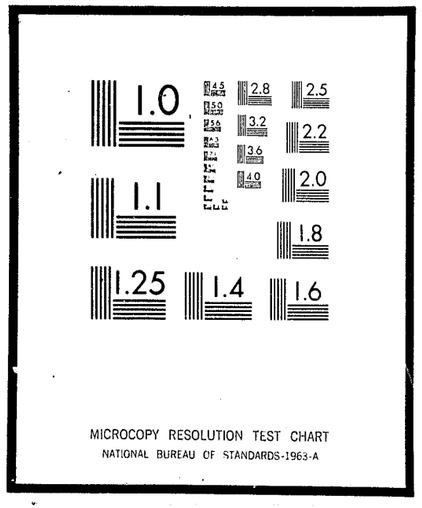

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EQUIPMENT SYSTEMS IMPROVEMENT PROGRAM

PERSISTENCE OF SELECTED GENETIC MARKERS IN DRIED BLOOD

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Approved

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Law Enforcement and Telecommunications Division

ABSTRACT

The persistence of selected genetically derived constituents in dried human blood was investigated over a six-month period. Blood drawn from 12 volunteer donors was deposited on six substrates, glass, wool, nylon, and three types of pure cotton (plain cotton, perma press, and denim), and the resulting dried blood specimens were aged at ambient temperature under 20 and 66% relative humidity conditions. A few samples were aged at -20°C. Analyses were performed at 1, 2, 4, 13, and 26 week aging periods.

Red cell antigen systems evaluated in this study included ABO, MN, Rh, Kidd, Duffy, and Kell. The antigens tested totalled 15. The most stable antigens among those investigated were A, B, and O of ABO; M, N, and s of MN; and D of the Rh system. These variants were identified in specimens that were aged for 26 weeks at the low and high relative humidity conditions. The least stable variants, which persisted for one week at either humidity level, were Jk^a of Kidd, Fy^a of Duffy, and K of Kell system. Of these antigens, only K aged at 20% humidity was detected at the two week test period. Other variants (S of MN and C, c, E, and e of Rh) persisted for varying lengths of time that ranged from 2 to 26 weeks depending on aging conditions. In particular, Rh factors, C, c, and E were adversely affected by high moisture environment. Storage of the specimens at -20°C generally resulted in a longer persistence time in comparison to those at room temperature. No significant differences in persistence due to substrate effect were observed.

Four enzyme systems also included in this study were AK, ADA, PGM, and EAP. AK and PGM polymorphs were still identifiable at the 26 week test period, and ADA and EAP at 13 weeks for the low and high humidity storage conditions. PGM polymorphs appeared to be somewhat more stable at the low humidity, and ADA and EAP at the high humidity environment. No obvious differences in persistence due to phenotype or to substrate were discernible. Again, the storage of the specimens at -20°C generally preserved the enzymes better than did aging at room temperature.

The probability for two randomly selected individuals to have the same combination of variants was calculated for each test period, based only on the persisting genetic markers included in the study, using blood frequency of occurrence data in the literature, and assuming no assay errors. Discrimination of one in 2500 is possible at one week for either humidity storage condition. At 26 weeks, however, the values are one in 65 and one in 28 for aging at ambient temperature under 20 and 66% relative humidity levels, respectively. Additional factors affecting the discrimination probability are included.

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PREFACE

The study of the persistence of genetic markers in dried blood described in this report was conducted as a part of the Blood and Bloodstain Analysis Program sponsored by the Law Enforcement Assistance Administration at The Aerospace Corporation. This program aims at the development of a blood individualization technique, based on identification of blood antigen and protein/enzyme phenotypes, and which is acceptable for use by crime laboratories and as evidence in our courts of law. In addition, blood type frequency data for the U.S. population are being sought.

The purpose of the persistence study was to obtain data that will enable the practicing criminalist to select only those genetic marker systems for analysis based on information on the history of the serological evidence, where the probability of a successful analysis is high. The results will also serve as a baseline to determine the increased sensitivity of new or improved analysis methods currently under development. The majority of the genetic marker systems evaluated in this study is being analyzed in those crime laboratories performing blood individualization. The persistence study should be expanded to include other systems of interest as they gain usage in forensic applications.

This report provides a summary and a glossary for the nonspecialist reader as well as detailed descriptions of the analysis methods used and the results obtained of primary interest to the practicing forensic serologist.

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SUMMARY

Human blood contains many inherited cell and serum constituents, called genetic markers or variants, which can be identified to show the blood's relatively unique characteristics. By the "individualization" of blood through such identification of genetic markers, serological evidence can be used to establish the presence of an individual at the crime scene. However, blood is most often found at crime scenes in the dried state, and the ability to detect variants diminishes with increasing age of the serological specimen. Some genetic markers in bloodstains can be identified after several years, whereas others cannot be successfully analyzed after even a few days. Since such large differences in the stability of these variants exist, a knowledge of the persistence of genetic markers in dried blood with time, environment, and substrate would be useful to the criminalist in the selection of the specific analyses to be performed on serological evidence.

There is a paucity of data in the literature regarding the persistence of genetic markers in dried blood. No systematic study encompassing many genetic marker systems has been reported, and relatively large discrepancies exist among the results obtained by different investigators. In particular, detailed information on the effects of humidity and substrate on persistence is not available.

In order to overcome, at least in part, this lack of data, the persistence of selected genetic markers in dried blood aged under 20 and 66% relative humidity conditions at ambient temperature as well as for specimens

stored at -20°C was determined over a six month period. The red cell antigen systems, ABO, MN, Rh, Kidd, Duffy, and Kell were analyzed using the absorption-elution method and the enzyme polymorph systems, adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (EAP) using electrophoresis on starch gel. The antigen systems were selected on the bases of availability of antisera and of specific analysis techniques that were well established. Furthermore, ABO, MN, and Rh are the major blood group systems most widely used in forensic applications. However, the effects of humidity, and to some extent substrate, are not known for these systems. The AK, ADA, PGM, and EAP systems were chosen for the same reasons.

Blood used for the aging study was obtained from 12 volunteer donors whose blood was typed to serve as the baseline. Six different substrates used for the deposition of blood were glass, nylon, wool, and three types of pure cotton, namely, plain cotton, perma press, and denim. Emphasis was placed on textile substrates as stains on fabrics are most often found at crime scenes or on a suspect's clothing. Blood analyses were conducted after storage for the following time periods: 1, 2, 4, 13, and 26 weeks.

For the dried blood specimens stored at 20 and 66% relative humidity levels, the maximum aging period at which the red cell antigens were successfully analyzed is given in Figure S-1. Similarly, the longest storage period at which the enzyme polymorphs were still identifiable is given in Figure S-2.

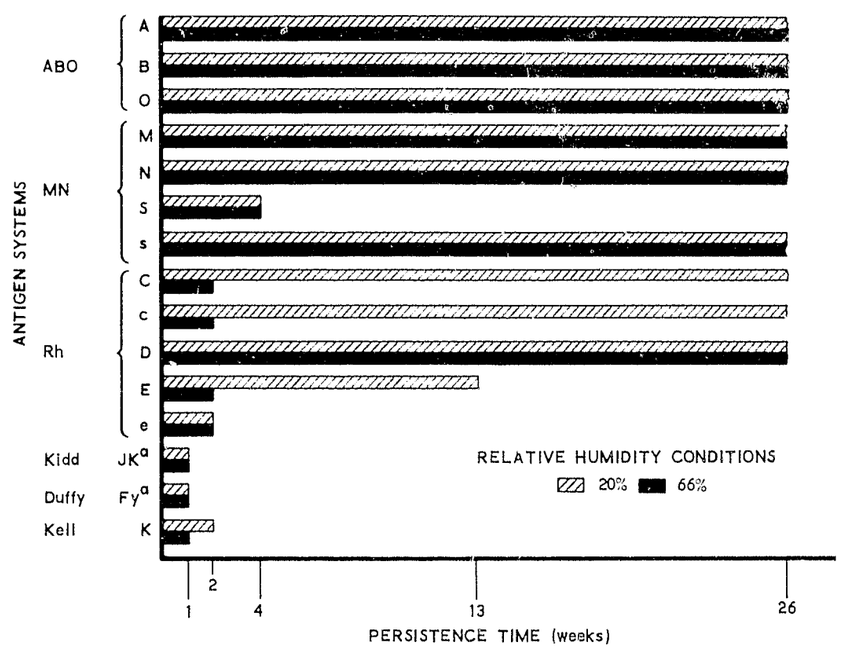

Figure S-1. Persistence of Red Cell Antigens in Dried Blood at Ambient Temperature

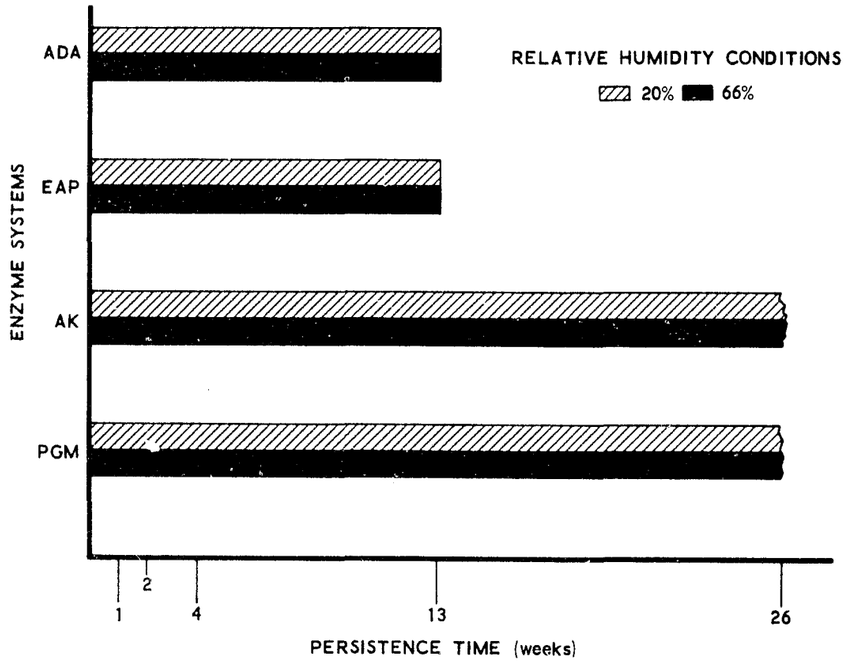

Figure S-2. Persistence of Enzymes in Dried Blood at Ambient Temperature

Based on the enzymatic activity still remaining in the dried blood, the storage of specimens at the higher humidity condition in comparison with the lower moisture level appeared to be beneficial for the preservation of ADA and EAP polymorphs, whereas the converse was observed for PGM polymorphs. No effect due to differences in humidity was seen for the AK system.

