Detectability of Selected Genetic Markers in Dried Blood Upon Aging

National Institute of Law Enforcement and Criminal Justice
Law Enforcement Assistance Administration
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ABSTRACT

The purpose of this detectability study is to provide the practicing criminalist with adequate data to permit the selection of those blood genetic marker systems for serological analysis that have a high probability of yielding significant results. In addition, the data provide a baseline for detectabilities obtained by present analytical techniques that can be used to test the value of new and improved techniques. This is the first comprehensive detectability study that takes into account, for a large number of genetic marker systems, the factors of bloodstain history, i.e., the material on which the blood was deposited and the humidity at which it was stored.

The detectability of selected, genetically derived constituents in human blood aged for up to six months was investigated. Blood drawn from 12 volunteer donors was deposited on six substrates, i.e., glass, wool, nylon, and three types of cotton (plain cotton, perma press, and denim). The resulting dried blood specimens were aged at ambient temperature at 20 and 66% relative humidities; a few samples were aged at -20°C. Analyses were performed on the samples kept blind for the investigators at one, two, four, 13, and 26 week aging periods.

Red cell antigen systems selected for this study were ABO, MN, Rh, Kidd, Duffy, and Kell. The most stable antigens 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 aged for 26 weeks at both low and high relative humidities. The least stable antigens, Jk^a of Kidd, Fy^a of Duffy, and K of the Kell systems, were detectable for only one week at either humidity level. Of these antigens, only Fy^a and K aged at 20% humidity were detected at the two week test period. Other variants (S of MN and C, c, E, and e of

Rh) were detected for varying lengths of time ranging from two to 26 weeks. In particular, Rh factors C, c, and E were affected adversely by high moisture environments. Storage of the specimens at -20°C generally resulted in a longer detectability time than storage at room temperature. No significant differences in detectability resulting from substrate were observed.

The four enzyme systems selected for this study were adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (EAP). AK and PGM isoenzymes 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 isoenzymes appeared to be more stable at low humidity, and ADA and EAP at high humidity. No obvious differences in detectability resulting from phenotype or substrate were discerned, except possibly for perma press and denim which appeared to shorten the detectability time of PGM. Again, storage of the specimens at -20°C generally preserved the enzymes better than storage at room temperature.

The discrimination probability was calculated on the basis of the genetic markers which were still detectable at the end of each aging interval. No test error was assumed, and frequency of occurrence data for each genetic marker were taken from the literature.

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PREFACE

The study of the detectability 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. The program was directed toward the development of a blood individualization technique, capable of identifying blood antigens and protein-enzyme phenotypes. This technique would be acceptable for use by crime laboratories and would provide admissible evidence in our courts of law. An additional program objective is to compile a blood type frequency data bank for the U. S. population.

The purpose of the detectability study was to obtain data that would aid the practicing criminalist to select only those genetic marker systems for analysis, which, on the basis of serological evidence, would be most likely to provide usable data. The results would also serve as a baseline to determine the sensitivities of new or improved analysis methods currently under development. The majority of the genetic markers evaluated in this study are being analyzed in crime laboratories where blood individualization tests are performed. The study should, however, be expanded to include other systems of interest as they gain usage in forensic applications.

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

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*This report is a revision of the one previously titled, "Persistence of Selected Genetic Markers in Dried Blood," ATR-75(7910)-1, April 1975.

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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 indicate the presence or absence of an individual at the crime scene. However, blood found at crime scenes is most often in the dried state, and the ability to detect variants diminishes with the age of the serological specimen. Some genetic markers in bloodstains can still be identified after several years, whereas others cannot be successfully analyzed after only a few days. Because of such large differences in the stability of these variants, a knowledge of the detectability 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 detectability 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 detectability is not available.

To help overcome this lack of data, the detectability of selected genetic markers in dried blood aged under 20 and 66% relative humidity conditions at ambient temperature, as well as for several 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 an absorption-elution method, and the isoenzyme systems

adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (EAP) were studied by means of electrophoresis on starch gel. The antigen systems were selected on the bases of availability of antisera and of specific analysis techniques that were well characterized. ABO, MN, and Rh are the blood group systems most widely used in forensic applications, yet, even for these, the effects of humidity and to some extent of substrate are unknown. The AK, ADA, PGM, and EAP isoenzyme systems were chosen on the bases of similar considerations.

