fiori first suggested the use of paper chromatography for the identification of blood by separating and locating hemoglobin and/or its derivatives. Whatman No. 1 filter paper was employed, using a solvent system of 2,4-lutidine:water: :2:1, in the presence of 20–30 mℓ concentrated ammonia. Spots were detected with alcoholic benzidine (pH 4.4–4.6 with acetic acid) spray reagent. R_f values were of the order of 0.56–0.57 but were highly temperature dependent, and hematoporphyrin could be detected by its fluorescence under 366 mn light prior to spraying. Using this method, 5 nℓ of blood could be detected (corresponding to about 1 μg Hb), though best results were obtained with 30 nℓ of blood (Fiori, 1957). The disadvantages of this system were the relatively long development time required (12-14 hrs), and the fact that pre-saturation was required. In 1961, Fiori reported a modified procedure which was considerably faster. The solvent system employed was methanol:acetic acid:water : :90:3:7, Whatman No. 1 filter paper was again used, and chromatography was carried out in the ascending direction. In this system the solvent front migrated about 12 cm in 1–2 hrs and pre-saturation was not required. After a run, the paper was dried in a 100° oven to inactivate vegetable peroxidases. The chromatogram was then examined under UV light to detect fluorescent materials which differed from hematin compounds. Examination at two wavelengths, 253.7 nm and 366 nm, was recommended. Hematoporphyrin could be identified at this stage. A two-step visualization reaction was used, the first consisting of spraying with 1% (w/v) benzidine in 96% alcohol, acidified with acetic acid. Spots which developed at this stage represented chemical oxidants. The second step was the spraying of the paper with 3% H_2O_2 solution to develop the hematin compound spots. This procedure was also described by Fiori in his review in 1962. In running unknown samples, it was recommended that several dilutions of extract be run in the system in a preliminary chromatogram to determine the optimal concentration of extract to be used. A second run was then made using the optimal concentration of extract, and the proper controls. The R_f values for hematin compounds are about 0.70–0.71. Hb-A and Hb-F are not differentiated, nor are a number of animal hemoglobins. Most chemical oxidants have different R_f values. A few, such as $CuNO_3$, NiCl and $CuSO_4$, have similar R_f's but are differentiated by the two-step spraying procedure. These also give different colored spots than does blood. Rust does not interfere. Hematoporphyrin formed long "comets" in this system, and Fiori recommended use of the earlier lutidine:water system if identification of this substance was required. A control spot of 10–20 μℓ of a 1:1000 dilution of whole human blood was run with each chromatogram. If oxidants are encountered, a second chromatogram sprayed with phenolphthalin H_2O_2 reagent, which might help to differentiate them, was recommended. This procedure, Fiori said, amounted to a method for rendering the benzidine test considerably more specific than it would be if it is carried out directly; but he suggested, nevertheless, that another, equally sensitive and specific test for blood be run in parallel on case materials. An anti-Hb precipitin test in agar gels was thought to be the best choice.

Farago (1966) reported a thin-layer chromatographic method for the identification of hematin compounds from bloodstains. Kieselguhr 250 μ TLC plates were employed with a solvent system of methanol:acetic acid:water:90:3:7. 5 to 10 μℓ of water or saline bloodstain extract was spotted on the plate, with 10 μℓ of 1% whole blood as control. At 20–24°, the plates were developed for 25 min, dried at 100° and sprayed with benzidine reagent and H_2O_2 in two successive steps. The R_f of the hematin compounds was 0.79, and the method could detect 3–4 μg blood.

There were many other studies on the paper chromatography of hemoglobins prior to Fiori's (1957a) paper, but they were designed to gather information about various human hemoglobin variants and not as forensic methods of identification (Andersch, 1953; Berlingozzi *et al.*, 1953a and 1953b; Kruh *et al.*, 1952; Penati *et al.*, 1954; Sansone and Usmano, 1950a and 1950b; Schapira *et al.*, 1953; and others). There have also been studies on the separation of human and animal hemoglobins, and on the applicability of the chromatographic patterns thus obtained to determination of species of origin of bloodstains. These are described in a subsequent section (section 17.3).

Chromatography has additionally been employed as a device for concentrating diluted bloodstains and samples, and for separating blood material from various debris. Frache (1939a, 1939b, 1941) used small alumina columns to concentrate the blood substances in diluted samples in preparation for subsequent identification and species of origin tests. In 1951, Kirk *et al.* proposed the use of paper chromatography for the separation of blood from debris. It was noted that blood may be encountered mixed with a variety of materials, such as soil, leaves, wood products, and so on. It may also be extremely diluted or diffuse on surfaces from washing, or present in small quantities which occupy large surface areas, as for instance on a car in a hit-and-run case. The technique which Kirk *et al.* described is applicable to many of these situations, and involves placing a wet piece of filter paper in contact with the moistened blood-containing material. Water was used as the solvent. The capillary action of the migrating solvent carries and concentrates the blood material in a particular region of the filter paper strip, where it may then be subjected to identification, species and blood grouping tests. Obviously, different situations call for different strategies in terms of the actual experimental set-up, and Kirk *et al.* discussed a number of these in the paper. Positive identification, species, and grouping tests could be gotten from very small amounts of material from irregular surfaces and/or which had been mixed with debris. Schaidt (1958) reported a very similar technique, which he had apparently devised independently. Fiori (1962) noted a clever modification of this approach. The filter paper strip was cut so that one end was pointed (V-shaped). The pointed end

was made the top, and the chromatography allowed to proceed in the vertical direction, thus concentrating the desired blood material in the point of the filter paper. The paper could then simply be inverted, so that the V-shaped point was the bottom, and the material eluted in a small volume into any desired container, essentially by descending chromatography.

Such methods could on occasion be very valuable. Hirose (1971a) studied the extractability of blood material from soil. Acetone, acidified with acetic acid, was found to be the best extraction medium. In a test where 0.1 mℓ blood in 8.9 mℓ water was mixed with 50 g soil, it was found that only 70% of the hemoglobin was recovered if extraction began within 5 min of mixing the blood with the soil. The recovery dropped to 22% after two days, and to 7% after 40 days. Soil apparently has absorptive properties for the blood proteins which accounted for this observation. Hirose (1971b) also found that a precipitin test with anti-human-serum or anti-human-Hb serum was positive up to 2–5 days after mixing of blood with soil, if the blood material was extracted with saline. It is possible that the chromatographic techniques could yield better results than extraction methods.

7.3 Electrophoretic Methods

Electrophoretic techniques have been used extensively for the investigation of human and animal hemoglobins, but seldom for the purpose of blood identification as such. Introna and Scudier (1960) used paper electrophoresis for the identification of blood. The technique is analogous to the paper chromatographic methods, and was said to be about as sensitive and very reliable. The method took advantage of two principles: (1) serum haptoglobin (Hp) is a hemoglobin-binding protein, the complex having a particular electrophoretic mobility being detectable with the usual peroxidase reagents, such as benzidine; (2) Met-Hb will form a complex with serum albumin, as will hematin, and these have about the same electrophoretic mobility as albumin in veronal buffers at pH 8.6. Thus, if bloodstain extracts are electrophoresed, the Hb-Hp complexes, which have the electrophoretic mobility of β_1-globulins, can be detected. It is known also (Allison, 1957) that if the Hb concentration exceeds the Hp binding capacity, the free hemoglobin is converted to met-Hb, which complexes with albumin. This complex migrates to the albumin position where it can be detected as a second benzidine-positive band. Hematin does not migrate, and older stains thus present a problem. If human serum is added, hematin can be induced to migrate to the Hb-Hp position, and if hematin is in excess, the second albumin band will appear as well. Human serum is unsuitable for this purpose, however, because it may contain small amounts of Hb. It was found, however, that addition of a small amount of a 10% human serum albumin solution to the hematin would give complex formation, and a benzidine positive band at the albumin position following electrophoresis. Haptoglobin exibits polymorphism in humans, but this aspect is not discussed here, and the method above apparently did not differentiate the iso-Hp proteins.

7.4 Heating Test

In 1938, Moody *et al.* reported the results of a series of experiments in which blood had been heated to very high temperatures and the residues examined. The experiments were begun to look into methods for identifying grossly contaminated, carbonized or incinerated blood samples. Blood specimens were heated to various temperatures, from 160° to 871°. It was found that the residues took on characteristic appearances at various temperatures. Dried blood melts at around 260°. The characteristics of the residues obtained after heating blood samples to 315°, 371°, 426°, 482° and 537.8° were described in detail. It was suggested that, in some cases, heating and examination of the residue formed could itself be used as a test for blood. A number of substances were subjected to similar treatment, and none gave residues resembling those of blood. It was further shown that none of the substances interfered with the formation of the characteristic blood residue if they were mixed with the blood during the heating. There were no differences observed in residue characteristics among a number of vertebrate bloods, and results could be obtained with starting quantities as small as 1 mg. The method, however, does not seem to have been put to use or tested further by other workers.

SECTION 8. IDENTIFICATION OF BLOOD FROM PARTICULAR SOURCES

8.1 Identification of Menstrual Blood

There sometimes arise occasions in which the identification of blood as being of menstrual origin is relevant to a particular case or situation. A number of investigators have devoted their attention to this problem at various times for over a century. A number of different methods have been suggested, and all attempt to take advantage of properties of, or cellular inclusions in, menstrual blood which are not characteristic of circulating blood.