In neither the red cell antigen nor the enzyme polymorph systems were significant variations observed in the persistence of genetic markers due to the stain substrate. For the limited tests conducted on specimens stored at -20°C, the persistence of the antigens and polymorphs was found to be generally better than for the specimens aged at room temperature.

Immunological analysis of dried blood by means of the currently available absorption-elution method is extremely difficult in comparison to whole-blood analysis. Even with relatively fresh stains, questionable results were obtained for a number of specimens. In these instances, analyses were repeated as many as four times before definitive results were obtained. Difficulties were encountered especially in the detection of C, c, and E antigens of the Rh system even for two week old stains and for Kell, Kidd, and Duffy systems after one week of aging. The problems in bloodstain typing of antigens are associated primarily with the limitations of the current analysis methods that often yield ambiguous results in the identification of the genetic markers in dried blood.

Persistence data obtained in this study, together with the known frequencies of occurrence of the blood variants, were used to calculate the discrimination probability, i.e., the probability that two randomly selected

individuals will have the same combination of genetic markers. This value was determined for each aging period using only those genetic markers that were still detectable. By the identification of selected genetic markers included in this study, an a priori discrimination of one in approximately 2500 is possible for a dried blood specimen exposed to either 20 or 66% relative humidity for one week. After 26 weeks storage, however, the values are one in 65 and one in 28 for aging under the low and high relative humidity levels, respectively.

Each experimental test has associated with it a finite probability of error. As this work has shown, some of the genetic markers in bloodstains deteriorate with time. As they become more and more difficult to detect, the probability of an error in their determination increases. Calculations have been made on the effects that such errors will have on the practical use of blood evidence. The calculations are:

1. The probability of a wrong identification as results of experimental error.
2. The probability of missing a match as result of experimental error.
3. The cumulative effect of separate errors in a series of measurements.

Data concerning the first two types of calculations will be published in a separate memorandum.

CHAPTER I. INTRODUCTION

The purpose of this study was to investigate the persistence of selected genetic markers in dried blood by using currently available analysis techniques and methods. The effects of humidity and substrate on persistence and discrimination probability are presented from the results of this study. This information will be useful to practicing forensic serologists and will provide a reference to determine the sensitivity of improved analysis techniques and methods that will be developed.

The persistence of genetic markers in dried blood is of significance because serological evidence is often found at the scene of major crimes (homicide, rape, and assault), and the capability to establish the involvement of a specific individual in the commission of a crime with a high degree of confidence is an important aspect of forensic science technology. Human blood contains many genetically derived cell and serum constituents, such as antigens, enzymes, and proteins. The identification of the inherited variants present in these constitutents would make it possible to establish the unique characteristics of a particular blood; this is known as "individualization."

Whereas the analysis of whole fresh blood are relatively simple and straightforward, dried blood analyses are considerably more difficult. Even with relatively fresh stains, e.g., two week old, questionable typing results were obtained for a number of specimens using the currently available absorption-elution method. In these instances, analyses were repeated as many as four times before definitive test results were obtained. The problems

in bloodstain typing of antigens are primarily associated with the limitations the current analysis methods that often yield ambiguous results in the identification of genetic markers. This difficulty in indentifying the presence of genetic markers increases with increasing age of the bloodstains. Nevertheless, some variants in dried blood can be successfully analyzed after several years, whereas, others cannot be identified after even a few days. Thus, large differences exist in the stability of blood variants.

A knowledge of the persistence of genetic markers in dried blood is valuable to the criminalists in the selection of the specific analyses to be performed on the serological evidence. Although the majority of the blood evidence reaches the smaller, local crime laboratories within a period of one week, centers such as the FBI and state crime laboratories that service large territories receive serological clue materials that are considerably older. Furthermore, the blood analyses may not be performed immediately upon receipt of the test samples depending on their caseload, seriousness of the offense, and significance of the serological clue. In some laboratories, a portion of the blood evidence is routinely set aside and stored for possible analyses at a later date should rechecking or additional testing become necessary. In addition to the crime laboratory needs, persistence data would also benefit any developer of new methods in selecting systems in which individualization can be achieved on a relatively old stain. For these reasons, and because of the increasing effort to obtain a higher degree of discrimination probability of the blood origin based on analyses of additional genetic marker systems, persistence data correlated with environmental and substrate effects are required.

The successful identification of variants in dried blood is related to the analytical methods used. With improvements in blood analysis techniques and methodologies resulting in more sensitive tests, detection of the variants may be extended over longer aging periods. Results of the persistence tests made by current analysis methods may serve as a reference to determine the sensitivity of improved analysis techniques and methods that will be developed in the future.

At present, only scattered information canb e found in the literature regarding the stability of genetic markers in stains and a wide range of values exists in the persistence data reported by different investigators. Furthermore, systematic studies that encompass many genetic marker systems have not been performed, and no detailed information on the effects of humidity and substrate is available.

The present study investigated the feasibility of identifying the variants in bloodstains on fabrics after they were aged under controlled low and high humidity conditions for varying lengths of time. Emphasis is placed on the analyses of stains on fabrics as over 90% of the serological clue materials are found on textiles.[1] The persistence of the variants of ABO, MN, Rh, Kidd, Duffy, and Kell red cell antigen systems and of adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), erythrocyte acid phosphatase (EAP) enzyme systems was determined over a six-month period. The red cell antigen systems were selected on the bases of availability of antisera and of specific analysis techniques that were well established. Furthermore, ABO, MN, and Rh systems are the major blood group systems most widely used in forensic application. However, the effects of

humidity and to some extent substrate are not known for these systems. The AK, ADA, PGM, and EAP systems were chosen for the same reasons.

CHAPTER II. BACKGROUND

The persistence of antigens and enzymes in dried blood varies from a few days to years depending on the conditions of specimen exposure and on the particular genetic marker systems being tested. Even within the same system, considerable discrepancy exists among the different investigators regarding the time limits for the detectability of variants. This large discrepancy is due in part to the extremely complex nature of bloodstain analysis and to the development of increasingly more sensitive techniques to identify genetic markers in older stains. Dried blood analysis, in comparison with the testing of whole blood, often yields less definitive results and requires considerable expertise on the part of the serologist. The reliability of the test results also diminishes with increasing age of stains. The current investigation was initiated because of the large discrepancy in the literature data and the paucity of information regarding the effects of substrate and humidity.

Although the detectability of variants in old, dried blood is generally correlated with the persistence of the markers in the case of red cell antigens, it is possible that the loss of detectability is not necessarily due to age degradation of antigenic sites. The inability to identify the variants may also be due to the inaccessibility of the antigenic sites present on cell surfaces resulting from cell shrinkage. This shrinkage, caused by continuing dehydration and "blockage" of the reactive sites, is aggravated with increasing age of blood. For the purpose of this study, however, no distinction is made between detectability and persistence.

Another important criterion affecting blood group typing of old stains is the number of antigenic sites per blood cell for each antigen type. The variants can be detected more readily when a greater number of antigenic sites per cell is available. Presumably, this is also true of detection on old stains. This is based on the assumption, however, that other factors, such as the strength of hemagglutinability and antisera specificity, are constant. Because these factors also play a significant role in the detectability of variants, the persistence of genetic markers in dried blood cannot be assessed based solely on the number of antigenic sites per cell. This value has been calculated for some antigens. For example, in the ABO system, A and B cells have approximately 1,000,000 and 700,000 sites, respectively.[2] For the Rh system, the number of sites per cell calculated is 30,000 for D (R_1 R_2 type),[3] 79,000 for c,[4] and 21,000 for e.[4]

The information in the literature regarding the detectability of genetic markers in dried blood with age for the systems studied is shown in Table 1. The data listed indicate the age of the stain when successful analyses were performed and may not necessarily represent the maximum time limit at which the variants could be identified. However, some variations in the persistence of red cell antigens such as the Rh variants are reported. The antigens of the ABO system are the most stable among those listed, and this system is, by far, the best known and most widely used in forensic serology. Among the red cell antigenic systems listed, Kell and Duffy are the least studied and least stable in comparison with variants of the other major blood group systems. Large differences in the reported persistence data were

Table 1. Persistence of Genetic Markers in Dried Blood Reported in the Literature

System	Variants	Age of Dried Blood	Reference
ABO	A, B	2–34 yr	5
MN	M, N	29–39 wk	5–7
	S	26 wk	
Rh	C, c, D	4–26 wk	7–9
	E	5–6 wk	7, 8
	e	4–5 wk	7, 8
Duffy	Fy^a	6 days	10
Kell	K	3–19 days	8, 11
AK		4–48 wk	12–15
ADA		2–22 wk	12, 14
PGM		4–22 wk	14–18
EAP		0.5–9 wk	14–21

found for the enzyme systems. In decreasing order of stability are AK, PGM, ADA, and EAP, with variations in persistence ranging for as long as 11 months to as short as a few days.

CHAPTER III. EXPERIMENTAL

A. Baseline Analyses on Whole Blood

Fresh blood drawn from 12 randomly selected volunteer donors at The Aerospace Corporation was analyzed by the Human Genetics Laboratory, University of California, Los Angeles. The 12 donors consisted of ten Caucasians and two Orientals. Blood group typings were performed by direct agglutination in test tubes, and the enzyme/protein polymorph identifications were by electrophoresis using starch gel. The results of the whole blood analyses served as a baseline for the study of the persistence of genetic markers in dried blood.

B. Preparation of Aging Test Specimens

Blood drawn from the same 12 donors was placed on six different substrates. The substrates selected were glass, wool, nylon, and three types of pure cotton that consisted of plain cotton, perma press, and denim. Each substrate was assigned to two individual blood donors, and the assignment was maintained throughout the course of this study. Based on the results of the baseline whole blood analyses, an attempt was made to pair individuals of dissimilar blood types on a single substrate so that possible variations, if any, in persistence of different factors due to the material could be detected.

Blood was deposited on glass microscope slides as controls as potential reactions with detergents, preservatives, dyes, and other materials possibly present in textiles would be absent. The glass slides were washed

with "Liqui-Nox" soap solution, rinsed, and air dried. Cloth samples were washed in a phosphate-type detergent (Tide), rinsed thoroughly, dried for 24 hours, and then cut into one-inch squares before deposition of blood. The perma press, wool, denim, and nylon were newly purchased materials; the plain cotton was a printed sheet that had been used for three years and had undergone repeated washings.