Blood used for the study was obtained from 12 volunteer donors. Glass, nylon, wool, and three types of cotton, i.e., plain, perma press, and denim, were used as substrates. Emphasis was placed on textile substrates because stains most often encountered in routine casework are on fabrics at crime scenes or on a suspect's clothing. The blood analyses were conducted after storage for one, two, four, 13, and 26 weeks.

For the dried blood specimens stored at 20 and 66% relative humidity, the average aging periods at which the red cell antigens were successfully analyzed on the various substrates are given in Figure S-1. Similarly, the storage periods at which the isoenzymes were identifiable are given in Figure S-2.

On the basis of the amount of enzymatic activity (see Appendix A, Tables A-2 through A-5) detected in dried blood, storage of specimens at the higher humidity condition appears to be better for the preservation of the ADA and EAP isoenzymes; the reverse appears to be true for the PGM isoenzymes. No effect resulting from differences in humidity was detected for the AK system.

Neither the antigen nor the isoenzyme systems exhibited significant variations in the detectability of genetic markers because of substrate differences. The

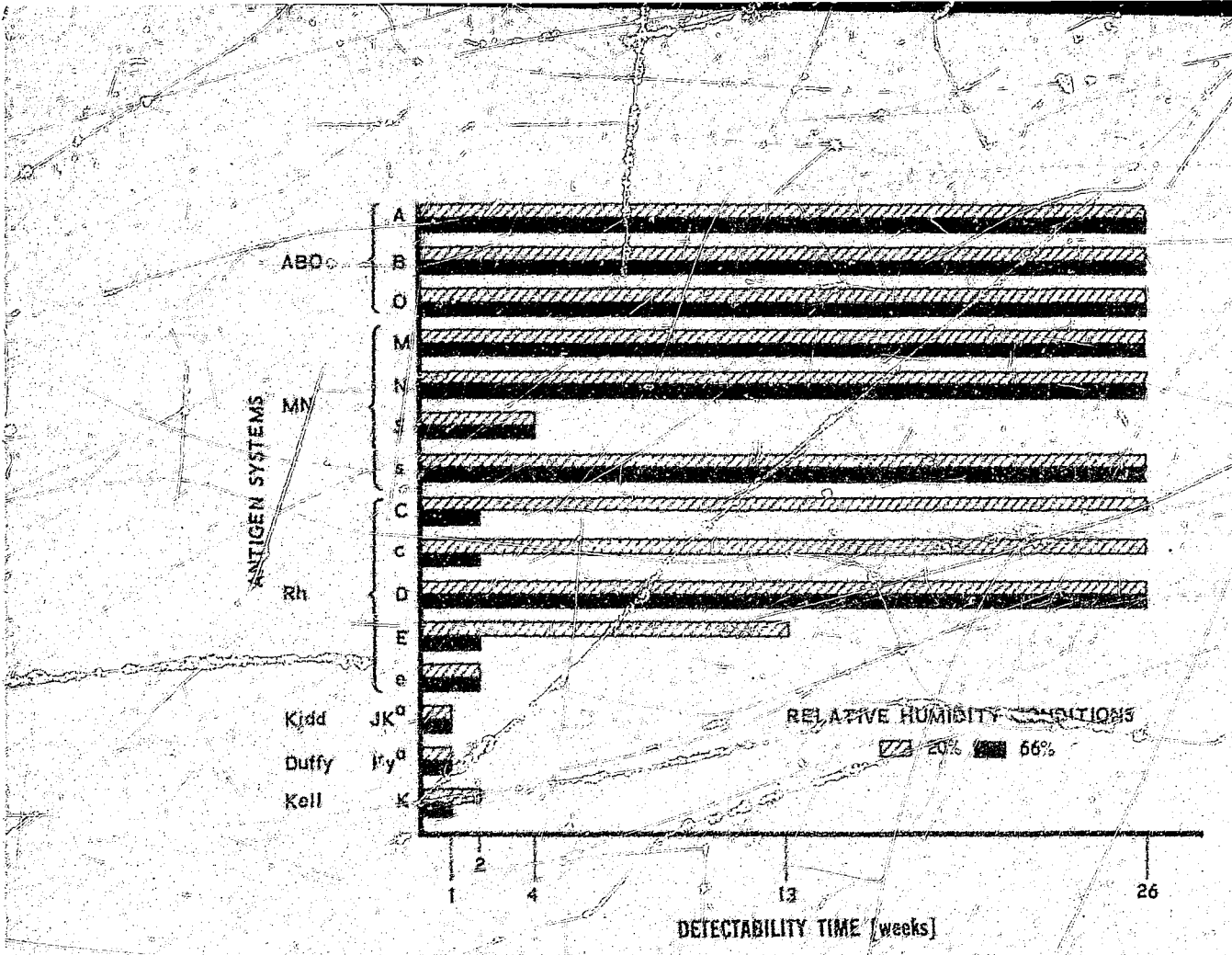

Figure S-1. Average Detectability of Red Cell Antigens in Dried Blood at Ambient Temperature