8.1.1 Microscopical and histological methods

One of the oldest methods of identifying menstrual blood is based on the fact that it contains endometrial and vaginal epithelial cells, which may be identified microscopically.

As early as 1848, however, Casanti, in a paper concerned primarily with bloodstain species differentiation, said that he could discriminate menstrual from circulating blood. The method consisted of treating a dried blood mass with concentrated phosphoric acid and carefully studying the characteristics of the resulting residue. The method did not enjoy any popularity.

In 1858, a definitive paper by Robin appeared in which it was said that careful microscopical study of the bloodstain enabled identification of characteristic exfoliated cells in menstrual blood, and thereby, identification of menstrual blood in stains.

Ewell noted this method in 1887, but said that he did not regard the finding of such cells as conclusive proof that the bloodstain was of menstrual origin. Formad (1888) also mentioned identification of characteristic epithelial cells in bloodstains as a method of diagnosing menstrual blood. He went on to note that this type of blood showed an "acid reaction". The latter observation was no doubt based on the now familiar fact that vaginal mucus is acidic. The finding of characteristic epithelial cells as a means of differentiating menstrual blood was recommended by Vibert (1911) as well. Strassman (1921) used a differential staining technique, employing a number of histological stains, which resulted in the simultaneous staining of spermatozoa and vaginal epithelial cells in samples containing both.

It has been known for a long time that characteristic bacteria occupy the normal human vagina, and these too may be found in menstrual bloodstains. For many years, these bacteria, or vaginal flora, were known as Döderlein's bacilli, after Döderlein (1892). In addition, vaginal epithelial cells are known to be rich in glycogen. There is a definite relationship between the flora, the epithelial cell glycogen, and the levels of hormones which control the menstrual cycle. This matter is discussed somewhat more fully in a subsequent section (10.2.7).

Some authorities have recommended microscopical identification of the characteristic bacterial cells, along with the epithelial cells, for the identification of menstrual bloodstains (Kerr, 1954; Smith and Fiddes, 1955). Smith and Fiddes used a methylene blue-eosin stain for the purpose, and thought that the presence of the specific bacterial cells constituted an important criterion for concluding that the bloodstain was of menstrual origin.

Wiegemann (1912) in his dissertation first established that vaginal epithelial cells characteristically contain glycogen, which can readily be detected by its reaction with iodine. This observation was confirmed by the Belgian investigator Lenger in 1911, and forms the basis of one of the most widely used techniques for identifying menstrual blood, which contains the glycogen-rich cells.

Merkel (1924) took advantage of the high glycogen content of vaginal epithelial cells, in devising a method for identification of menstrual blood and vaginal secretions. He employed Lugol's iodine solution to stain the cellular glycogen. It may be noted that the use of iodine stain for glycogen detection dates to Claude Bernard's studies of diabetes (1877). Berg (1957a) recommended Merkel's technique, using a Lugol's solution of 0.2 g iodine and 0.3 g KI in 45 mℓ water. Vagnina (1955) pointed out that he regarded the presence of glycogen-containing epithelial cells as good evidence that the stain was of menstrual blood. In 1943, Mack introduced a very simple method for the iodine staining of vaginal smears, which he called the iodine vapor technique. This consisted very simply of placing a microscope slide containing a vaginal cell smear over a container of Lugol's iodine solution with the side containing the cells toward that solution. The iodine vapors from the solution stained the glycogen in the cells. Sakamoto (1957) used this method with success in examining menstrual bloodstains. Ota *et al.* (1965) soaked fibers containing menstrual blood in a 2% bicarbonate solution, then subjected the dried smear to the Mack technique. Detectability of glycogen-containing epithelial cells in menstrual bloodstains as a function of the conditions to which the stain had been exposed was studied as well. Ota *et al.* found that some conditions had an adverse effect, the highest detection rate being with stains in dry air, and the lowest rate being with stains which had been in running water. Stains in normally and highly humid air, and in still water, showed intermediate rates of detectability.

Menstrual bloodstains do not always give positive results with the glycogen detection techniques in epithelial cells.

Furuya and Inoue (1966a) said that their rates of detection were variable. Neumann (1949) found that 23 examples of menstrual bloodstains showed negative results out of 248 examined. False positive results are apparently also possible. Oral cavity epithelial cells, which are morphologically very similar to vaginal epithelium, may contain small amounts of glycogen, although because the amounts of glycogen in the two types of cells are so different, it is not expected that this would present a practical problem (Mueller, 1953; Sakamoto, 1957). Popielski (1949), after some fairly careful investigations came to the conclusion that the finding of glycogen-positive epithelial cells was not necessarily diagnostic for menstrual blood. He showed that morphologically similar cells could be obtained from the male urethra, and that substantial numbers of them (25 to 50 percent) contained glycogen. This fraction decreased if smears were taken at times of sexual excitation, but was even higher in the cells from post-gonorrheal urethral discharge. Cells from the female urethral orifice also showed the characteristic epithelial morphology, and many contained glycogen. Thus, if any of these types of cells were to be mixed with blood, the presence of glycogen-containing cells could easily be misinterpreted. Other types of blood than menstrual also contain glycogen-positive cells. Furuya and Inoue (1966a) obtained positive results in about 54% of 133 cases of bloodstains from menstrual blood, and blood shed during labor and abortion. They also showed (1966b) that blood shed during the puerperium contained glycogen-positive cells. The puerperium is the period of approximately 40 days from birth to the complete involution of the maternal uterus. Blood shed during this time is sometimes called lochial blood. Glycogen-containing epithelial cells were found in bloodstains obtained from recently delivered women up to puerperal day 17. Thus, if nothing at all is known of the history of a bloodstain submitted for examination, some care must be exercised in interpreting the finding of glycogen-containing epithelial cells.

8.1.2 Methods based on fibrinolytic properties

That menstrual blood does not clot has been known for many years. Luginbuhl and Picoff (1966) quoted Hunter as having written in 1794 that: "In healthy menstruation, the blood which is discharged does not coagulate, in the irregular or unhealthy it does". Formad (1888) also noted the property of incoagulability as being characteristic of menstrual blood. The property is of no direct value, of course, in bloodstains. Methods have been proposed, however, to take advantage of it indirectly. To help clarify the current thinking about the reasons for the incoagulability of menstrual blood, and the forensic methods of identification based upon this property, some background on the fibrinolytic enzyme system will be presented. The fibrinolytic system is actually quite complicated, still not completely understood, and its literature is immense. In 1959, now already some 20 years ago, Sherry *et al.*, in their review, said that studies of fibrinolytic activity had resulted in a literature ". . . so vast that it would be impossible to attempt a complete survey within the confines of this report". Even at that, the review ran 40 pages and dealt only with the then current aspects. 425 references were cited. The interested reader must, therefore, be referred to the specialized literature if more detailed information on this interesting subject is wanted (Hahn, 1974; Marder, 1968; McNicol and Douglas, 1976; Sherry *et al.*, 1959; Verstraete *et al.*, 1971).

Blood clotting is an extremely complex process. Many factors are now known to be involved, and the nomenclature and terminology were most complicated and confusing until the early 1960's. At that time, a universal roman numeral designation was adopted for use by the international community, largely through the results of an international committee chaired by Dr. I. S. Wright (MacFarlane, 1976). The last step in the clotting reaction involves the conversion of fibrinogen to fibrin. The fibrinolytic system, which is of interest to the present discussion, is quite obviously related to the clotting system, and in a way, represents its physiological converse. The fibrinolytic system acts to dissociate previously formed blood clots, or to prevent their formation in the first place. The relationship between these physiological systems is still not very well understood, but their functions clearly have enormous consequences for the health and well-being of the organism. There is a fairly widely held, but unproven, hypothesis that the fibrinolytic system is in a dynamic equilibrium with the clotting system.

In its barest essentials, the fibrinolytic system is represented in Fig. 8.1. Plasminogen is a heat-stable plasma protein, the molecular weight of which has been reported to be as low as 81,000 and as high as 143,000. Its activation to plasmin, the active fibrinolysin, is a proteolytic reaction, analogous to the activation of trypsinogen to trypsin, pepsinogen to pepsin, etc. A limited number of peptide bonds are split in the activation reaction, and the reaction is irreversible. Activation is effected by so-called plasminogen activators, which may function in one of three ways:

- Acting directly on plasminogen e.g. tissue activator, and urokinase from urine
- Proteolytic enzymes acting normally e.g. trypsin and plasmin itself
- By converting normally inert proactivator to activator e.g. streptokinase (SK) and tissue lysokinase

Natural activators occur in blood plasma and in tissues. The tissues contain a soluble activator as well as a much more insoluble one. The latter may be extracted with thiocynanate and has been called fibrinokinase. Activator may be found in milk, tears, saliva, bile and semen, but not in sweat. In addition, there are known to be naturally-occurring anti-plasmins and anti-activators. The active plasmin (fibrinolysin) is responsible for the proteolytic degradation of fibrin *in vivo*.