The bloodstained specimens were air dried for 24 hours in a fume hood, and they were then placed in a constant humidity chamber for aging under ambient laboratory temperature. Two sets of conditions that represented exposure of the specimens to low and to high moisture environment were selected. These were 20 and 66% relative humidities maintained by a saturated aqueous solution of potassium acetate and sodium nitrite, respectively. Variations in relative humidities due to temperature changes were minimal since the fluctuations was ±3°C in ambient laboratory temperature (23°C). In addition, two bloodstain specimens prepared on pure cotton were aged in the freezer at -20°C. Thus, 26 specimens were available for analyses at each aging period.

C. Immunological Typing of Dried Blood

The method used in this study for the blood typing of antigens in stains was the absorption-elution technique. This technique was employed because of its much greater sensitivity over the absorption-inhibition method. The absorption-elution technique has been described by Kind,[23,24] Nickolls and Pereira,[25] Outteridge,[26] and Fiori.[5] As time did not permit the selection of the optimum operating conditions, the procedures followed in this study

represent an eclectic composite and modification of those published in the literature for each antigen. Negative controls run in this study consisted of pieces of unstained cloth subjected to conditions identical to the bloodstained cloth. Positive controls were run the same way as the tests, with the exception that the antigen type was known beforehand. The tests were run, in 10 × 75 millimeter test tubes on 3 millimeter square specimens of the bloodstained cloth that had been aged under the previously mentioned conditions of substrate and humidity. Table 2 is a compilation of the experimental procedures carried out for the immunological typing of the antigens.

Sources of antisera used in this study were as follows: Hyland Laboratory for anti-A, anti-B, anti-H, anti-M, anti-N, anti-S, anti-s, anti-c, anti-C, anti-D, anti-E, anti-e, and anti-K; Spectra Biologicals for anti-Fy^a; and Dade for anti-Jk^a and anti-human globulin.

1. ABO and MNs antigens. To each test tube containing a cloth specimen, two drops of cold physiological saline solution were added, followed by one drop of the appropriate anti-serum (A, B, H, M, N, or s). The mixture was capped and incubated in a refrigerator over night. The saline solution and the excess anti-serum were withdrawn with a pipette attached to an aspirator. The cloth specimen was then washed six times with cold saline; 15 to 20 minutes standing was allowed between successive washes. Care was taken to remove all of the saline solution, especially after the last wash. Then, for the elution, two drops of saline were added, and care was taken to submerge the cloth specimen in the saline solution. The test tubes with the specimens were capped and placed in a circulating

Table 2. Experimental Procedures for Antigen Typing

Antigen	Antiserum Added (drops)	Procedures						
		Incubation		Washing[a] Medium	Diluent for Elution[b]	Indicator Cells		Reabsorption[c] Temp. (°C)	Antihuman Serum Added[d] (drops)
		Temp. (°C)	Time (hr)			Suspension (%)	Medium		
ABO	1	5	12-18	Cold saline	Saline	0.5	Saline	5	0
MN	1	5	12-18	Cold saline	Saline	0.5	Saline	5	0
s	1	5	12-18	Cold saline	Saline	0.5	Saline	5	1
S	2	37	12-18	RT[e] saline	Saline	0.5	Saline	37	1
Rh (C, c, D, E, e)	2	37	12-18	RT[e] saline	BSA[f]	0.5-1.0	BSA[f]	37	0
Fy^a, Jk^a, K	1	37	32-48	RT[e] saline	Saline	0.5	Saline	37	1-2[g]

[a]Washed six times with a 15 to 20 min standing between each wash.

[b]Elution was carried out at 56°C for 15 min in a circulating water bath.

[c]Reabsorption was allowed to occur for approximately 1 hr.

[d]Subsequent centrifugation at 1500 rpm for 3 min followed by reading for agglutination with concave mirror and light.

[e]Room temperature saline solution.

[f]Bovine serum albumin in 0.3% concentration.

[g]Dependent on specific instructions accompanying the batch of antiserum used.

warm water bath at 56°C for 15 minutes. Immediately upon removal of the cloth specimens from the test tubes, a drop of 0.5% saline suspension of the appropriate indicator cells was added to the solution remaining in the test tubes. After reabsorption for one hour in the refrigerator, one drop of anti-human serum was added when necessary (the Coombs test such as for the s antigen). The solution was centrifuged for three minutes at 1500 revolutions/minutes (rpm). Finally, reading for agglutination was performed using a concave mirror and a light.

2. Rh (C, c, D, E, e) and MN (S) antigens. The procedure followed was similar to that described for the ABO-MNs antigens with these changes: (1) two drops of the appropriate antiserum (C, c, D, e, or S) was added at the initial stage; (2) incubation was carried out at 37°C overnight in a circulating water bath; (3) the cloth specimens were washed with room temperature saline; (4) 0.3% bovine serum albumin was added in the case of the Rh antigens just prior to elution; (5) the corresponding 0.5-1% indicator cells suspended in bovine serum albumin were added in the case of the Rh antigens; (6) reabsorption was allowed to occur at 37°C for one hour; and (7) one drop of the antihuman serum was added prior to centrifuging in the case of the S antigen.

3. Kidd (Jk^a), Duffy (Fy^a), and Kell (K) antigens. The method used was essentially that of Ruffie and Ducos[11] with some adaptations.

Two drops of saline were added to each test tube containing a cloth specimen. One drop of the appropriate antiserum (Jk^a, Fy^a, or K) was placed into the tube, and the solution was incubated at 37°C in a circulating

water bath for 32 to 48 hours. After absorption, all excess antiserum and saline were removed. The cloth specimens were washed six times with room temperature saline, allowing a 15 to 20 minute soaking between washings. Care was taken to remove all washings, especially after the last wash. Two drops of saline were added, and care was taken to submerge all cloth specimens in the liquid. The test tubes were then capped, and elution was permitted to occur for 15 minutes at 56°C. The cloth specimens were quickly removed, and one drop of the appropriate indicator cells, in 0.5% saline suspension, was added to the remaining liquid. After reabsorption for one hour at 37°C, one drop of antihuman globulin was added.

The samples were spun at 1500 rpm for 3 minutes, and agglutination was read macroscopically using a concave mirror and light.

D. Enzyme Analysis of Dried Blood by Electrophoresis

These analyses were conducted on cuttings of the same specimens used for the red cell antigen typing. Electrophoresis was performed at the Human Genetics Laboratory, University of California, Los Angeles. The general procedure followed in the preparation for analysis involved the soaking of the dried blood specimen for one hour in two drops of a 1% β-mercaptoethanol solution in the corresponding gel buffer (see Table 3). Each specimen extract was absorbed on a Whatman No. 3 filter paper (8 mm × 5 mm) that was then inserted directly into the starch gel for the respective electrophoretic runs.

The isoenzyme separations were routinely carried out using 10.5% Electrostarch (Otto Hiller) gel in a Grafar electrophoresis chamber (13 cm × 30 cm × 0.6 cm) equipped with a Buchler power supply (model No. 3-1014A),

Table 3. Experimental Procedures for Blood Enzyme Analysis

Enzyme System	Procedures							
	Electrophoresis[a]					Staining		
	Bridge Buffer		Gel Buffer: Dilution Factor of Bridge Buffer	Constant Voltage Setting (V/cm)	Stain Mixture Ingredients[b]	Overlay Material	Time[c] (min)
	System	pH					
ADA	Citric acid	5.0	1/20	5	Adenosine-MTT, xanthanine oxidase, nucleoside, phosphorylase	Agar	60
AK	Citric/NaOH	7.0	1/10[d]	4	MTT, ADP, NADP, PMS, G-6-PD, hexokinase	Agar	30-45
EAP	Citrate-phosphate	6.3	1/40	5	Phenolphthalein, monophosphate, NH_4OH	Filter paper	120
PGM	Harris	7.4	1/10	5-6[e]	TRIS, MTT, G-1-P, NADP, PMS, G-6-PD	None	60

(a)Run in refrigerator with cooling plate for 18 hrs on starch gel.

(b)MTT 3-(4,5-dimethyl-thiazolyl-2)-2,5-diphenyl tetrazolium bromide
ADP Adenosine diphosphate
NADP Nicotinamide adenine dinucleotide phosphate
PMS Phenazine methosulphate
G-6-PD Glucose-6-phosphate dehydrogenase
TRIS TRIS-(hydroxymethyl)-aminomethane
G-1-P Glucose-1-phosphate
G-1, 6 di P Glucose-1, 6-diphosphate

(c)Staining was allowed to occur at 37°C.

(d)Histidine buffer (0.005 M) was used instead of bridge buffer.

(e)Constant current, 50 ma, was used.

0 to 1000 volts, 0 to 250 milliamps. The electrophoresis conditions for the enzyme polymorphs are compiled in Table 3.

1. Adenosine deaminase (ADA). The determination of ADA in the aged bloodstains was performed using a cooling plate according to the method described by Spencer et al.[27]

2. Adenylate kinase (AK). Starch-gel electrophoresis followed the procedure of Fildes and Harris[28] using a cooling plate in the refrigerator at 180 volts (4 V/cm) and with no addition of magnesium chloride to the staining agar overlay.

3. Erythrocyte acid phosphatase (EAP). Starch-gel electrophoresis was carried out at 4°C according to the method of Swallow and Harris[29] with the following modification in the staining procedure of Sparkes et al.[30] A solution containing 0.05 grams phenolphthalein monophosphate in 25 milliliters of citrate buffer at pH 5.5 was poured over thick filter paper on the cut gel and incubated for two hours at 37°C. The filter paper was then removed and the gel flooded with ammonium hydroxide until the color developed.

4. Phosphoglucomutase (PGM). Electrophoresis was carried out at 4°C according to the method described by Spencer, Hopkinson, and Harris.[31]

CHAPTER IV. RESULTS AND DISCUSSION

The results of the red cell antigen and enzyme polymorph analyses conducted on 12 volunteer blood donors of whom ten were Caucasian and two Oriental are given in Tables 4 and 5, respectively. These analyses were performed on whole blood by the Human Genetics Laboratory, University of California, Los Angeles, to serve as a baseline for dried blood analyses.

A large number of tests was required to determine the persistence of genetic markers in dried blood. The details of the experiment are in Chapter III. Analyses of 15 antigens and four enzymes (included in this study) would require approximately 500 tests to be performed at each aging test period. However, not all variants were tested at all storage times.