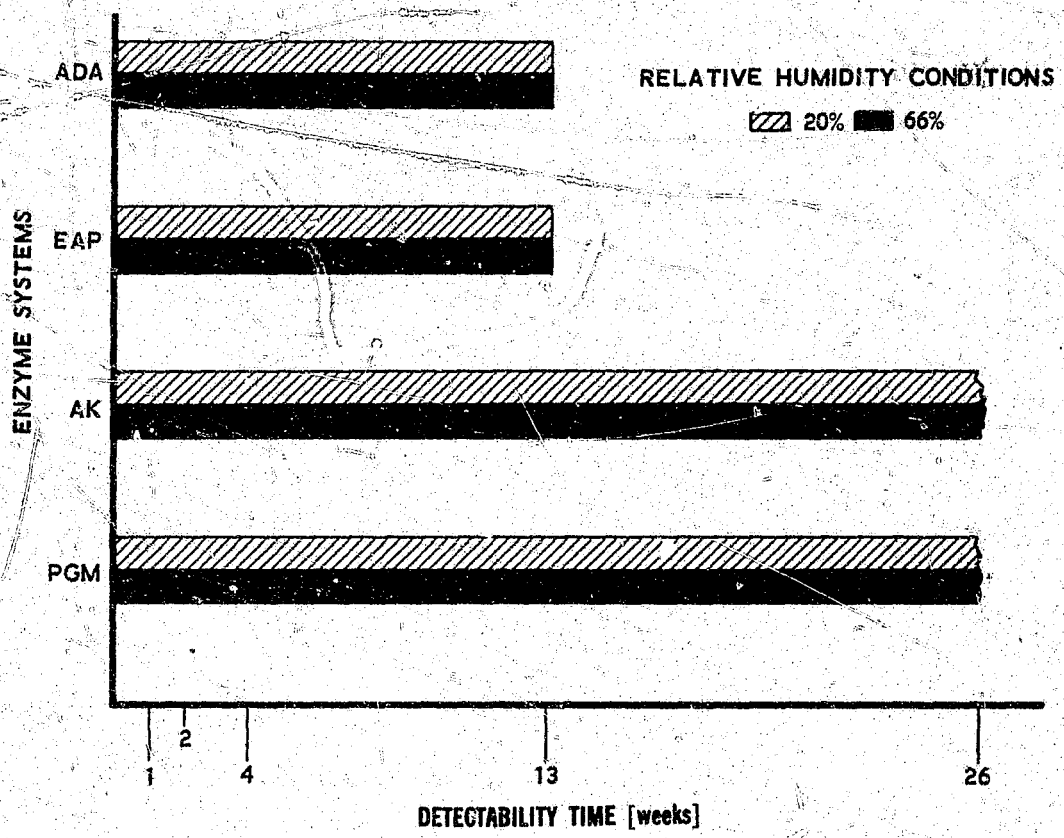

Figure S-2. Detectability of Enzymes in Dried Blood at Ambient Temperature

detectability of the antigens and isoenzymes for the specimens stored at -20°C, however, was found to be generally better than that for the specimens aged at ambient temperature.

Detectability data obtained in this study, together with the known frequencies of occurrence of the blood variants for the British population,* were used to calculate the discrimination probability (the probability that two randomly selected individuals will have the same combination of genetic markers) for each bloodstain aging period. For example, a discrimination probability 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.

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*Because the blood frequency data are unknown for most of the U. S. population, data for the British population were used to approximate possible discrimination values. Some U. S. crime laboratories use the same data for their calculations and court presentations.

CHAPTER I. INTRODUCTION AND BACKGROUND

The purpose of this study was to investigate the detectability with time of selected genetic markers in dried blood by using currently available analysis techniques and methods. The effects of humidity and substrate on genetic marker persistence and on the discrimination probability are presented, and should be useful to practicing forensic serologists, as well as to provide a reference to evaluate the sensitivity of improved analysis techniques and methods that may be developed.