The incoagulability of menstrual blood is apparently due to the presence of activator. Menstrual discharge has no fibrin; it contains activator, plasmin, large amounts of proactivator, and no plasminogen (Albrechtsen, 1956). Using

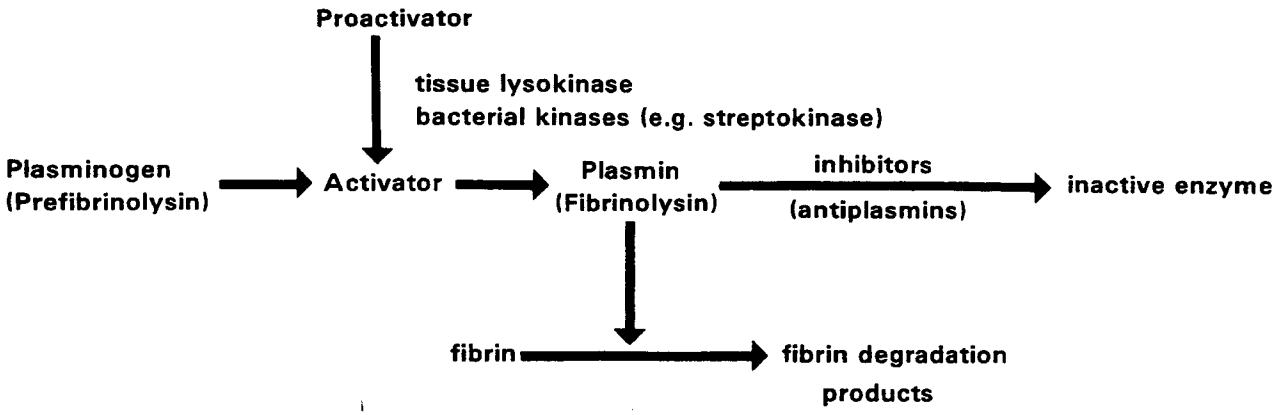


Figure 8.1 The Fibrinolytic System

histological techniques, Luginbuhl and Picoff (1966) found that proactivator was present throughout the endometrial stroma at all stages of the menstrual cycle, but that the presence of activator was confined to the superficial layers of tissue at around the time of menstruation. There was little activator present during other phases of the cycle, and virtually none at midcycle. Beller (1971) noted that the fibrinolytic breakdown products present in menstrual discharge showed the characteristics of fibrinogen degradation products, rather than of fibrin degradation products.

In 1949, Popielski had noted that the absence of fibrin threads in a bloodstain would indicate that it was not of menstrual origin, but said that this simple device could not be used if the stains had soaked into any type of absorbent substrate. Berg (1954) was apparently the first medico-legal investigator to take advantage of the fibrinolytic activity of menstrual blood for identification purposes. His method consisted of incubating suspected stain extract with fresh human fibrin at 37° for 24 hrs or so. At the end of the incubation period, a micro-Kjeldahl assay for nitrogen was carried out to assay for the fibrinolytic products. If the value was sufficiently different from a nonfibrinolytic control, menstrual blood could be diagnosed. This method was also discussed by Berg (1957b), and in this latter paper, he said that he had been using this method since 1952.

In 1959, Culliford reported an electrophoretic method based on the same principle as Berg's assay, but easier to carry out in terms of product detection. A strong extract of suspected stain was made, along with a similarly strong extract of capillary bloodstain as control. These samples (30 $\mu\ell$ minimum) were incubated with human fibrin at 37° for 24 hrs. 20 $\mu\ell$ of each sample was then applied to Whatman #1 filter paper and electrophoresed at 120–150 V for 16 hrs. The bridge buffer consisted of 33 g sodium barbital, 19.5 g sodium acetate, 205 mℓ of 0.1N HCl, all in a 3ℓ final final volume with water. The strips were dried at 105° and stained with azocarmine B. An extra band, due to the fibrinolytic products, could usually be seen in the menstrual blood samples. The presence of the band was noted to be variable, however, even in known menstrual bloodstains, and samples were encountered in which the band was absent altogether. Kamimura (1961) confirmed Culliford's observations using almost the same technique, except that he employed bromphenol blue as a protein stain. Positive results were obtained in stains up to 6 months old, and in putrefied bloodstains, but bloodstains that had been heated to 60° for 30 min, or that had been in water, gave negative results. Kamimura also noted that bloodstains from blood shed at parturition in induced abortions was indistinguishable from menstrual blood by this method.

In 1947, Permin first reported the use of what came to be called the fibrin plate method for the assay of fibrinolytic activity. The method was refined by subsequent workers (Astrup and Müllertz, 1952; and others), and can be used for the assay of any of the components of the fibrinolytic system. The method consists simply of preparing purified fibrinogen, and suspending a quantity of it in buffer in the presence of a small amount of thrombin. This mixture is placed in a Petri dish, and allowed to form a gel. The material whose fibrinolyitc activity is to be tested is placed on the surface of the plate, directly on the gel. After incubation, usually about 24 hrs at 37°, the digested fibrinogen can readily be seen. If semiquantitative results are wanted, the sample can be placed on the gel in such a way that the digested area is amenable to measurement. It has been common practice to use beef or ox plasma to prepare the purified fibrinogen. In 1962, Shiraishi utilized this method to demonstrate the fibrinolytic activity of menstrual blood, and to identify menstrual bloodstains. The fibrin plate was prepared by dissolving 1 mℓ of fibrinogen solution in 2 mℓ barbiturate buffer (0.1M, pH 7.8) and 20 $\mu\ell$ thrombin in a small (4.5 cm diameter) Petri dish, shaking gently for 3–5 sec to mix, and allowing formation of the white gel. The plate was then incubated 30 min at 37° before use. The fibrinogen was prepared from beef plasma, washed, and dissolved in the barbiturate buffer. In the first paper (1962a), Shiraishi

showed that menstrual blood serum contained large amounts of plasmin, it being able to dissolve the fibrin plate even at dilutions as high as 1:1000. The maximum dilution of menstrual blood serum which would dissolve the plate varied with the day of the menstrual period on which the sample was collected. The 1:1000 value occurred on the second day and was the highest, while the lowest dilution, 1:100, occurred in samples from Day 5. It could similarly be shown that circulating blood was always negative in the fibrin plate test unless streptokinase was added (see Fig. 8.1). In the presence of streptokinase, circulating blood serum would dissolve the fibrin plate at dilutions as high as 1:640,000. Similarly, streptokinase greatly increased the dilutions at which menstrual blood serum would dissolve the plate. The second paper (1962b) dealt with the identification of menstrual bloodstains. One thread from a 1 cm^2 stain was adequate to demonstrate fibrinolysis on the plate. The technique was extremely simple: the thread was placed onto the fibrin plate surface, the plate incubated, and examined. Stains that were kept two years at room temperature gave positive results, as did stains left a month under water and stains heated to 70–100° for an hour. Shiraishi said that neither blood shed at abortion or delivery, nor lochial blood gave a positive test.

Considerably less encouraging results were obtained by Schleyer (1963) in his study of the fibrin plate techique for menstrual blood identification. Only about half the 39 menstrual bloodstains examined gave positive results on the fibrin plate described by Astrup and Müllertz (1952). The presence of natural plasmin inhibitors was discussed as a possible reason for the false negative results. Schleyer was, therefore, less enthusiastic about the techique than Shiraishi had been, and stated that the results only had any meaning when they were positive. In 1973, Whitehead and Divall conducted experiments to determine the amounts of "soluble fibrinogen", that is, high MW fibrinogen breakdown products, in menstrual blood. They used a tanned red cell hemagglutination inhibition immunoassay (TRCHII) (Fox *et al.*, 1965; Merskey *et al.*, 1966; Hahn, 1974). It was found that menstrual blood contained significantly more soluble fibrinogen, expressed as a percentage of total protein, than did capillary blood. Mixtures of capillary blood and semen or capillary blood and vaginal secretions in stains were also higher in soluble fibrinogen than capillary blood alone, but not has high as menstrual blood stains. Whitehead and Divall continued this work (1974), conducting an immuno-electrophoretic study of the controlled degradation of fibrinogen and fibrin by plasmin, and its applicability to the identification of menstrual bloodstains. Their experiments were based on earlier work by Nussenzweig and Seligmann (1960), who carried out the first systematic immunoelectrophoretic study of fibrinogen and fibrin degradation by plasmin, and by Berglund (1962) and Dudek *et al.* (1970). The last-mentioned investigators used CM-Cellulose chromatography to separate the products. Marder (1968 and 1971) has also studied the problem extensively. The scheme for the degradation of fibrinogen which emerges from these studies is indicated in Fig. 8.2 (Marder, 1971).

Whitehead and Divall's experiments gave results in accord with the scheme in Fig. 8.2. The first stage of degradation (fibrinogen conversion to Fragment X, plus A, B and C) was reached after 4 min digestion. Fragments Y and D could be seen after 10 min of digestion, and after 45 min, Fragment Y had disappeared and only Fragments D and E were visible. The digestion of fibrin was studied as well, and followed a pattern quite similar to that of fibrinogen, except that the rate was slower. Fragments A, B and C do not react with the antiserum to fibrinogen, and thus are not detected in this immunoelectrophoretic system (Nussenzweig and Seligmann, 1960). 102 menstrual bloodstains were examined in the system. First stage degradation products were found in 56, and second stage products in 18. No third stage degradation patterns were observed, and 28 stains gave no precipitin arcs at all. The absence of precipitin arcs in 28 of the 102 stains, it was said, might be due to the low levels of degradation products in some stains. The predominance of Stage I and Stage II patterns, and the absence of Stage III ones, in the positive sample might be explained by the intervention of plasmin inhibitors present in endometrial tissue. 24 samples of circulating bloodstains were tested, and none gave precipitin arcs.