In order to reduce the total number of analyses, and, thus, reduce cost and time, specimens aged for four weeks were evaluated first. The test approach used in this study is shown in Figure 1. Those antigens and enzyme polymorphs that were detectable at this time were analyzed at 13 weeks, and, if still detectable, again at 26 weeks. The two week tests, however, were usually not performed. For the variants that could not be identified at four weeks, tests were performed at two weeks, and, if still not detectable, at one week. Thus, the selection of the variants that were analyzed at the two and 13 week aging periods depended on the results of the four week tests. Similarly, the one and 26 week analyses were influenced by the two and 13 week test results, respectively.

Table 4. Blood Group Phenotype[(a)] of Donors

Donor ID No.	Red Cell Antigen System						Stain Substrate
	ABO	MNSs	Rh	Jk^a	Fy	K	
I	O	MNSs	cde	-	a - b +	-	Cotton
II	B	Ms	CcDEe	+	a - b +	-	Denim
III	O	Ns	cDEe	-	a - b +	+	Glass
IV	O	Ms	CDe	-	a + b -	-	Wool
V	O	Ns	CcDe	+	a - b +	-	Denim
VI	A_1	MNs	CcDe	+	a - b +	-	Perma press
VII	O	Ns	CcDEe	-	a + b -	-	Cotton
VIII	O	MNSs	CcDe	-	a + b -	-	Glass
IX	O	MNSs	CcDe	-	a + b -	-	Perma press
X	A_2	Ms	CcDEe	-	a - b +	-	Nylon
XI	O	MNs	cDEe	-	a - b +	-	Nylon
XII	O	MNSs	CcDe	+	a + b -	-	Wool

(a) Analyses performed by the Human Genetics Laboratory, University of California, Los Angeles.

Table 5. Blood Enzyme/Protein Phenotype[(a)] of Donors

Donor ID No.	Enzyme/Protein System					
	AK	ADA	PGM	EAP	GPT[(b)]	Hp[(b)]
I	1-1	1-1	2-2	B	2-2	2-2
II	1-1	2-1	1-1	BA	1-1	2-2
III	1-1	1-1	1-1	B	2-2	1-1
IV	1-1	2-1	2-1	B	1-1	2-1
V	1-1	2-1	2-1	B	2-1	1-1
VI	1-1	1-1	2-1	BA	2-1	2-1
VII	1-1	1-1	1-1	BA	2-1	2-1
VIII	1-1	1-1	1-1	B	2-1	2-1
IX	1-1	1-1	1-1	B	3-2	2-2
X	1-1	1-1	1-1	B	2-1	2-2
XI	1-1	1-1	1-1	BA	1-1	1-1
XII	1-1	1-1	1-1	BA	2-1	2-1

(a) Analysis performed by the Human Genetics Laboratory, University of California, Los Angeles.

(b) Aging analysis not performed because of difficulties encountered.

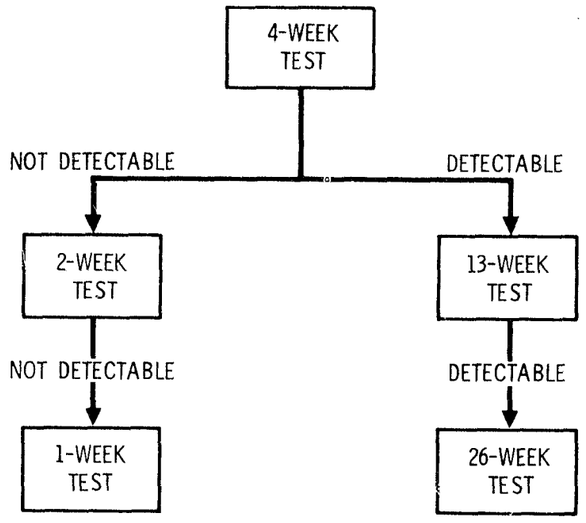

Figure 1. Design Used in Dried Blood Analysis

The number of analyses required during this study was, thus, considerably reduced. Nevertheless, because a necessary part of bloodstain analysis involves performing tests on controls simultaneously with the specimens, the number of analyses conducted remained large, totaling over 2100. The number and the types of tests performed at each aging test period are listed in Table 6. The values listed in this table represent conservative numbers as they do not include analyses that were repeated when questionable results were obtained. If one assumes that a single test, on the average, required 20 minutes, the total time expended for the analyses of dried bloodstain alone amounted to over 700 hours.

Table 6. Number of Analytical Test Performed

System Antiserum	Aging Test Period, weeks						Total No. of Tests
	0 Whole Blood	1	2	4	13	26	
ABO							
Anti-A	12	-	-	36	32	36	116
Anti-B	12	-	-	36	32	35	115
Anti-H	12	-	-	36	34	36	118
MN							
Anti-M	12	-	34	32	31	36	145
Anti-N	12	-	34	34	34	36	150
Anti-S	12	-	-	35	34	-	69
Anti-s	12	34	34	34	34	35	183
Rh							
Anti-C	12	-	15	35	17	19	98
Anti-c	12	-	15	36	16	18	97
Anti-D	12	-	-	36	36	36	120
Anti-E	12	-	32	36	17	-	97
Anti-e	12	-	32	35	-	-	79
Kidd							
Jk^a	12	32	34	36	-	-	114
Duffy							
Fy^a	12	32	32	34	-	-	112
Kell							
K	12	32	32	34	-	-	112
AK	12	-	-	26	26	26	90
ADA	12	-	-	26	26	26	90
PGM	12	-	-	26	26	26	90
EAP	12	-	26	26	26	26	116
						Total	2111

A. Red Cell Antigen Persistence

The results of the persistence of the red cell antigens of the ABO, MN, Rh, Kidd, Duffy, and Kell systems investigated in the present study are schematically represented in Figure 2. In this graph, the antigens are depicted as persisting at the specified aging period when more than half of the total number of specimens containing this specific variant yielded a positive test. Some variants, e.g., A and B of the ABO and K of the Kell system, were present in the blood of only one or two individuals among the 12 volunteer blood donors participating in this study. Thus, the results for these antigens were obtained on relatively few specimens in comparison with the other variants that were more prevalent among the donors. It can readily be seen from Figure 2 that persistence varies considerably with the specific antigen and, in some cases, with the relative humidity levels during storage. Greater details regarding the results obtained for each variant as well as substrate effects are given in Appendix A.

The presence of the ABO antigens in dried blood was found to be detectable after aging for 26 weeks. The two A, one B, and nine O types of blood present in the 12 donors were successfully identified after storage for this time period under both low and high relative humidity conditions at ambient temperature. Similarly, the two O type stains stored at -20°C in the freezer were readily identifiable. No obvious substrate effect was observed, although nylon and denim were found to yield false positive tests in several instances. Difficulties in typing bloodstains on nylon had been reported by de Ren et al.[32] The results of the detectability of the ABO

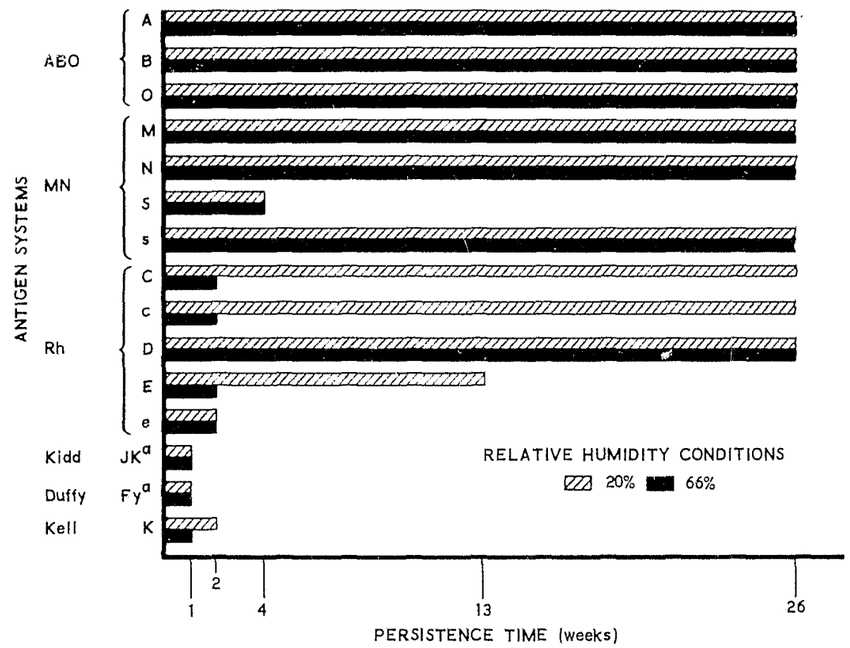

Figure 2. Persistence of Red Cell Antigens in Dried Blood at Ambient Temperature

system found in our present study are consistent with those reported by Fiori[5] in that A and B antigens can be readily detected on bloodstained fabric after two years at ambient conditions. In fact, type A stain was successfully typed by the same investigator even after 34 years. These analyses were conducted using the absorption-elution method on stains prepared on different fabric substrates that included cotton, wool, silk, and synthetic materials such as nylon and rayon. Nonspecific absorption-elution was stated not to occur with any of these materials.

Considerable difficulties were encountered in the dried blood analyses of the MN antigen system. Antisera concentration and typing conditions must be rigidly controlled to obtain reproducible results. For the specimens exposed to the two humidity levels, M, N, and s antigens could be identified for the majority of the samples after 26 weeks. However, at this test period false negative tests were obtained for three out of nine N type stain specimens conditioned at 66% relative humidity. S antigen, which could be detected at four but not at 13 weeks, was found to be the least stable among the variants tested in this system. Among the several false positive or negative tests obtained in this system, it was not possible to single out a particular substrate as being more prone to yielding erroneous results.

Both M and N antigens in stained cloth have been reported by Fiori[5] to be detectable after aging for about 29 weeks under ambient conditions. Tests were not conducted by this investigator for longer storage times. These same antigens were still identifiable after 39 weeks aging in a study conducted by Pereira.[6] Lincoln and Dodd[7] were able to type S antigens with

stains aged for 26 weeks, but were unable to type s antigens even in relatively fresh stains. It would appear that the ability to identify these antigens is greatly dependent on the procedure and the quality of the antisera used. Even for the antisera obtained from the same supplier, considerable batch-to-batch variations exist that can result in differences in the detectability of a specific antigen.

Large differences in persistence of the Rh antigens, with the exception of D and e, were observed depending on the exposure of the specimens to either the low or the high moisture level. The former environment was found to be beneficial to the preservation of most of the Rh antigens. For the specimens stored at 20% relative humidity, both C and c could be typed at 26 weeks and E at 13 weeks, whereas, at 66% relative humidity, all three of these antigens were detected at two but not at four weeks. D antigen was identifiable after aging at both relative humidity levels for 26 weeks and e after aging for two weeks. The persistence of the Rh antigens when stored at -20°C was generally better than that observed for aging at the low relative humidity level and room temperature. Again, no significant effect on persistence caused by the substrate was observed. Note, however, that questionable results were obtained in Rh antigen typing. Even with relatively fresh stains, e.g., two weeks, inconclusive tests especially for C, c, and E were obtained. These tests were then repeated, sometimes, as many as four times until unambiguous results were achieved.