Detectability of genetic markers in dried blood is significant because serological evidence is often found at the scene of major crimes (homicide, rape, and assault). The ability to establish the involvement of specific individuals in the commission of such crimes with a high degree of confidence is an important aspect of forensic science. Human blood contains many genetically controlled cell and serum constituents such as antigens, enzymes, and proteins. The inherited variants present in these constituents serve to characterize and identify blood; determination of these variants is known as blood "individualization."

The present study investigated the feasibility of determining the variants in bloodstains on fabrics after they were aged under controlled low and high humidity conditions for varying lengths of time. Emphasis was placed on the analyses of stains on fabrics because more than 90% of serological clue materials are found on textiles.[1*] Detectability of the variants of ABO, MN, Rh, Kidd, Duffy, and Kell red

*Values established by an Aerospace Corporation survey of 27 crime laboratories.

cell antigen systems and of adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (EAP) enzyme systems was studied 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. Although ABO, MN, and Rh systems are the blood group systems most widely used in forensic applications, the effects on them of humidity and, to some extent, of substrate are unknown. The AK, ADA, PGM, and EAP isoenzyme systems were chosen on the basis of similar considerations.

Detectability of antigens and enzymes in dried blood ranges from a few days to years, depending on the conditions of specimen exposure and on the particular genetic marker system being tested. Even for a single system there is little agreement among investigators on time limits for variant detectability. These discrepancies are due in part to the extremely complex nature of bloodstain analysis, to the development of more sensitive techniques for identifying genetic markers in stains, and to a lack of standard analytical procedures. Dried blood analysis often yields less definitive results than whole blood analysis and requires considerable expertise on the part of the serologist. Moreover, the reliability of the test results diminishes with the age of the stains.

The current investigation was initiated because of the large discrepancies in available data, and because of the paucity of information on the effects of substrate and humidity. Additionally, no systematic study encompassing many genetic marker systems has yet been conducted, and at the present time crime laboratories do not have a clear basis for determining which genetic markers should be tested to obtain meaningful results.

Loss of variant detectability in old, dried blood results from degradation and inaccessiblity of the antigenic sites with time, the latter being caused by cell shrinkage through continuous dehydration.[2] Hence, the number of antigenic sites per cell surface for each type of antigen is an important factor in the blood group determination of old stains and may help explain differences in detectability with age. The number of antigenic sites per cell has been calculated for some antigens. In the ABO system, A and B cells have approximately 1,000,000 and 700,000 sites, respectively.[3] In the Rh system, D (R_1 R_2 type) cells have 30,000,[4] c cells 79,000,[5] and e cells 21,000 sites.[5]

Other factors that play a significant role in variant detectability include the specificity of antisera and the strength of hemagglutinability; requirements for these may also change with the age of the stains.

Information in the literature regarding detectability of genetic markers in dried blood with age for the systems studied is given in Table 1. The data listed indicate the age of the stains when the genetic markers were detected through the use of several analytical techniques. Variations in the persistence of such red cell antigens as the Rh variants are reported. ABO system antigens are the most stable of those listed, and this system is by far the best known and most widely used in forensic serology. The Kell and Duffy systems are the least studied and least stable of the systems listed. Large differences in the reported persistence data were also found for the enzyme systems. In decreasing order of stability are AK, PGM, ADA, and EAP, with variations in persistence ranging from a few days to 11 months.

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	9
MN	M, N	29–39 wk	9-11
	S	26 wk	10
Rh	C, c, D	4–26 wk	10, 18-19
	E	5–6 wk	10, 18
	e	4–5 wk	10, 18
Duffy	Fy^a	6 days	20
Kell	K	3–19 days	12, 18
AK		4–48 wk	20, 22, 31-33
ADA		2–22 wk	31-32
PGM		4–22 wk	22, 24-26, 32
EAP		0.5–9 wk	22, 24-29, 32

Some comments on the difference in meaning between detectability and persistence are in order. Detectability is a function of the sensitivity of current analytical techniques, whereas persistence is a function of the rate of the denaturation or chemical degradation of the genetic markers. Thus, if a genetic marker is detected in a bloodstain aged for a certain length of time, it is known to persist for at least that amount of time. If, however, the marker is not detectable by present techniques, its persistence for that time remains unknown. It may still persist, but at a presently undetectable level.

The absolute persistence of genetic markers, in principle, can never be determined. However, for all practical purposes, the persistence may be considered to be the time the material takes to deteriorate to the extent that it becomes undetectable with current analytical techniques. This definition of persistence is operational and is commonly used. Operational persistence changes as the sensitivity of analytical methods increases. In this sense detectability and persistence are interchangeable terms.