8.1.3 Immunological methods

A few attempts have been made to prepare specific antisera for the differentiation of menstrual blood. Sudo (1957) reported that he could prepare antisera to menstrual blood in rabbits or sheep, and by suitable absorptions, obtain an antiserum which detected menstrual blood in stains up to 2 years old. Domestic fowl were not suitable for making this antiserum, he said, because the anti-hemoglobin titer was too high. Harada (1960) made antisera to human and horse fibrinogen. The anti-human serum reacted with menstrual blood, and not with capillary blood, but it also reacted with the blood of victims of sudden death.

8.1.4 Methods based on menstrual blood toxicity

There have been a few reports that menstrual discharge contains various toxins, and a few investigators have indicated that these might be used as the basis for a test for the presence of menstrual blood in stains. In 1927, Böhmer noted that menstrual blood contained a substance which greatly retarded the growth of seedlings of *Lupinus mutabilis* plants (1927a). Neither cord blood nor cadaveric blood had this effect. He further indicated (1927b) that menstrual blood inhibited glucose fermentation by yeast cells, but cord serum showed this effect as well. Yamaguchi (1958) reported that menstrual blood serum contained a substance that was toxic to mice, and that the concentration of the toxin increased during successive days of the menstrual period. It does not appear that Yamaguchi was describing the same substance that had been reported earlier by Smith and Smith (1940 and 1944). The latter toxin resided not in

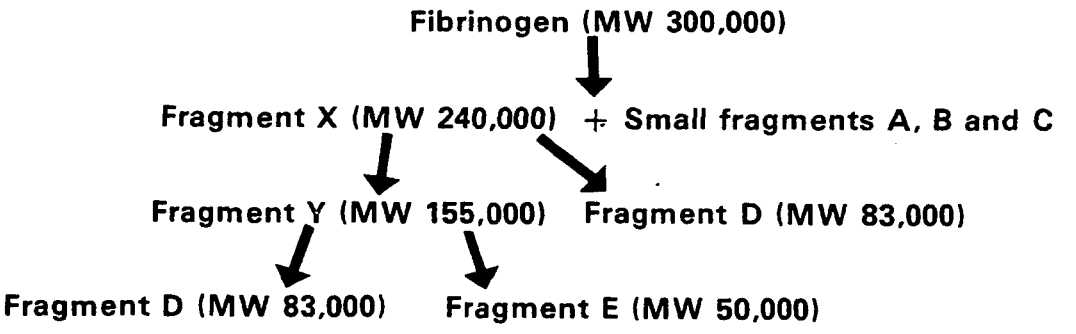
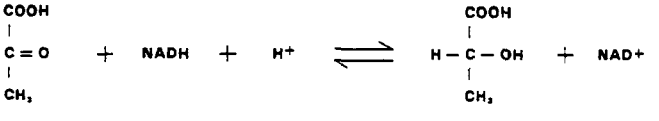


Figure 8.2 Fibrinogen Degradation by Plasmin (after Marder, 1971)

the menstrual blood, but in the endometrial cells and debris of the menstrual discharge. It had a toxic effect in rats, primarily female rats, that depended on the time of estrus of the rat at which the injections were given. There do not seem to have been many additional studies on these menstrual blood toxins. There has been very little interest in them as the basis of identification tests for menstrual blood, and hence no attempt has been made to review this leterature in detail.

8.1.5 Lactic dehydrogenase isoenzyme determination

Lactic dehydrogenase (or LDH) was the first enzyme that was recognized as exhibiting multiple molecular forms, or isoenzymes. Isoenzymes will be discussed in more detail in a subsequent unit. They comprise an extremely important category of genetic markers in blood and body fluids, and more will be said about LDH in the introductory discussion of the unit. LDH is systematically called L-lactate:NAD oxidoreductase (EC 1.1.1.27), and catalyzes the reversible conversion of pyruvic acid to lactic acid with NAD as cofactor:



LDH is an extremely important branchpoint enzyme in carbohydrate metabolism. The metabolic route followed at the level of pyruvate determines whether the cell will oxidize the starting material, glucose, to the level of lactate (or ethanol), or through the Krebs Cycle and the mitochondrial electron transport chain to the level of CO_2 and water. At pH 7, the equilibrium in the reaction as written above lies far to the right. The enzyme is a tetramer, consisting of four polypeptide chains. Two types of polypeptide chains can be present in the intact molecule. One of these, which is characteristic of muscle, is designated "M", while the other, which predominates in heart, is designated "H". Using M and H chains to form tetrameric molecules, the possible structures for LDH become: H_4, H_3M, H_2M_2, HM_3 and M_4. These five structurally different, but catalytically identical molecules constitute the five isoenzymes. LDH is found in most tissues, and all could theoretically contain the five isoenzymes. It is found, however, that different isoenzyme patterns tend to be characteristic of different tissues. In pathological states, the affected tissue sometimes synthesizes abnormal quantities of its characteristic isoenzymes, and these can be found circulating in plasma. Such altered patterns from the serum of patients can be of diagnostic value, and are used in clinical situations.

LDH isoenzymes differ sufficiently in charge at moderately alkaline pH that they are readily separated by electrophoresis on a variety of support media. At pH 8.6, the proteins migrate anodically, the fastest migrating isoenzyme often being designated LDH-1, the next-fastest LDH-2, and so forth. The most cathodic (slowest) band is called LDH-5. In 1971, Asano *et al.* reported that the LDH pattern of menstrual blood differed from that of peripheral blood, in that the LDH-4 and LDH-5 isoenzymes were markedly elevated. Electrophoretic separations were carried out on Cellogel at pH 8.6. A similar pattern, though less intense, was seen in blood shed at delivery and in cadaveric blood. Stains up to 2 weeks old could be diagnosed by this technique. Asano *et al.*(1972) thought that the source of the LDH-4 and LDH-5 isoenzymes was either the leucocytes or the endometrial tissue debris, or both. If densitometric methods were used, the increased intensity of the sum of the LDH-4 and LDH-5 was considered characteristic. These results were confirmed by Dixon and Gonsowski (1974), who carried out the separations on Sartorius cellulose acetate membranes in veronal buffer at pH 8.6.

In 1978, Stombaugh and Kearney conducted extensive studies on the use of elevated LDH-4 and LDH-5 as a means of discriminating peripheral, menstrual, cadaveric and other types of blood from one another. The LDH enzymes were separated on cellulose acetate membranes, stained for activity and the isoenzymes quantitated densitometrically. Both the range and the mean of LDH-4 and LDH-5 as a percentage of total LDH activity in peripheral, cord, cadaveric

and menstrual bloods were studied. There was considerable overlap in the ranges of activity seen in cord, cadaver and menstrual bloods, especially the latter two. Further, there was variation in the LDH-4 and LDH-5 activities in cadaver blood depending upon the cause of death. In cases of wounds with associated tissue damage, the levels tended to be higher. In four cuttings from the same menstrual bloodstain, the LDH-4 and LDH-5 activity percentages varied from 9.1 to 33.3%. Stombaugh and Kearney concluded that the use of LDH-4 and LDH-5 isoenzyme levels was not satisfactory in the discrimination of blood specimens from different sources.

8.2 Identification of Retroplacental Blood, Blood Shed at Parturition and the Forensic Diagnosis of Pregnancy in Bloodstains

As in the case of menstrual blood identification, there may be occasions on which it is desirable or necessary to try to identify bloodstains as being of retroplacental or lochial origin, of having come from a pregnant woman, or of having been shed at abortion. A number of methods and techniques have been proposed for these situations, most of which take advantage of hormones, or of various proteins present only during pregnancy and/or the puerperium. These kinds of blood are closely related, as are many of the substances thought to characterize them, so they are considered here all together.

8.2.1 Methods based on the pregnancy hormones

During pregnancy, the trophoblastic cells of the placenta secrete a hormone, similar to the pituitary gonadotropins, which is called human chorionic gonadotropin, or HCG. The hormone is believed to be physiologically important in maintaining the integrity of the conceptus during the first trimester of pregnancy, and may be found in detectable amounts in the serum and urine of pregnant women from very early in pregnancy through parturition.

One of the oldest tests for pregnancy is the so-called Aschheim-Zondek test. In this test, an ether extract is made of the urine of the pregnant woman, evaporated down, and redissolved in buffer. This material contains HCG, and if injected into immature female animals, will cause abnormal ovarian development to occur within a few days. The injected animals are sacrificed in the test, the ovaries examined at autopsy, and compared with a non-pregnant-urine-injected control. In 1932, Goroncy reported that, with minor modifications, the technique could be used to detect the presence of the hormone in extracts of blood or urine stains. Mice were used as test animals. He thought that the technique could be of value in forensic investigations on occasion, but did point out that he had sometimes observed false negatives.