The Rh antigens, C, c, and D, have been successfully typed on 26 week old stains by Lincoln and Dodd.[7] These same antigens were identified

in specimens aged for four to eight weeks by other investigators.[8-10] The persistence of E and e has been reported to range from four to six weeks.[7,8] However, inability to type the latter antigen even in relatively fresh stains because of the poor quality of the antiserum has also been reported.[10]

The antigens of the Kidd, Duffy, and Kell systems are extremely difficult to type in that the agglutination between the antigen and antibody is relatively weak. Thus, clear-cut results were often not obtained. Nevertheless, by repeating the analyses on the same specimens, it was possible to identify the presence of Jk^a of Kidd, Fy^a of Duffy, and K of Kell system after aging for one week under the two relative humidity levels tested. After this time period, only the K antigen aged at 20% humidity was detected at the two week test period. However, this result was based on tests performed on blood from the one individual among the participating donors who has the K positive blood. Tests were not performed on the frozen specimens for these three systems.

The persistence of Kidd, Duffy, and Kell antigens has not been studied in detail according to the literature. However, it has been reported that Fy^a can be detected after six days and K after 19 days.[8] In comparison to the majority of the antigens of ABO, MN, and Rh systems, the variants of the above mentioned three systems appear to be considerably less stable.

B. Enzyme Polymorph Persistence

The electrophoretic separation and identification of polymorphs of AK, ADA, PGM, and EAP systems in dried blood aged under 20 and 66% relative humidity conditions were conducted at the Human Genetics Laboratory,

University of California, Los Angeles. The analyses were performed on the same specimens used for the immunological tests. Although polymorph analyses of GPT and Hp were conducted on whole blood, they were not performed on the dried blood specimens because of difficulties encountered in electrophoretic analyses of stains. The results of the persistence of iso-enzymes of AK, ADA, PGM, and EAP systems are summarized graphically in Figure 3. Again as in the case of red cell antigens, the polymorph systems are shown as persisting when half of the specimens tested could be identified.

The electrophoretic plate after development with a staining agent was read by qualitatively characterizing the resultant polymorph band pattern, if any, into five categories. Readings of 2 or more were interpreted as positive results. Although such readings based on the judgment of the analyst are somewhat subjective, relative values can be used to determine the effects of storage conditions. Further details of the electrophoresis results including these reading values are presented in Appendix B.

For the AK system, all 12 donors participating in the present study were of 1-1 phenotype. The polymorphs were faintly detectable and identifiable at 26 weeks for both the low and the high relative humidity conditions and for the frozen samples. However, at the 13 week period, the band patterns were clearly defined for all storage conditions. No observable differences among aging conditions or substrates were distinguishable.

The activity of AK enzymes was reported by Welch[15] to decrease somewhat on aging of the stain from 15 to 30 days, although the pattern was

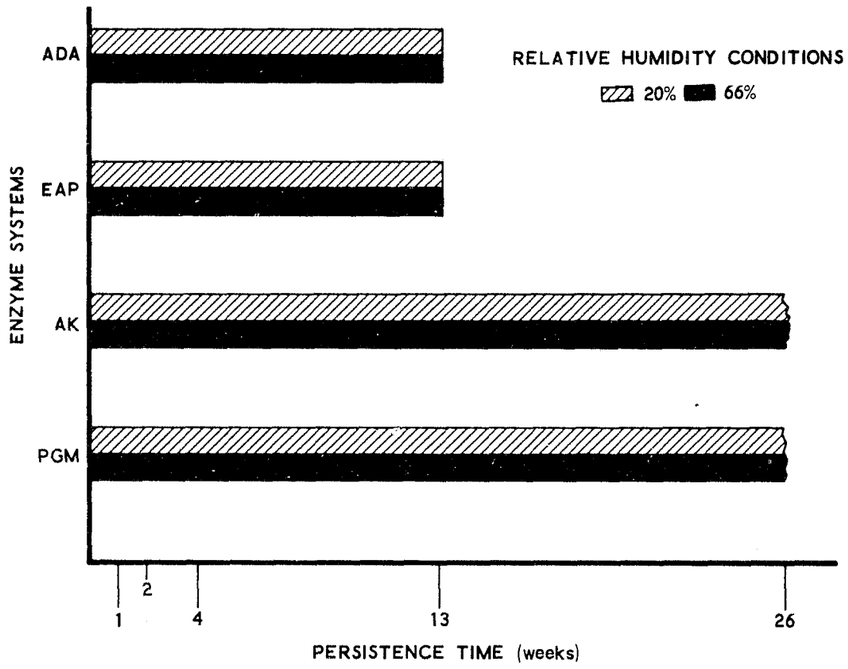

Figure 3. Persistence of Enzymes in Dried Blood at Ambient Temperature

still clear and readily discernible. Brinkmann et al.[13] and Rothwell[16] were able to analyze the isoenzymes after 11 months storage at room temperature. Although the band patterns were not interpretable, the former investigators reported that the activity could still be detected on six year old stains. The latter investigators were able to identify the AK polymorphs after storage of samples at -20° C for two years.

The ADA polymorphs included in this study consisted of nine individuals of 1-1 and three 2-1 phenotypes. At the 26 week analysis period, although the activity of the enzyme was faintly detectable, only one of the two frozen specimens was identifiable. All phenotypes were distinguishable at 13 weeks, and the patterns obtained on the specimens stored at the high humidity condition were more clearly defined than those exposed to the lower moisture level (see Appendix B, Table B-3). It would appear from these results that the ADA isoenzymes retain somewhat greater activity when aged under the higher humidity level. Differences due to the effect of the fabric substrates or of the two phenotypes tested on the persistence of this blood enzyme were not observed. The results of this study, which indicate that ADA polymorphs are detectable in dried blood at 13, but not 26 weeks, are consistent with 22 week persistence reported by Brinkmann and Dirks.[13] In contrast, Welch[15] found that the ADA isoenzymes in bloodstains on cotton and exposed to air and ambient temperatures could be identified after 15 but not 20 days.

Of the three phenotypes of the PGM system tested in this study, nine of the 12 specimens were identifiable at the low and seven of the 12 at the high relative humidity levels at 26 weeks. Both frozen samples were success-

fully analyzed at this time period. All specimens aged for 13 weeks yielded distinguishable band patterns with greater clarity of the patterns observed for the specimens stored at the lower level of humidity. In contrast to the ADA isoenzymes, the exposure of dried blood to 66% relative humidity appeared to result in a decreased enzymatic activity (see Appendix B, Table B-4). The persistence characteristic of eight specimens of 1-1 phenotype and three of the 2-1 type investigated in this study was approximately the same. However, the 2-2 type present in only one individual appeared to be somewhat less stable. PGM polymorphs have been reliably detected in dried blood after storage for time periods ranging from four to 13 weeks,[15-19] but the percentage of the specimens that could be analyzed successfully after longer storage was decreased. Occasionally, however, polymorphs present in five month old samples have been identified.[16-19]

Seven blood specimens used in this study were of the B and five of the BA phenotype of the EAP system. Essentially, all specimens could be analyzed successfully at 13 week storage period. It is interesting to note that a greater enzymatic activity was generally observed for the specimens aged under the high humidity level in comparison with those exposed to a lower level of moisture (see Appendix B, Table B-5). For the 26 week tests, only two specimens (one of each phenotype) from a total of 12 could be identified after conditioning at the high relative humidity, none at the low humidity, and one (B type) of two specimens (consisting of both phenotypes) for the frozen samples. In a study conducted by Brinkmann et al.,[20] B and BA phenotypes were identified in stains up to nine and six weeks, respectively. No significant difference in persistence between these phenotypes was observed

in this study. However, a difference in persistence of a few weeks would not be seen in the current study as the analyses were not performed at frequent intervals after four weeks storage. When the specimens stored at 20 and 66% relative humidity levels were compared, the latter condition appeared to be beneficial for the retention of the EAP enzymatic activity. Although somewhat inconclusive, storage of the sample in the cold was deemed to be better than conditioning at room temperature.

A large discrepancy exists in the literature data regarding the persistence of the EAP polymorphs in stains. The reported time limits for the identification vary from a few days,[21] 22 days[15] 30 days[22] and up to eight or nine weeks.[20] The results of this study showed that it is possible to identify the EAP polymorphs at a longer aging time, i.e., 13 weeks under the three storage conditions investigated.

C. Discrimination Probability

Based on the results of this aging study and on the known frequencies of occurrence of the different variants, it is possible to calculate the discrimination probability achievable by the analyses of the genetic markers persisting at each test period. This value as used in this report is defined as the probability that two randomly selected individuals will have the same combination of blood variants. Whenever a variant becomes not identifiable due to age of stain, the degree of individualization resulting from the analysis of that system is decreased. The overall discrimination probability for each aging test period is obtained by multiplying the values for each system. A high degree of individualization is desirable as the blood analysis results

can then be used to exclude positively a suspect from involvement in a crime, or alternatively, to identify an individual from a closed group of suspects. The results calculated for the detectable variants among those tested for the two relative humidity conditions are shown in Figure 4. Discrimination probabilities of individual systems for the different test periods as well as the method of calculation are given in Appendix C.

An a priori discrimination of one individual out of a total of approximately 2500 is possible after one week storage at both 20 and 66% relative humidity conditions. At 26 weeks, the values are one in 65 and one in 28 for aging under low and high moisture levels, respectively. It can readily be observed that the degree of individualization possible decreases rapidly with increasing age of dried blood especially when exposed to a more humid environment. It may be possible that development of more sensitive analysis techniques would make possible the detection of these genetic markers in old dried blood on specimens aged in an adverse environment.