CHAPTER II. EXPERIMENTAL PROCEDURE

A. Whole Blood Analyses

Fresh blood drawn from 12 volunteer donors at The Aerospace Corporation was analyzed by the Human Genetics Laboratory, University of California, Los Angeles. Blood group typings were performed by direct agglutination in test tubes, and polymorphic enzyme identifications made by electrophoresis with starch gel. The blood specimens provided 15 different red cell antigens and eight different isoenzymes for detectability studies.

B. Preparation of Aging Test Specimens

Blood drawn with 20 cc syringes from the same 12 donors was directly deposited from the syringes onto six different substrates: glass, wool, nylon, and three types of cotton, i.e., plain, perma press, and denim. Before the deposition of blood, the cloth samples were washed in a phosphate-type detergent (Tide), thoroughly rinsed, dried for 24 hours, and cut into one inch squares. The nylon, wool, perma press, and denim were purchased new, whereas the plain cotton was a printed sheet that had been used for three years and had undergone repeated washings.

Glass (in the form of microscope slides) served as a control substrate. Unlike textiles, glass is considered to be inert to blood constituents, being void of detergents, preservatives, dyes, and other possible interferences. However, because of sample losses encountered during the washing procedure of the analyses for antigens, testing of the glass specimens was limited to determination of enzymes.

The 26 bloodstained specimens were air dried for 24 hours in a fume hood. They consisted of duplicate sets of 12 specimens each, plus two (stained cotton specimens) reserved for freezing. The duplicate sets were separated into two constant humidity chambers for aging at ambient laboratory temperature (23°C). One chamber was maintained at 20% relative humidity by a saturated aqueous solution of potassium acetate, the other at 66% relative humidity by a similar solution of sodium nitrite. One hundred percent relative humidity at 23°C corresponds to a water vapor pressure of 21 mm Hg; variations in this value as a result of ambient temperature fluctuations ($\pm$ 3°C) amount to $\pm$ 4 mm Hg. The humidity chambers consisted of glass dessicators, and were exposed to fluorescent lighting throughout the aging period. The remaining two bloodstained specimens, prepared on plain cotton, were aged in a freezer at -20°C, in the dark, at a humidity value of 0.9 mm Hg.

To reduce the total number of analyses, specimens aged for four weeks were evaluated first. The test approach used in this study is shown in Figure 1. Antigens and enzyme polymorphs detectable at four weeks were analyzed at 13 weeks and, if still detectable, at 26 weeks. For variants that could not be identified at four weeks, the experiment was repeated, and tests to detect the genetic markers were performed at two weeks. The experiment was repeated again for undetectable variants, and the tests performed at one week.

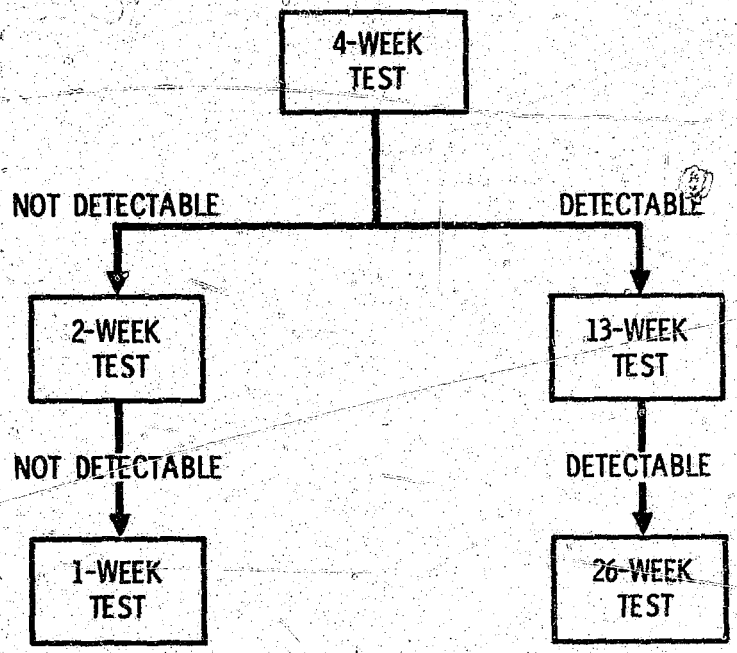

Figure 1. Design Used in Dried Blood Analysis

The types and numbers of analytical tests performed in this study are presented in Table 2. The number of tests listed, altogether totaling over 2100, does not include the repeated analyses performed when questionable results were obtained. With an average of 20 minutes per test assumed, the time expended on dried bloodstain analyses alone exceeded 700 hours.