In 1948, Berg employed a similar bio-assay in attempting to differentiate between blood shed at normal delivery (retroplacental) and blood shed at abortion. The assay was based on the experiments of Friedman (1932), who had noted that post-partum rabbits could be used as test animals in much the same way as immature animals. Berg obtained positive results from both types of blood, although the amounts of material required (an amount of stain containing the equivalent of 2–3 mℓ blood) were large by present-day standards. His results were apparently always positive with retroplacental blood, but variable in the case of abortion blood. The variability was a function of the progress of the pregnancy at the time of the abortion, and whether the blood being examined was shed prior to or after the expulsion of the fetus.

A more recent assay technique for HCG is hemagglutination inhibition. It is known that, under certain conditons, red cells can be sensitized with various proteins. If the cells are then incubated with specific antisera to the protein, agglutination occurs. If, however, the antiserum is incubated with an antigen containing solution prior to adding the sensitized test cells, some or all of the antibody will be bound, and subsequent agglutination will be correspondingly inhibited. This method will be discussed in more detail in a subsequent section (section 16.3). In the situation presently being discussed, red cells may be sensitized with HCG and used as test cells. An extract of the suspected stain, and a control extract, are incubated with a suitable titer of anti-HCG. Test cells are then added, and agglutination read after a suitable waiting period. Inhibition of agglutination, relative to the control, means that the test material reacted with some or all of the antisera, i.e., that it contained HCG. Semi-quantitation of the HCG may easily be had by carrying out the test on a series of doubling dilutions of the antiserum, i.e., a titration.

Abelli *et al*. (1964) utilized this procedure to try to diagnose bloodstains from terminated pregnancies. They employed a commercial test kit in which sensitized cells and antisera are provided. Stains were found to give positive reactions for short periods following pregnancy termination. In stains stored at 4°, positive results could be obtained in all samples up to 13 days old, and in some up to 18 days old. Stains stored at room temperature gave positive results only up to 8 days of age, and if kept at 60°, gave positive results only up to six days of aging. HCG is thus heat-labile in the stain. Tesar (1967), using a different kind of commercial test kit based on the identical principle, showed that positive results could be obtained from bloodstains up to 20 days old from pregnant women. He occasionally got positive results on stains as much as 3 months old. Göring *et al*. (1968) did a study of the effect of the presence of cleansing agents in textiles on the ability to detect HCG in bloodstains from pregnant women by the hemagglutination-inhibition technique. Stains deposited on fabrics that contained the washing agents were tested weekly for HCG. Two detergents were tested. In one case, detection was possible to 5 weeks, and in the other, to 7 weeks. The control stains gave positive results to 3 months of age. The agents tested were called "Fit" and "Persil", and were apparently marketed in Germany. Low-Beer and Lappas (1980) have recently described a crossed electroimmunodiffusion procedure for

detecting HCG in bloodstains representing 100–200 μl blood. The hormone could be detected in bloodstains from pregnant women from 45 days following the last menstrual period to parturition.

8.2.2 Methods based on pregnancy-associated proteins

The subject of pregnancy-associated proteins (as distinct from pregnancy-associated enzymes, which are considered in sections 8.2.3 and 8.2.4) was opened by Smithies in 1959. In some 10% of serum samples from recently delivered women, or women in late pregnancy, a protein band was observed in the haptoglobin region following starch gel electrophoresis. This observation was confirmed by Afonso and De Alvarez (1963) who noted that the protein was not equivalent to cystine aminopeptidase. They also pointed out that Giblett had independently observed the protein at about the same time as Smithies. Smithies called the region of the gel to which the protein migrated the "pregnancy zone", and the term "pregnancy zone protein" has persisted. The protein is especially characteristic of the 2nd and 3rd trimesters of pregnancy. Afonso and De Alvarez (1964) found the protein in 10% of sera from the first trimester, 69% of those from the 2nd, and more than 80% of those from the third trimester. The protein was found to be an α_2-globulin, and was not observed prior to the ninth week of gestation. It was not identical to ceruloplasmin, thyroxin-binding protein, nor transcortin.

Following the initial observations, the subject became somewhat complicated because, not only were additional pregnancy-associated proteins discovered, but many of the observations were independent, each group applying its own separate nomenclature. Some of the confusion has recently been cleared up, but the field is very active, and rapidly developing,

In 1966, Bundschuh reported a new antigen in serum, detected by variously absorbed horse immune antisera, which could be found in almost 78% of women, but in only about 22% of men. The antigen appeared to be inherited, but its expression was under the influence of sex hormones in some way. This antigen was named "Xh". MacLaren *et al.* (1966) found a similar protein, and called it Pa-1. Kueppers (1969) studied Xh further, noting that he had found it in 97% of women and 88% of men, using anti-female-serum serum absorbed with pooled male serum. He also found that the purified protein had a sedimentation coefficient of 12.2S and an isoelectric point of about 4.75. Dunston and Gershowitz (1973) studied the antigen and found that, in addition to the sex dependence of its expression, there was some age dependence of expression in females as well. They argued for the Xh designation, saying that the "Pa-1" designation implied more about the protein in terms of standard nomenclatures than was actually known.

In 1969, Margolis and Kenrick reported that "pregnancy zone protein" occurred not only in pregnant women, but also in those taking estrogenic oral contraceptives. They found that the protein had a MW of 450,000. Beckman *et al.* (1970) found that the protein was not present in cord blood, and they confirmed (1971) that women taking estrogenic oral contraceptives had the protein in their sera. Bohn (1971) reported that four proteins could be detected in pregnant serum by immunodiffusion with antisera made from placental extracts. One of these was identical to human placental lactogen (HPL). Two others were β_1-glycoproteins and one was an α_2-glycoprotein. One of the β_1-glycoproteins was pregnancy-specific, occurring in urine and in serum. The other β_1-glycoprotein and the α_2-glycoprotein could be found in non-pregnant serum but were elevated in pregnancy or in the presence of estrogenic oral contraceptives. The α_2-glycoprotein, Bohn said, was identical to pregnancy zone protein, and to Xh. In 1972, having found that the nonpregnancy-specific β_1-glycoprotein and α_2-glycoprotein were elevated in some disease states as well, he proposed to name them β_1-AP-glycoprotein and α_2-AP-glycoprotein, where "AP" stood for "acute phase". By 1973, Bohn was referring to the pregnancy-specific β-glycoprotein as "SP_1", while the non-pregnancy-specific β-glycoprotein was "SP_2" and the α_2-glycoprotein was "SP_3". "SP" in these designations stood for "Schwangerschaftsprotein", i.e., "pregnancy protein".

By 1975, the number of different names being applied to the α_2-glycoprotein by the above-mentioned and still other authors had reached such absurd proportions that a large group of workers in the field jointly agreed that this protein would henceforth be called "pregnancy associated α_2-glycoprotein" or "α_2-PAG" (Berne *et al.*, 1975). This protein is the same one that has been called "pregnancy zone protein", pregnancy-associated α_2-globulin (Hasukawa *et al.*, 1973), pregnancy-associated globulin (Horne *et at.*, 1973), alpha-2-pregnoglobulin (Berne, 1973), pregnancy-associated α-macroglobulin (Stimson and Eubank-Scott, 1972), and has been described additionally by Than *et al.* (1974) and by von Schoultz and Stigbrand (1974). The MW has been reported to be as low as 360,000 (von Schoultz and Stigbrand, 1974; Bohn and Winckler, 1976), and as high as 760,000 (Than *et al.*, 1974).

In 1972, Gall and Halbert first reported finding four antigens in pregnancy serum by immunodiffusion using anti-pregnancy-plasma serum, exhaustively absorbed with non-pregnant plasma. These were soon named "pregnancy-associated plasma proteins A, B, C and D", or PAPP-A, -B, -C and -D (Lin *et al.*, 1973). B and C were β-globulins while A and D were α_2-globulins. The MW of A, C and D were determined to be 750,000, 110,000 and 20,000, respectively. It became clear almost immediately that PAPP-D was human placental lactogen (HPL), and that PAPP-C was identical to Bohn's SP_1 protein (Lin *et al.*, 1974a, 1974b). None of the PAPP's were equivalent to the pregnancy associated α_2-glycoprotein (Lin and Halbert, 1975), and the amounts present in the placenta were shown to be, from greatest to least, D (or HPL) > B> C> A (Lin *et al.*, 1976). PAPP-A and PAPP-C, as well as HPL, are probably synthesized by the placental trophoblast cells (Lin and Halbert, 1976). Lin *et al.* (1976) showed, finally, that PAPP-B and PAPP-D (i.e. HPL) disappear within a day of delivery.

The level of PAPP-A drops rapidly within a few days of delivery and becomes undetectable at 4–6 weeks postpartum. PAPP-C levels fall rapidly too, being undetectable at 3–4 weeks postpartum.