Each experimental test has associated with it a finite probability of error. As this work has shown, some of the genetic markers in bloodstains deteriorate with time. As they become more and more difficult to detect, the probability of an error in their determination increases. Calculations have been made on the effects that such errors will have on the practical use of blood evidence. The calculations are:

1. The probability of a wrong identification as results of experimental error.

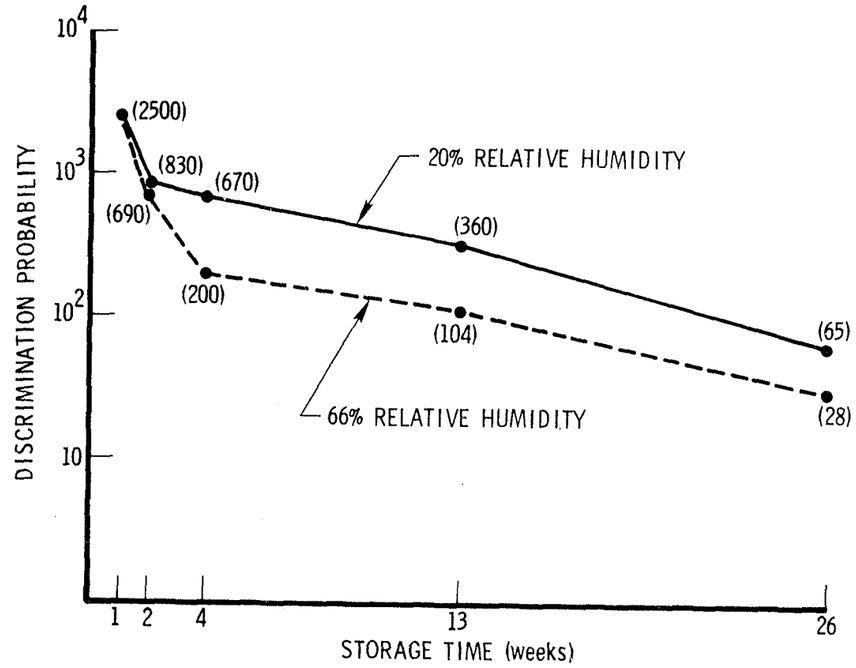

Figure 4. Discrimination Probability with Age of Dried Blood (The discrimination probability is the total number of individuals of whom two would be expected to have the same combination of variants.)

2. The probability of missing a match as result of experimental error.

3. The cumulative effect of separate errors in a series of measurements.

Data concerning the first two calculations will be published in a separate memorandum.

Since the majority of the serological evidence reaches most crime laboratories within a week, the discrimination probability value calculated for the one week aging test period is applicable in most cases. However, in order to achieve a greater discrimination of relatively old dried blood, it is necessary that analyses of additional genetic marker systems other than those included in the present study be performed. Among the systems that offer promise are the immunological typings of Gm and HL-A* antigens and electrophoretic analyses of Hp, GPT, and Gc. With the exception of Hp, the techniques for analyses of these systems applicable to crime laboratory use still need development, and information on the persistence of these genetic markers is required.

*The Aerospace Corporation sponsored a persistence study at the Department of Surgery, University of California, Los Angeles. The results "Identification of Dried Human Blood Samples by HL-A Antigens," M. Tagasugi, D. Akira, and P. I. Terasaki, were presented at The California Association of Criminalists 43rd Semiannual Seminar at Long Beach, California, 9-11 May 1974.

CHAPTER V. CONCLUSIONS

This study confirmed literature reports that large differences in the persistence of genetic markers in dried blood can be observed. Among the variants investigated, the aging time at which successful analyses could be performed varied from 1 to 26 weeks. In general, the persistence of the genetic variants was enhanced when the dried blood specimens were stored at room temperature under low relative humidity level, in comparison with poorer results when aging under high humidity environment. This effect was especially apparent for C, c, and E antigens of the Rh system. Since storage of the specimens at -20°C was generally more beneficial than aging at room temperature, this condition should be maintained, whenever possible, after receipt of the serological specimens to reduce further deterioration. No significant difference in persistence resulting from various textile substrates was observed.

Even the limited set of blood variants investigated in this study yielded discrimination probabilities considerably greater than commonly obtained by current routine bloodstain analyses. For the genetic markers analyzed in this study, the probability of two randomly selected individuals having the same combination of variants is one in 2500 after one week for both humidity conditions. Previous criminalistic laboratory surveys have shown that 95% of clues are available for analysis within one week.[1] This value decreases after 26 weeks to one in 65 and in 28 for the low and high relative humidity levels, respectively.

Although in this study it was shown that A, B, and O of the ABO, M, N, and s of the MN, D of the Rh system, and the polymorphs of AK and PGM systems can be identified in dried blood aged at room temperature for 26 weeks under the low and high relative humidity conditions, these tests were conducted on clean specimens free of impurities and that the conclusions cannot necessarily be extrapolated to actual practice. Actual serological evidence often contains impurities such as perspiration, urine, soil, and bacterial contamination. Depending on the nature and degree of contamination, it may not be possible to identify variants that are readily distinguishable in clean specimens. Further, the maximum elapsed time at which analyses could be successfully performed may be considerably shortened by the presence of contamination.

The results of this study have shown that false test results, either positive or negative, can be obtained even when using the most dependable techniques currently available. False negative tests were obtained, e.g., even though the antigens were shown to persist for longer time periods in subsequent tests. The large number of analyses that were conducted at each aging test period precluded the repetition of these tests in our study. However, in actual practice where serological evidence is being analyzed, the importance of conducting tests at least in triplicate, and with the proper controls, cannot be overemphasized. This is necessary if the results are to be used in court testimony so that the validity of the ensuing conclusions will not be challenged.

The persistence data obtained in this study should be of use to forensic serologists in selecting the variants to be analyzed when the approximate age and exposure conditions of the serological evidence are known. With the development of improved, more sensitive, analysis techniques, it may be possible to increase the time limits for variant detectability in older dried blood. Such techniques will be especially beneficial to crime laboratories in those cases where serological evidence is obtained after some time has elapsed since the commission of the crime.

It is strongly recommended that additional persistence studies be undertaken for all other genetic marker systems offering good discrimination probabilities.

NOTES

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APPENDIX A. RED CELL ANTIGEN ANALYSES

A summary of the immunological analyses of red cell antigens in dried blood specimens for the different blood types is given in Table A-1. The number of positive tests obtained versus the number that should have been obtained is given for each aging test period. For example, the value 9/10 indicates that nine out of a possible ten specimens yielded positive tests and one gave a false negative result. The number of expected positives varied somewhat with each test period because of the unavailability of the donor having the particular blood type during sample preparation or of loss of the specimen during analyses. The readings of agglutination reactions were interpreted as being positive when a two plus or more reaction was observed.

The effect of the substrate on the ability to type the presence of a specific antigen is shown in Tables A-2 through A-6. With the anti-A and anti-B sera, false positive tests were obtained in several instance with denim and nylon, especially with the former material. In the MN and Rh systems, false readings were observed in a few specimens involving wool, denim, and nylon substrates. However, no single material consistently gave erroneous results.

Table A-1. Summary of the Red Cell Antigen Persistence in Dried Blood

System Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)									
	1		2		4		13		26	
	Low	High	Low	High	Low	High	Low	High	Low	High
ABO										
A					2/2	2/2	2/2	2/2	2/2	2/2
B					1/1	1/1	1/1	1/1	1/1	1/1
O					8/8	7/8	8/8	9/9	9/9	8/8
MN										
M			9/9	7/8	8/8	8/8	8/8	7/7	9/9	8/8
N					7/7	7/7	8/8	8/8	8/8	6/9
S					3/3	2/3	1/3	0/3		
s					11/11	8/10	11/12	8/12	11/12	9/11
Rh										
C				7/7	9/9	0/8	7/8		8/8	
c				7/9	10/10	1/10	7/8		7/8	
D					11/11	11/11	10/11	10/11	9/9	9/9
E			3/4	4/4	4/5	1/5	3/4		0/4	
e			9/10	9/10	1/12	4/12				
Kidd										
Jk^a	3/3	3/3	0/3	1/3	0/4	0/4				
Duffy										
Fy^a	4/4	4/4	1/5	0/4	0/5	0/5				
Kell										
K	1/1	1/1	1/1	0/1	0/1	0/1				

Table A-2. Typing Results on Bloodstained Cotton

System Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)														
	1			2			4			13			26		
	Low	High	F[a]	Low	High	F	Low	High	F	Low	High	F	Low	High	F
ABO															
A	-[b]	-	-	-	-	-	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
O	NT[c]	NT	-	NT	NT	NT	2/2[d]	1/2	NT	2/2	2/2	NT	2/2	2/2	2/2
MN															
M	NT	NT	NT	1/1	1/1	1/1	2/1[e]	1/1	NT	1/1	1/1	NT	1/1	NT	1/1
N	NT	NT	NT	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2	NT	2/2	1/2	2/2
S	NT	NT	NT	NT	NT	1/1	1/1	0/1	1/1	0/1	0/1	0/1	NT	NT	NT
s	2/2	2/2	NT	2/2	1/2	2/2	2/2	2/2	1/2	2/2	1/2	-	2/2	1/2	2/2
Rh															
C	NT	NT	NT	NT	1/1	NT	1/1	0/1	1/1	1/1	NT	1/1	1/1	NT	1/1
c	NT	NT	NT	NT	2/2	NT	2/2	1/2	1/2	1/1	NT	2/2	2/2	0/2	2/2
D	NT	NT	NT	NT	NT	NT	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1	1/1
E	NT	NT	NT	1/1	2/1[e]	NT	1/1	0/1	1/1	1/1	0/1	1/1	0/1	NT	0/1
e	NT	NT	NT	2/2	2/2	2/2	0/2	1/2	0/1	NT	NT	0/2	NT	NT	-
Kidd															
Jk^a	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Duffy															
Fy^a	1/1	1/1	-	0/1	0/1	-	0/1	0/1	-	NT	NT	-	NT	NT	-
Kell															
K	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a] Frozen specimen stored at -20° C.

[b] Test specimens of this blood type were not prepared.

[c] Not tested.

[d] Numerator is the number of positive test(s), and the demoninator is the maximum theoretically possible.

[e] One false positive test was obtained.

Table A-3. Typing Results on Bloodstained Nylon

System / Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)									
	1		2		4		13		26	
	Low	High	Low	High	Low	High	Low	High	Low	High
ABO										
A	NT[a] (d)	NT	NT	NT	1/1[b]	1/1	1/1	1/1	2/1[c]	1/1
B	-	-	-	-	-	-	-	-	-	-
O	NT	NT	NT	NT	1/1	1/1	1/1	1/1	1/1	1/1
MN										
M	NT	NT	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2
N	NT	NT	NT	NT	1/1	1/1	1/1	1/1	1/1	2/1[c]
S	-	-	-	-	-	-	-	-	-	-
s	2/2	2/2	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2
Rh										
C	NT	NT	NT	NT	1/1	0/1	1/1	NT	1/1	0/1
c	NT	NT	NT	1/2	2/2	0/2	2/2	NT	2/2	0/2
D	NT	NT	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2
E	NT	NT	2/2	2/2	2/2	0/2	1/2	0/2	0/2	NT
e	NT	NT	2/2	2/2	0/2	0/2	NT	NT	NT	NT
Kidd										
Jk^a	-	-	-	-	-	-	-	-	-	-
Duffy										
Fy^a	-	-	-	-	-	-	-	-	-	-
Kell										
K	-	-	-	-	-	-	-	-	-	-

(a) Not tested.

(b) Numerator is the number of positive test(s), and the denominator is the maximum possible.

(c) One false positive test was obtained.

(d) Test specimens of this blood type were not prepared on this substrate.

Table A-4. Typing Results on Bloodstained Wool

System / Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)									
	1		2		4		13		26	
	Low	High	Low	High	Low	High	Low	High	Low	High
ABO										
A	-[a]	-	-	-	-	-	-	-	-	-
B	-	-	-	-	-	-	-	-	-	-
O	NT[b]	NT	NT	NT	2/2[c]	2/2	2/2	2/2	2/2	2/2
MN										
M	NT	NT	2/2	2/2	2/2	2/2	1/1	2/2	2/2	2/2
N	NT	NT	2/1[d]	1/1	1/1	1/1	1/1	1/1	1/1	0/1
S	NT	NT	NT	NT	0/1	0/1	-	-	NT	NT
s	1/1	1/1	0/1	2/2	2/2	1/2	1/2	1/2	2/2	2/2
Rh										
C	NT	NT	NT	2/2	2/2	0/2	1/2	NT	2/2	0/2
c	NT	NT	NT	0/1	1/1	1/1	1/1	NT	0/1[d]	0/1
D	NT	NT	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2
E	-	-	-[d]	-	-	-	-	-	-	-
e	NT	NT	2/2	2/2	0/2	0/2	NT	NT	NT	NT
Kidd										
Jk^a	-	-	-	-	0/1	0/1	-	-	-	-
Duffy										
Fy^a	1/1	1/1	1/2	0/2	0/2	0/2	NT	NT	NT	NT
Kell										
K	-	-	-	-	-	-	-	-	-	-

(a) Test specimens of this blood type were not prepared.

(b) Not tested.

(c) Numerator is the number of positive test(s), and the denominator is the maximum possible.

(d) One false positive test was obtained.

Table A-5. Typing Results on Bloodstained Perma Press

System / Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)									
	1		2		4		13		26	
	Low	High	Low	High	Low	High	Low	High	Low	High
ABO										
A	NT[a]	NT	NT	NT	1/1[b]	1/1	1/1	1/1	1/1	1/1
B	-[c]	-	-	-	-	-	-	-	-	-
O	NT	NT	NT	NT	1/1	1/1	1/1	1/1	1/1	1/1
MN										
M	NT	NT	2/2	2/2	2/2	2/2	2/2	1/1	2/2	2/2
N	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2	2/2	1/2
S	-	-	NT	NT	1/1	1/1	1/1	0/1	NT	NT
s	2/2	2/2	1/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
Rh										
C	NT	NT	NT	2/2	2/2	0/2	2/2	NT	2/2	1/2
c	NT	NT	NT	2/2	2/2	1/2	1/2	NT	1/1	1/2
D	NT	NT	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2
E	-	-	-[d]	-[d]	-	-	-	-	-	-
e	NT	NT	2/2	2/2	0/2	0/2	NT	NT	NT	NT
Kidd										
Jk^a	-	-	0/1	0/1	0/1	0/1	NT	NT	NT	NT
Duffy										
Fy^a	1/1	1/1	1/1	0/1	0/1	0/1	NT	NT	NT	NT
Kell										
K	-	-	-	-	-	-	-	-	-	-

[a] Not tested.

[b] Numerator is the number of positive test(s), and the denominator is the maximum possible.

[c] Test specimens of this blood type were not prepared.

[d] One specimen yielded a false positive test.

Table A-6. Typing Results on Bloodstained Denim

System / Stain Type	Stain Age (weeks)/Relative Humidity (20 and 66%)									
	1		2		4		13		26	
	Low	High	Low	High	Low	High	Low	High	Low	High
ABO										
A	-[a]	-	-	-	-	-	-	-	-[b]	
B	NT[c]	NT	NT	NT	1/1[d]	1/1	1/1	1/1	2/1[e]	2/1[e]
O	NT	NT	NT	NT	1/1	1/1	1/1	1/1	1/1	1/1
MN										
M	NT	NT	1/1	0/1	1/1	1/1	2/1[f]	1/1	1/1	1/1
N	NT	NT	1/1	2/1[f]	1/1	1/1	1/1	1/1	1/1	1/1
S	-	-	-	-[f]	-	-	-	-	-	-
s	2/2	2/2	2/2	2/2	2/2	1/2	2/2	1/2	2/2	2/2
Rh										
C	NT	NT	NT	2/2	2/2	0/2	2/2	NT	2/2	0/2
c	NT	NT	NT	2/2	2/2	0/2	2/2	NT	2/2	0/2
D	NT	NT	NT	NT	2/2	2/2	2/2	2/2	2/2	2/2
E	NT	NT	0/1	1/1	0/1	0/1	1/1	0/1	0/1	NT
e	NT	NT	1/2	2/2	1/2	1/2	NT	NT	NT	NT
Kidd										
Jk^a	1/2	2/2	0/2	1/2	0/2	0/2	NT	NT	NT	NT
Duffy										
Fy^a	-	-	-	-	-	-	-	-	-	-
Kell										
K	-	-	-	-	-	-	-	-	-	-

[a] Test specimens of this blood type were not prepared on this substrate.

[b] One specimen (type B) yielded a false positive test.

[c] Not tested.

[d] Numerator is the number of positive test(s), and the denominator is the maximum possible.

[e] One specimen (type O) yielded a false positive test.

[f] One false positive test was obtained.

APPENDIX B. ENZYME POLYMORPH ANALYSES

The results of electrophoretic separation and identification of AK, ADA, PGM, and EAP polymorphs in dried blood aged under 20 and 66% relative humidity conditions at ambient temperature and also for the specimens stored at -20°C are summarized in Table B-1. The fractional values represent the number of specimens identifiable over the total number analyzed. It can readily be seen that AK and PGM polymorphs were identifiable after 26 weeks of storage and, consequently, are more stable than the ADA and EAP isoenzymes, which persisted for only 13 weeks.

The enzymatic activity, if present, after development of the band pattern was interpreted as follows: 0, no detectable enzymatic activity; 1, faintly detectable but not identifiable; 2, faintly detectable and identifiable; 3, identifiable; and 4, clearly identifiable. These reading values together with the substrate and phenotype are given in Tables B-2 through B-5. All the AK and the majority of the PGM analyses of specimens stored for 26 weeks yielded identifiable band patterns. It can be seen from the 13 week reading values for the PGM system that clearer band patterns and, thus, greater enzymatic activities were present in specimens exposed to the low humidity. In contrast, exposure to the higher moisture level appears to be beneficial for the preservation of ADA and EAP polymorphs (Tables B-3 and B-5, reading values at 13 weeks). In general, the persistence of the isoenzymes stored at -20°C was generally better than those aged at room temperature under either relative humidity level. Again, no significant differences

resulting from substrate effect or to the phenotypes were evident. Such differences may possibly be detectable only if analyses were conducted at more frequent time intervals.

Table B-1. Summary of Enzyme Polymorph Persistence in Dried Blood

System	Stain Age (weeks) and Storage Condition											
	2			4			13			26		
	Low[a]	High[b]	F[c]	Low	High	F	Low	High	F	Low	High	F
AK				12/12[d]	12/12	2/2	12/12	12/12	2/2	12/12	12/12	2/2
ADA				11/12	12/12	2/2	12/12	12/12	2/2	0/12	0/12	1/2
PGM				11/12	12/12	2/2	12/12	12/12	2/2	9/12	7/12	2/2
EAP	11/12	12/12	1/2	11/12	12/12	2/2	11/12	11/12	2/2	0/12	2/12	1/2

[a] 20% relative humidity.

[b] 66% relative humidity.

[c] Frozen at -20°C.

[d] Numerator is the number of specimens yielding readable results, and the denominator is the total tested.

Table B-2. Persistence of AK Polymorphs in Dried Blood

Substrate	Donor ID No.	Phenotype	Stain Age (weeks)/Relative Humidity (20 and 66%)					
			4		13		26	
			Low	High	Low	High	Low	High
Glass	III	1-1	4	4	4	4	2	2
	VIII	1-1	4	4	4	4	2	2
Cotton	I	1-1	4	4	4	4	2	2
	VII	1-1	4	4	4	4	2	2
Nylon	X	1-1	4	4	4	4	2	2
	XI	1-1	4	4	4	4	2	2
Wool	IV	1-1	4	4	4	4	2	2
	XII	1-1	4	4	4	4	2	2
Perma Press	VI	1-1	4	4	4	4	2	2
	IX	1-1	4	4	4	4	2	2
Denim	II	1-1	4	4	4	4	2	2
	V	1-1	4	4	4	4	2	2
Cotton (Frozen)	I	1-1	4		4		2	
	VII	1-1	4		4		2	

0 No detectable enzymatic activity.

1 Faintly detectable but not identifiable

2 Faintly detectable and identifiable

3 Identifiable

4 Clearly identifiable

Table B-3. Persistence of ADA Polymorphs in Dried Blood

Substrate	Donor ID No.	Phenotype	Stain Age (weeks)/Relative Humidity (20 and 66%)					
			4		13		26	
			Low	High	Low	High	Low	High
Glass	III	1-1	4	4	3	4	1	1
	VIII	1-1	4	4	3	4	1	1
Cotton	I	1-1	4	4	3	4	1	1
	VII	1-1	4	4	3	4	1	1
Nylon	X	1-1	4	4	3	4	1	1
	XI	1-1	1	4	3	4	1	1
Wool	IV	2-1	4	4	3	4	1	1
	XII	1-1	4	4	3	4	1	1
Perma Press	VI	1-1	4	4	3	4	1	1
	IX	1-1	2	4	3	4	1	1
Denim	II	2-1	4	3	3	4	1	1
	V	2-1	4	4	3	4	1	1
Cotton (Frozen)	I	1-1	4		3		4	
	VII	1-1	4		4		1	