In summary, the proteins which have had more than one name, but which are identical, are: (1) pregnancy-associated α_2-glycoprotein, with many former names; (2) PAPP-D = HPL; (3) PAPP-C = SP_1. PAPP-A is apparently pregnancy specific, and is not immunologically identical to other known pregnancy-associated proteins. SP_2, a β-glycoprotein, remains unique, though not pregnancy-specific. Curiously, Than *et al.* (1974) reported a MW of 760,000 for their protein, which they said was identical to SP_3, i.e., α_2-PAG. All other reports of the MW of α_2-PAG have been in the neighborhood of 500,000 except that Bohn and Winckler (1976) reported 360,000 for the purified protein. The 760,000 value of Than *et al.* (1974) is very close to the MW of 750,000 reported for PAPP-A although this could certainly be a coincidence. Lin *et al.* (1974b) insisted that PAPP-A is not equivalent to any of the SP proteins. Towler *et al.* (1976) showed that the levels of HPL and of the specific β_1-glycoprotein (probably equivalent to SP_1) correlate well with gestation stage. These, as well as perhaps PAPP-A, seem to offer the best prospects for application to the forensic diagnosis of pregnancy in bloodstains.

8.2.3. Methods based on leucine aminopeptidase and cystine aminopeptidase

Leucine aminopeptidases (EC 3.4.1.1) are α-aminopeptide amino acid hydrolases, which hydrolyze L-peptides, splitting off N-terminal leucine residues which have a free α-amino group. The enzymes hydrolyze a fairly broad range of substrates, and may also show esterase activity as well. Cystine aminopeptidases are very similar enzymes, except that, as the name implies, they prefer substrates having N-terminal cystine residues. These enzymes have usually been assayed using artificial substrates, L-leu-β-naphthylamide for leucine aminopeptidase, and L-cys-S-S-cys-β-naphthylamide for cystine aminopeptidase. The cystine aminopeptidase (hereinafter, CAP) enzymes do show leucine aminopeptidase (hereinafter, LAP) activity, but LAP does not hydrolyze the cystine substrate.

In 1961, Page *et al.*, using vertical starch gel electrophoresis, noted that LAP could be detected in all sera, but that two CAP enzymes in serum, CAP_1 and CAP_2, were characteristic of pregnancy. CAP was not found in nonpregnant, nor in fetal serum. The CAP is believed to function physiologically as an oxytocinase. Oxytocin is a hormone, elaborated by the mammalian neurohypophysis. A cystine-containing nonapeptide, its function is to elicit smooth muscle contraction, as of the uterus during birth, and the ejection of milk in lactating females. It is very closely related structurally to another neurohypophyseal hormone called vasopressin. The various reports of "LAP isoenzymes" in pregnancy, in addition to the enzyme found in all normal sera, may actually represent reports of the apparently pregnancy-specific CAP enzymes (Rowlessar *et al.*, 1961; Smith and Rutenberg, 1963). This is partucularly a possibility in the studies in which CAP was not separately assayed, because, as noted above, CAP possesses LAP activity. The serum LAP pattern is also altered in various pathological states, especially in hepatic and biliary diseases (Kowlessar *et al.*, 1961). Robinson *et al.* (1966) noted that the CAP enzymes were found only in pregnant serum, and not in the sera of non-pregnant women, even those on estrogenic oral contraceptive therapy.

In 1970, Gladkikh reported that bloodstains from pregnant or puerperal women could be discriminated on the basis of an additional, slow LAP band following electrophoresis. It seems probable that the slow band represented one of the CAP enzymes. The band could be seen in bloodstain extracts from women, from the 8th to 10th week of gestation until about 30 days postpartum. The enzyme could be detected in stains up to 50 days old. The slow band was not seen in bloodstains from men or from non-pregnant women. Some fetal sera, but not bloodstain extracts from fetal blood, showed a similar but qualitatively different slow band. Oya and Asano (1971) reported very similar results after electrophoresis on Oxoid cellulose acetate membranes, noting that the enzyme appeared in the last half of pregnancy, and also characterized retroplacental blood. In 1974, Oya enlarged his studies, noting that the resolution of the usual and pregnancy-specific LAP enzymes was not terribly good on cellulose acetate membranes, but that L-methionine inhibited the usual serum enzyme, but not the pregnancy-specific one, with L-leu-β-naphthylamide as substrate. By running paired samples, and staining with and without L-met, the bands could be resolved. Oya *et al.* (1975a) employed polyacrylamide disc gel electrophoresis to examine these enzymes in serum and in placental extracts. In this study, a CAP assay was incorporated, and it was clear that the two slower bands had CAP activity as well as LAP activity, while the fastest band had only LAP activity. The fast LAP band was the enzyme found in all normal sera, was heat-stable and L-met inhibitable. The CAP bands were heat-labile, not inhibited by L-met, and seemed to originate in the placental lysosomes. The bands were detectable in bloodstains after about the 4th month of pregnancy, and in stains up to about 2 weeks old (Oya *et al.*, 1975b). It was recommended that a phosphocellulose column be employed to remove the excess hemoglobin when examining bloodstains for CAP enzymes.

8.2.4 Method based on alkaline phosphatase

Alkaline phosphatases (EC 3.1.3.1) are widely occurring enzymes with broad substrate specificities. They catalyze the hydrolysis of orthophosphoric monoesters at alkaline pH optima, and are systematically named orthophosphoric monoester phosphohydrolases (alkaline optimum). Serum contains a number of alkaline phosphatase enzymes derived from different tissues, including liver, intestine, bone, and in pregnancy, placenta. Some aspects of the alkaline phosphatase enzymes are not pertinent to the present discussion,

and are not taken up here. These include the relationship between intestinal enzyme expression and the ABO blood groups and secretor loci (Schreffler, 1965; Beckman and Zoschke, 1969), and the fact that placental alkaline phosphatase exhibits polymorphism in human beings (Beckman and Beckman, 1968; Beckman, 1970).

That plasma contains alkaline phosphatase activity has been known since the work of Kay (1930a, 1930b). Fishman and Ghosh (1967) said that the French investigator, Coryn, first noted in 1934 that the activity of the enzyme is elevated in pregnancy. The enzyme in maternal circulation during pregnancy was shown to differ from the other alkaline phosphatases in its immunological properties, K_m for various substrates, electrophoretic mobility, inhibition by L-phenylalanine and heat stability (Posen *et al.*, 1969). By 1967, it had become clear that the heat-stable alkaline phosphatase of pregnancy plasma was of placental origin (Fishman and Ghosh, 1967; Posen, 1967). The placental alkaline phosphatase is stable to at least 55° for 2 hours, is almost 90% inhibited by 10 mM L-phe, has a pH optimum of 10.6 in 18 mM phenylphosphate and a K_m of 0.51 for phenylphosphate (Ghosh, 1969).

In 1973, Oya *et al.* showed that the heat stable alkaline phosphatase could be detected in bloodstains from the blood of women in the latter half of pregnancy, the blood shed at delivery, or puerperal blood. The assay of total alkaline phosphatase and heat-stable alkaline phosphatase (that which survived 56° for 30 min), using p-nitrophenylphosphate as substrate, was recommended for the medico-legal diagnosis of pregnancy from bloodstains. Stafunsky and Oepen (1977) recommended a slightly modified, but similar procedure. Results could be obtained in bloodstains stored up to 19 months. A disadvantage of the method is the relatively large amounts of sample required. Stafunsky and Oepen extracted stains from 1 to 3 cm^2 in size. Fishman *et al.* (1972) said that the level of the placental enzyme increased exponentially in maternal serum as a function of gestation time. Thus the amount of enzyme to be expected will depend on the progress of the pregnancy. In bloodstains, the age of the stain is probably inversely related to the amount of active enzyme as well. Older stains, or stains from persons whose pregnancies have not progressed very far, or a combination of these circumstances, might, therefore, require prohibitively large amounts of sample in order to obtain unequivocal results with the usual spectrophotometric assay technique.

There is another consideration, which is of enormous importance in the interpretation of results of heat-stable alkaline phosphatase assays in bloodstains of unknown origin. Fishman *et al.* (1968a, 1968b) first described an alkaline phosphatase in the serum of a patient named Regan with a bronchiogenic carcinoma. This enzyme in every way resembled the placental enzyme, and was found in serum, in primary tumor tissue and in its metastases. The enzyme in the plasma of patients with neoplastic disease came to be called the Regan isoenzyme, and is immunologically and biochemically indistinguishable from the heat-stable alkaline phosphatase of the placenta (Fishman, 1969). Fishman has recently (1974) reviewed this entire subject excellently.

8.3 Identification of Fetal and Blood from Children

8.3.1 Fetal hemoglobin

Fetal hemoglobin (Hb F) is best distinguished from the hemoglobin of adults (Hb A) by electrophoresis. The test for Hb F is probably the simplest way of diagnosing fetal, or early childhood, blood. Before an electrophoretic method had been worked out, it was common practice to discriminate Hb F from Hb A on the basis of their differential sensitivity to alkali denaturation. Hb A is quite alkali-labile, whereas Hb F is relatively resistant, the denaturation usually being detected spectrophotometrically. This method was applicable to bloodstains (Huntsman and Lehmann, 1962; Culliford, 1964; Watanabe, 1969). Pollack *et al.* (1958) successfully separated Hb A from Hb S (sickle-cell hemoglobin) in a medico-legal case in Massachusetts using paper electrophoresis, and suggested that it would be very desirable to have such a method for the separation of Hb A and Hb F as well. Wraxall provided such a method in 1972, which was simple, reliable and was performed on Sartorius cellulose acetate membranes. Wilkens and Oepen (1977a) fully confirmed the usefulness of Wraxall's method with bloodstains from 160 cord blood specimens on glass, wood, paper and textiles. The technique is also fully described by Culliford (1971). Immunological methods, using anti-Hb F, have been employed as well (Baxter and Rees, 1974b), and were fully discussed in Section 7.1. It must be noted that there are a few adults whose red cells contain abnormal amounts of fetal hemoglobin, and this fact must be kept in mind in interpreting the results of tests for Hb F.