0 No detectable enzymatic activity.

1 Faintly detectable but not identifiable.

2 Faintly detectable and identifiable.

3 Identifiable.

4 Clearly identifiable.

Table B-4. Persistence of PGM Polymorphs in Dried Blood

Substrate	Donor ID No.	Pheno-type	Stain Age (weeks)/Relative Humidity (20 and 66%)					
			4		13		26	
			Low	High	Low	High	Low	High
Glass	III	1-1	4	4	3	2	3	2
	VIII	1-1	4	4	3	2	3	3
Cotton	I	2-2	4	3	2	2	0	0
	VII	1-1	4	3	3	2	3	2
Nylon	X	1-1	4	4	3	2	3	2
	XII	1-1	4	4	3	2	3	2
Wool	IV	2-1	4	4	3	2	3	2
	XII	1-1	4	3	3	2	3	3
Perma Press	VI	2-1	1	4	3	2	3	0
	IX	1-1	3	4	3	2	0	0
Denim	II	1-1	3	4	3	2	2	0
	V	2-1	3	4	3	2	0	0
Cotton (Frozen)	I	2-2	3		4		2	
	VII	1-1	4		4		4	

0 No detectable enzymatic activity.

1 Faintly detectable but not identifiable.

2 Faintly detectable and identifiable.

3 Identifiable.

4 Clearly identifiable.

Table B-5. Persistence of EAP Polymorphs in Dried Blood

Substrate	Donor ID No.	Pheno type	Stain Age (weeks)/Relative Humidity (20 and 66%)							
			2		4		13		26	
			Low	High	Low	High	Low	High	Low	High
Glass	III	B			3	4	3	4	0	0
	VIII	B	3	4	3	3	3	3	0	0
Cotton	I	B	3	4	4	4	0	0	0	0
	VII	BA	3	4	3	4	3	4	0	0
Nylon	X	B	3	4	4	4	3	4	0	0
	XI	BA	3	4	4	4	3	4	0	0
Wool	IV	B	4	4	4	4	3	4	1	0
	XII	BA	3	4	4	4	3	4	1	0
Perma Press	VI	BA	3	4	0	4	3	4	1	2
	IX	B	1	4	2	4	3	4	1	0
Denim	II	BA	3	4	4	4	3	4	0	0
	V	B	3	4	4	4	3	4	0	2
Cotton (Frozen)	I	B	3		3		3		3	
	VII	BA	1		3		4		1	

0 No detectable enzymatic activity.

1 Faintly detectable but not identifiable.

2 Faintly detectable and identifiable.

3 Identifiable.

4 Clearly identifiable.

APPENDIX C. CALCULATION OF DISCRIMINATION PROBABILITIES

The discrimination probability, i.e., the probability that two randomly selected individuals will have the same phenotype, was calculated for the combination of variants that can be identified at each aging test period. For each individual genetic marker system, the value was obtained by summation of the squares of its phenotypic frequencies of occurrence. The overall discrimination probability was obtained by multiplying the values available for each system.

Depending on the persistence of the specific genetic markers, it was necessary to divide the identifiable phenotypes into different categories. For example, when both E and e factors of the Rh system could be typed, the identifiable phenotypes for these alleles are E, Ee, and e. However, when only E could be typed, distinguishable phenotypes are E (+) and E (-). The former includes the values for homozygous and heterozygous genotypes of E. Because the alleles are not independent of other factors within a genetic marker system, the published frequency data for the various combinations of identifiable E and e phenotypes with C, c, and D factors were used in the calculation of the discrimination probability.

Discrimination probabilities of the genetic marker systems achievable by the analyses of the variants detectable at each aging test period are given in Table C-1 for the two relative humidity storage conditions. For example, M, N, S, and s antigens of the MN system could be typed successfully in four week old dried blood, yielding a discrimination probability of 0.163. This

Table C-1. Discrimination Probability with Age of Dried Blood

Relative Humidity Storage Time (weeks)	ABO[a]	MN[b]	Rh[a]	Kidd[b]	Duffy[b]	Kell[b]	AK[c]	ADA[c]	PGM[c]	EAP[c]	Discrim. Prob. X10^4	1/Discrim. Prob.
Low (20%)												
1	0.396	0.163	0.211	0.644	0.552	0.836	0.836	0.818	0.470	0.329	4	2500
2	0.396	0.163	0.211	-	-	0.836	0.836	0.818	0.470	0.329	12	830
4	0.396	0.163	0.218	-	-	-	0.836	0.818	0.470	0.329	15	670
13	0.396	0.309	0.218	-	-	-	0.836	0.818	0.470	0.329	28	360
26	0.396	0.309	0.324	-	-	-	0.836	-	0.470	-	156	65
High (66%)												
1	0.396	0.163	0.211	0.644	0.552	0.836	0.836	0.818	0.470	0.329	4	2500
2	0.396	0.163	0.211	-	-	-	0.836	0.818	0.470	0.329	14	690
4	0.396	0.163	0.745	-	-	-	0.836	0.818	0.470	0.329	51	200
13	0.396	0.309	0.745	-	-	-	0.836	0.818	0.470	0.329	96	104
26	0.396	0.309	0.745	-	-	-	0.836	-	0.470	-	358	28

[a]Frequency data found in R. R. Race, and R. Sanger, "Blood Groups in Man," Oxford and Edinburgh, Blackwell Scientific Publications (1968).

[b]Frequency data found in P. D. Issitt, "Applied Blood Group Serology," Spectra Biologicals, Division of Beckton, Dickinson and Co. (1972).

[c]Frequency data found in R. L. Williams, "Forensic Science - the Present," Analytical Chemistry, Vol., 45, 1973, pp. 1076-1089A.

values increases to 0.309 at 13 weeks because of the inability to type S so that s and Ss phenotype are indistinguishable. The larger number represents a lower degree of individualization.

Example calculations for the MN system, where all four antigens (at 1 to 4 weeks) and where M, N, and s (at 13 and 26 weeks) were detectable, are performed by summation of the squares in Table C-2.

Table C-2. Discrimination Probability Calculations

Phenotype	At 4 Weeks		At 13 Weeks	
	Frequency	Frequency^2	Frequency	Frequency^2
MS	0.06	0.0036	0.06	0.0036
MSs	0.14	0.0196	0.22	0.0484
Ms	0.08	0.0064		
MNs	0.04	0.0016	0.04	0.0016
MNSs	0.24	0.0576	0.46	0.2116
MNs	0.22	0.0484		
NS	0.01	0.0001	0.01	0.0001
NSs	0.06	0.0036	0.21	0.0441
Ns	0.15	0.0225		
	Total: 0.1634		Total: 0.3094	

In the Rh system, the probability value when all five antigens, C, c, D, E, and e, are typed is 0.211. For low relative humidity storage conditions,

this value increase to 0.218 (unable to type e) at four weeks and finally to 0.324 (unable to type E) at 26 weeks. For high relative humidity conditions, the initial probability of 0.211 is increased to 0.745 when only the D (+) or D (-) could be determined at 4, 13, and 26 weeks. Jk^a of Kidd, Fy^a of Duffy, and K of Kell were identifiable only at one week with the exception of K, which was typed successfully at two weeks (low humidity condition).

As no obvious difference in persistence of enzyme polymorphs within a given system was observed for the aging periods tested, the discrimination probabilities remained constant as long as the isoenzymes were identifiable. Both AK and PGM polymorphs were successfully analyzed at 26 weeks, but ADA and EAP polymorphs were only identifiable at 13 weeks for the two relative humidity conditions investigated in this study.

GLOSSARY

ABSORPTION. Removal of antibodies from serum by adsorption onto red cells bearing the appropriate antigen receptor sites.

AGAR. A sulfuric acid ester of a complex galactose polysaccharide obtained from seaweed. Employed as support medium and gel in electrophoresis.

AGGLUTINATION. The specific clumping of red cells by an antibody (agglutinin).

ALBUMINS. Protein constitutents in blood serum.

ALLELE (allelomorph). One of two or more alternative forms of a gene occupying the same locus on homologous chromosomes. The expressed character of allelic genes are antithetical, because they are never (normally) inherited together from a single parent.

ANTIBODY. An immunoglobulin molecule with specific receptor sites formed in response to an antigenic stimulus. The term is usually used collectively to refer to molecules with similar specificity within a serum specimen.

ANTIGEN. Any substance that can stimulate neutralizing antibody production when introduced into a vertebrate.

ANTIGENIC DETERMINANT. The individual site on the antigen molecule that combines with a specific antibody.

ANTISERUM. Blood serum in which there are specific antibodies.

BUFFER. Any substance or chemical compound that tends to keep pH constant when acids or bases are added.

DISCRIMINATION PROBABILITY. The probability that two randomly selected individuals will have the same combination of genetic markers.

ELECTROPHORESIS. A technique for the separation of molecules through their migration on a support medium under the influence of an electric potential.

ENZYME. A protein substance produced by living cells capable of speeding up specific chemical transformations, such as hydrolysis, oxidation, or reduction, but is unaltered itself in the process; a biological catalyst.

ERYTHROCYTE. Red blood cell.

FORENSIC SEROLOGY. Applying methods of physiological fluid differentiation in criminalistics.

FREQUENCY OF OCCURRENCE. The percentage of occurrence of a genetic factor in a population.

GENETIC MARKER. A readily recognizable gene that can be used in family and population studies.

GENETICS. The branch of biology dealing with heredity and variation in animal and plant species.

GENOTYPE. An entire set of genes in a particular system thought to be present in an individual from the results of the typing tests.

HEMAGGLUTINABILITY. Ability of red blood cells to agglutinate.

HETEROZYGOUS. Having two different allelic genes on the two corresponding loci of a pair of chromosomes. If different alleles are present on the two chromosomes, the cell is said to be heterozygous for that gene.

HOMOZYGOUS. The presence of two apparently identical alleles at a given locus on paired (homologous) chromosomes.

IMMUNOLOGICAL ANALYSES. Analyses of antigens by their reaction with antisera.

INCOMPLETE ANTIBODY. An antibody that does not react visibily with red cell containing corresponding antigen. Other terms for such antibodies are: late antibodies, blocking antibodies, glutinoids, glutinins, albumin antibodies, and hyperimmune antibodies.

ISOENZYMES (Isozymes). Multiple molecular forms of an enzyme in a single species.

pH. The negative logarithm of the hydrogen ion concentration in solution; a measure of solution acidity or basicity.

PHENOTYPE. The measurable characteristics of an organism that reflect the genotype in cooperation with the environment.

POLYMORPHS. Genetically determined alternative forms of enzymes and proteins.

RELATIVE HUMIDITY. The ratio of the amount of water vapor in the air to the amount that would saturate it at the same temperature.

SERUM. The plasma of blood that separates on clotting; the liquid that separates from the blood when a clot is formed.

STARCH GEL. An electrophoresis medium.

SUBSTRATE. The support medium upon which electrophoresis is run, or material on which blood was deposited.

TITER (ANTISERUM). The effective strength or concentration of antibody in an antiserum.

END