8.3.2 Methods based on α_1-fetoprotein

In 1956, Bergstrand and Czar reported that they had found a high concentration of a protein in fetal serum that did not occur in maternal serum. This protein eventually became known as α_1-fetoprotein, or α-fetoprotein. Nishi and Hiraki (1971) reported that the protein had a MW of 64,600 and gave its amino acid composition. Masopust *et al.* (1971) said that the MW was 76,000, the isoelectric point 5.08, and that the protein had no associated sialic acid or lipid. The protein occurs normally in fetal serum, but is found in the serum of adult patients suffering from malignant tumors, especially hepatomas. It is clinically useful in the latter regard as a diagnostic tool (Abelev, 1971).

Patzelt *et al.* (1974) detected α-fetoprotein in bloodstains by what they referred to as "immunoelectroosmophoresis" (crossed-over electrophoresis), using a specific rabbit immune serum. The test was positive in bloodstains stored up to 3 months, and was carried out on 1% agar gels in veronal-sodium acetate buffer at pH 8.1. Thomsen *et al.* (1975) noted that there was a definite, inverse relationship between gestational age and the serum concentration of α-fetoprotein, and that this fact could be used as an aid to the

estimation of fetal age in medico-legal investigations. Wilkins and Oepen (1977b) showed that α-fetoprotein could be detected in fetal bloodstains up to 8 months old by crossed-over electrophoresis on Biotest cellulose acetate membranes. Stains on glass, wood, paper and textile substrates all gave positive results.

Pietrogrande *et al.* (1977) have described a quantitative immunoelectrophoresis (rocket electrophoresis) procedure for α-fetoprotein with a high sensitivity at serum concentrations of about 50 $\mu g/\ell$. Breborowicz and Majewski (1977) gave a direct radio-immunoassay procedure which detected 2 to 500 ng/mℓ of the protein in 20 $\mu\ell$ serum samples. Katsumata *et al.* (1979) used a commercially available RIA assay to detect alpha-fetoprotein in bloodstains. They said that this assay would detect from 21 to 320 ng per 9 mm^2 stain, and that the protein was detectable in stains on filter paper 1 month old. Tomasi (1977) has recently reviewed the subject of alpha-fetoprotein.

8.3.3 Miscellaneous methods

It is not intended to discuss the serum Gc (group specific component) polymorphism here, but it may be mentioned that Forster and Joachim (1968) reported that there were occasional differences in the Gc 2-1 immunoelectrophoretic patterns between the blood of adults and that of newborn or young children. The differences were such that the "variant" pattern could be mistaken for a Gc 1-1. They thought that antisera raised in goats were the best for discriminating the patterns. Occasionally, differences were observed in the Gc 2-2 patterns between adult and children's blood as well, but no differences were detected in the Gc 1-1 types.

In 1975, King and Whitehead reported that it was possible to identify with reasonable confidence the blood of persons over 15 years of age by virtue of natural antibodies that were present in sera. These antibodies reflected the individual's acquired immunities, and were expected, therefore, to be age-dependent. They tested for antibodies to *Mycobacterium tuberculosis, Vibrio cholerae, Candida albicans* and *Treponema pallidum,* using an indirect fluorescent antibody technique. The antigen was fixed on a slide, and incubated with bloodstain extract. If the serum of the bloodstain extract contained antibodies to the antigen being tested, binding would occur. The samples were then washed, and fluorescent-labelled antihuman IgG was added. This labelled antibody would bind to any IgG antibody which was bound to antigen being tested, and which would have to have been present in the bloodstain. After washing away excess antihuman IgG, the slides were examined in a fluorescence microscope. Good correlations with the age of the bloodstain donor were found for *M. tuberculosis* and *V. cholerae*, but not with *C. albicans* or *T. pallidum.*

SECTION 9. DETERMINATION OF THE AGE OF BLOODSTAINS

Aging bloodstains tend to undergo characteristic changes, and various attempts have been made to devise methods for estimating the elapsed time between the deposition of a bloodstain and its analysis in the laboratory. Although this information would be exceedingly valuable in some instances if it could be reliably obtained, there are many difficulties with all the methods. The major factor causing interpretive difficulty, regardless of the method chosen, is that a variety of different environmental factors, including heat, light, humidity, washing, putrefaction and the presence of contaminants, influence the rate at which the changes occur.

The majority of methods that have been suggested have to do with the transformations of hemoglobin into its derivatives, and the changes in color and solubility which accompany them. Tomellini (1907) devised a color chart with which unknown stain color could be compared to get an approximate estimate of stain age. Leers (1910) used solubility as a guide, noting that fresh stains dissolve readily in water and show an HbO_2 spectrum, while day old stains dissolve slowly and show the spectra of metHb. Older stains become water-insoluble but will dissolve in 2% KOH (week-old) or 33% KOH (month-old). Rauschke (1951) used water and 2% KOH solubility as guides to age. Minette (1928) tested for solubility in saliva, water and glycerine:water::1:4 at 37°, and correlated the solvation time with stain age. Bujan (1948) studied the Hb-metHb transformation in dried stains, noting that it could be complete within 2–3 hrs at ordinary temperatures, and even faster at higher temperatures. He tried to correlate the luminescence intensity of luminol in NaOH and Na_2CO_3 (in the presence of H_2O_2) with the age of the stain (see Section 6.7). Patterson (1960) showed that reflectance measurements carried out on experimental stains on filter paper, using a standardized color index, correlated with the stain age. The greatest rate of change in color occurred in the first few hours, decreased somewhat up to about 72 hours age, and was fairly gradual after that time. He noted that the role of environmental influences to which an unknown stain may have been subjected was a complicated matter, and that a variety of "standard curves" would have to be constructed if the method were to be employed in actual practice.

Schwarzacher (1930) proposed a method wherein stains were artificially aged under UV light. A third of the stain was irradiated for 30 min, the other ⅔ being covered. A second third was then irradiated along with the first third, the last third being non-irradiated. Color comparisons could then be made between the artificially aged and non-irradiated zones, and curves constructed as standards to be used with unknown stains. Rauschke (1951) said that the method was fairly precise for stains that were quite fresh, but that information about environmental influences would be needed.

Weinig (1954) applied a method to blood and semen stains that had been used by Metzger *et al.* (1931) to determine the age of inks. Bloodstains on paper and cloth can be examined in this way. The age of the stain is correlated with the progressive diffusion of Cl^- around the stain, which can be fixed as AgCl, and upon reduction forms a black border around the stain. Fiori (1962) gave the complete details of this method. A border forms around stains which are more than 2 months old, and its size increases in small increments as an approximate function of the age of the stain up to about 9 months. Humidity is a factor in this method, but aging results are independent of temperature and sunlight effects.

Schwarz (1936) determined catalase and "peroxidase" in bloodstains as a method of determining age. The "peroxidase" was determined using guaiacum, and was undoubtedly a determination of the amount of hemoglobin remaining in the stain which would catalyze the guaiacum bluing reaction with H_2O_2. In this sense, the study was very similar to that of Bujan (1948). The guaiacum blue color was said to vary inversely with stain age, as would be expected. Leucocytes possess peroxidase activity as well, and Undritz and Hegg (1959) carried out a microscopical study of peroxidases in the various leucocyte types as a function of bloodstain age (see Section 5.3).

Direct spectral determination of metHb is not very valuable in assessing stain age, because the hemoglobin is so rapidly converted to metHb (Bujan, 1948). Somewhat more involved spectral methods have been proposed, in which relationships between changes at several wavelengths have been studied. Funao and Maeda (1959) monitored the changes in optical density at 544 and 577 nm in the visible region, 345 nm in the near UV and 275 nm in the UV region in aging bloodstains. The visible bands decreased steadily up to 2 months of age. The UV bands decreased, and shifted to shorter wavelengths up to about 2 months of aging. The 345 nm band continued to shift for up to 3 years. These changes took place more rapidly in a lighted room than in the dark. In 1967, Kleinhauer *et al.* reported two methods of determining bloodstain age. In one, differential extractability in water as against ammonia solutions was measured, along with differential extractability in ammonia solution as against "transformation solution." The last mentioned consisted of 200 mg KCN and 200 mg potassium ferricyanide in 1ℓ H_2O, and effected the transformation of hemoglobin the stain to metHbCN. The differential extractabilities were expressed as ratios which they called "quotients," and these could be related to stain age. Both

quotients were higher in fetal bloodstains than in adult bloodstains. In the other method, the ratio of the OD of the 540 nm peak to the 500 nm trough in metHbCN, obtained by treatment with "transformation solution," was correlated to stain age up to 15 weeks. Kind *et al.* (1972) proposed a method whereby the spectrum of the stain was determined in the visible region from 490 to 610 nm. Vertical perpendicular lines were then dropped at wavelength values of 490, 560, 578 and 610 nm, and the points of intersection of the perpendiculars with the spectral trace at 490 and 610 connected by a straight line (as shown in Fig. 9.1). Points of

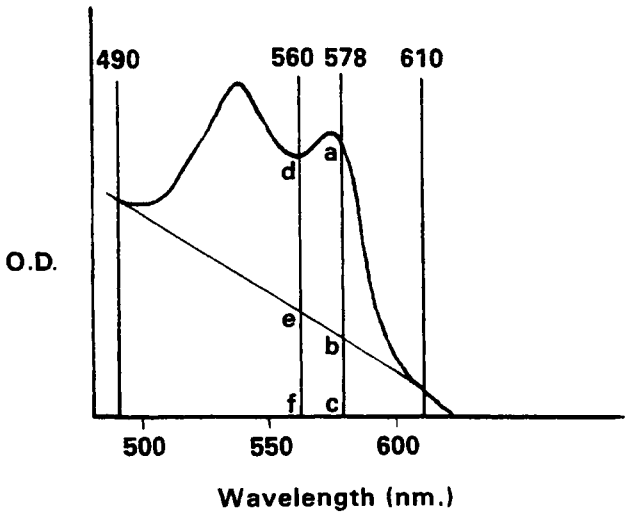


Figure 9.1 Scheme for Determination of α- ratio (after Kind et al., 1972)

intersection were identified by letters as shown, and a quantity called α could be calculated, according to:

$$\alpha = \frac{OD_{ac} - OD_{bc}}{OD_{df} - OD_{ef}}$$

where OD_{ac}, OD_{bc}, etc., represented the changes in optical density represented by line segments ab, bc, etc. The α ratio was found to decrease in some 200 bloodstains, which had been stored at room temperature in the dark for up to 8 years. The ratio underwent a change of from about 1.50 to about 0.80 over the course of some 10^5 hours of aging. There was considerable scatter in data points from samples of the same age group, however. An improved ratio, called α_s, was proposed by Kind and Watson (1973). α_s is calculated in precisely the same way as is α, except that two of the wavelengths at which the perpendiculars are dropped are changed as shown in Fig. 9.2. The spectra were determined in ammoniacal extracts of bloodstains, rather than directly in stains mounted in paraffin, as had been done in the 1972 experiments. The α_s ratio was seen to decrease in a consistent way for bloodstains aged up to 15 years, and there was less scatter in samples of the same age group. Köhler and Oepen (1977) reported a lengthy series of experiments on 85 blood-

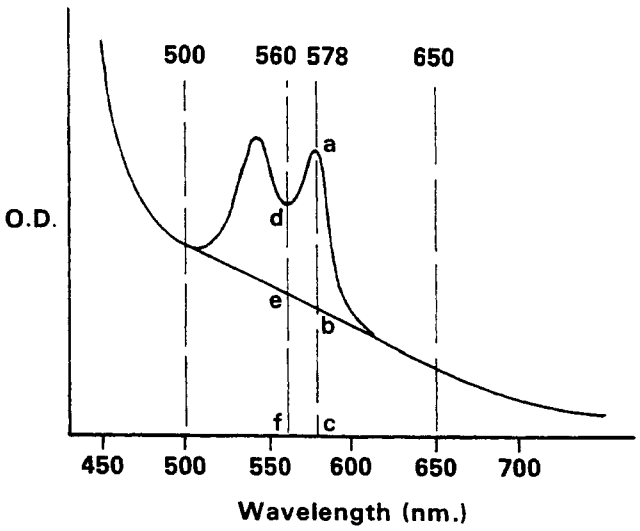


Figure 9.2 Scheme for Determination of α_s - ratio (after Kind and Watson, 1973)

stains on various substrates aged up to 5 years in which the methods of Kind *et al.* (1972) and of Kleinhauer *et al.* (1967) were tested. It was found that the variations of the parameters defined by the previous workers within an age group exceeded those between samples of different age groups. Köhler and Oepen concluded, therefore, that neither of these methods was suitable for reliable estimation of bloodstain age in practice.

Nuorteva (1974) pointed out that in decaying samples, which are found to be maggot-infested, an estimate of the age of the material could be made on the basis of knowledge of the length of the life cycle stages of the insects whose larvae were present.

Rajamannar (1977) looked at the serum protein profile by immunoelectrophoresis in stains as a function of their age, from 15 days to one year. The presence of precipitin arcs representing γ-, β_2M-, β_2C-, β_2B-, β_1-, α_1- and α_2-globulins and albumin was followed with time. A characteristic pattern of disappearance of various proteins at test points along the time line could be constructed. Albumin, α_1- and α_2-globulins were absent at 15 days of age. At 30 days, β_2M-globulin disappeared, with β_2C-globulin disappearing at 60 days, β_2B-globulin at 150 days, and β_1-globulin at 300 days. All the proteins were undetectable at 365 days of age. It should be pointed out that Sensabaugh *et al.* (1971) found albumin to be detectable by its immunological reaction in a dried blood sample eight years old.

The apparent disappearance of albumin in a comparatively fresh bloodstain in Rajamannar's (1977) experiments can be accounted for on the basis of a change in the electrophoretic mobility of albumin in aging bloodstains. Beginning almost immediately when blood dries, and progressing steadily with time, the amount of albumin which appears at the "albumin position" on electrophoretic support media de-

creases, while there is an apparent increase in the amount of "gamma globulin." Using monospecific precipitating sera, it can be shown that albumin does not denature; rather it migrates differently, and appears at the γ-globulin position. Quantitation of the amount of protein detectable with precipitating antisera in the "albumin" position and the gamma globulin "position" indicates that the ratio is approximately proportional to the age of the stain from which the extract was obtained. A preliminary account of this work has appeared (Lee and De Forest, 1978). Further studies are in progress in our laboratory, using two-dimensional immunoelectrophoresis (Laurell electrophoresis technique), to gather additional data about this interesting change in the albumin molecule (Lee, H.C., R. E. Gaensslen, B. Novitch and R. Fossett, in preparation). The alteration in electrophoretic mobility of albumin, and perhaps of other serum proteins, does not appear to be restricted to aging bloodstains. We have noticed that it can occur in aging serum samples as well, and others (e.g. Heftman *et al.*, 1971) have reported the same effect. Antisera specific for a particular serum protein must be used in examining bloodstain extracts because one cannot use the electrophoretic mobilities of these proteins in fresh serum as a guide to identity.

Shinomiya *et al.* (1978) used immunoelectrophoresis to estimate the age of bloodstains. They found that stain age could be correlated to the number of precipitin arcs detectable in stain extract following immunoelectrophoretic analysis.

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§ 1 *Hoppe-Seyler's Zeitschrift für Physiologische Chemie.* Began 1877, and published as *Zeitschrift für Physiologische Chemie* (*Z. Physiol. Chem.*) from volume 1 to volume 21.

§ 2 *Japanese Journal of Legal Medicine* (*Jpn. J. Leg. Med.*) has Japanese title *Nippon Hoigaku Zasshi*

§ 3 *Archiv für Experimentelle Pathologie und Pharmalkologie* (*Arch. Exp. Pathol. Parmakol.*), published 1873–1925, volumes 1–109 under above title. Volume 110–263 as *Naunyn-Schmiedeberg's Archiv für Experimentelle Pathologie und Pharmakologie,* often cited as *Naunyn-Schmiedeberg's Archiv.* Volume 264–271 as *Naunyn-Schmiedeberg's Archiv für Pharmakologie,* and from Volume 272 as *Naunyn-Schmiedeberg's Archives of Pharmacology.*

§ 4 *Archiv für Pathologische Anatomie und Physiologie und Klinische Medizin* (*Arch. Pathol. Anat. Physiol. Klin. Med.*), published 1847–1902, volumes 1–170, after which title changed to *Virchow's Archiv für Pathologische Anatomie und Physiologie und Klinische Medizin.* Often cited as *Virchow's Archiv,* or as *Arch. Path. Anat.*

§ 5 *Journal of the Kurume Med. Assoc.* (*J. Kurume Med. Assoc.*) has Japanese Title *Kurume Iggakai Zasshi*

§ 6 *Schweiz Wochenschrift für Chemie und Pharmacie* (*Schweiz Wochenschr. Chem. Pharm.*) has alternative title *Journal Suisse de Chemie et de Pharmacie.*

§ 7 *Vierteljahrschrift für Gerichtliche Medizin und öffentliches Sanitätswesen* (*Vierteljahrschr. Gerichtl. Med. Oeff. Sanitaetswes.*) began 1852, vol. 1 through new series vol. 15, 1871, published as *Vierteljahrschrift für gerichtliche und öffentliche Medizin.* Ended 1921, superceded by *Deutsche Zeitschrift für die gesamte gerichtliche Medizin.* Sometimes cited in the old literature as *Casper's Vierteljahrschrift.*

§ 8 *Annalen der Physik und Chemie* (*Ann. Phys. Chem.*) sometimes cited in the old literature as *Poggendorff's Annalen* (*Pogg. Annalen*).

§ 9 English edition of Nippon-no-ikai

§ 10 *Acta Criminologiae et Medicinae Legalis Japonica* (*Acta Crim. Med. Leg. Jpn.*) has Japanese title *Hanzaigaku Zasshi.* Began 1928, and had alternative German title *Archiv für gerichtliche Medizin und Kriminologie* from 1928–1944.

¶ Symbol indicates that a translation of the paper appears in Unit IX of this book