Table 2. Number of Analytical Tests 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

C. Antigen Typing of Dried Blood

Of the several methods that can be employed for the determination of antigens in dried bloodstains, absorption-elution is the most sensitive and the most widely employed. Described by Kind, [6,7] Nickolls and Pereira [8] Outteridge [9] and Fiori, [10] absorption-elution has proved to be markedly more sensitive than the absorption-inhibition method; [10] it has also been reported to be more successful than mixed agglutination for certain antigens. [12] For grouping in bloodstains, in contrast to grouping in saliva or seminal stains for secretors, absorption-elution is the method of choice.

Because there are numerous variations of the absorption-elution method in use, the specific technique employed for each antigen was adapted from those described in the literature. The procedures followed for the typing of the 15 antigens are summarized in Table 3. Although variations, such as the use of papain-, ficin-, or trypsin-treated indicator cells, were attempted, no significant improvements were obtained.

All antisera except anti-Fy^a and anti-Jk^a were obtained from Hyland Laboratory. Anti-Fy^a was obtained from Spectra Biologicals, and anti-Jk^a, as well as the antihuman globulin, were obtained from Dade. Because significant variability in their titers was observed, the antisera were tested, at times extensively, for their suitability for use by the absorption-elution technique. Antiserum that is too concentrated may produce agglutination in negative controls, and antiserum that is too dilute may result in insufficient absorption and subsequent false negatives. Therefore, negative controls consisting of unstained samples of all five textile substrates, subjected to procedures identical to those of the bloodstained substrates, were run

Table 3. 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
M,N	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.

under both humidity conditions as well as in the freezer. The antisera were diluted until they gave negative results with these controls. In doubtful cases they were tested with specific fresh blood samples to prevent false negatives.

Certain antigen systems present particular problems. MM cells are known to react with many samples of anti-N sera, causing them to be misgrouped as MN. For this reason exhaustive screening of every anti-N serum was performed to ensure that the serum used in the testing gave negative results with type MM cells. Additionally, in the Rh system, anti-C sera are known to be often contaminated by anti-D serum. Therefore, the anti-C sera were tested in the elution procedure with type Rh D positive, C negative cells, as negative controls. Negative results ensured the absence of anti-D contamination.

1. ABO (A, B, H) and MN (M, N, s) antigens. Fifteen 3 x 3 mm squares were cut from each of the bloodstained specimens tested at every aging period and placed in 10 x 75 mm glass test tubes. To each test tube containing a cloth specimen, two drops of cold physiological saline solution (4°C, pH 7.4) were added, followed by one drop of the appropriate antiserum (A, B, H, M, N, or s) (see Table 3). The mixture was stoppered and incubated in a refrigerator (4°C) overnight. The saline solution and excess antiserum were withdrawn from the tube and sucked from the cloth with a pipette attached to an aspirator. The cloth specimens were then washed six times with cold saline, the tubes being filled with saline and left standing at room temperature for 15 to 20 minutes for each wash. Care was taken to remove all of the saline solution, especially after the last wash.

For the elution, two drops of saline were added to each tube and the cloth specimens were carefully submerged in the solution. The tubes were stoppered and placed in a circulating water bath at 56°C for 15 minutes. The tubes were then removed from the water bath, the cloth specimens quickly removed from the tubes, and a drop of 0.5% saline suspension of the appropriate indicator cells was added to each tube without delay. After reabsorption for one hour in the refrigerator (4°C), one drop of antihuman serum was added (Coombs technique) when necessary (see Table 3). The tubes were centrifuged for three minutes at 1500 rpm, and their contents examined for agglutination macroscopically with a concave mirror (producing 1.2X magnification) and a light; microscopic examinations were made for confirmation when necessary.

2. MN (S), Rh (C,c, D, E, e), and Kidd (Jk^a), Duffy (Fy^a), and Kell (k) antigens. The procedure followed was basically as described in the foregoing, the modifications for each antigen being clearly noted in Table 3. For Kidd, Duffy, and Kell systems, the method was adapted from Ruffie and Ducos.[13]

D. Enzyme Analysis of Dried Blood by Electrophoresis

One-inch square cuttings of all bloodstain specimens tested at every aging period were submitted to the Human Genetics Laboratory, University of California, Los Angeles, for electrophoretic analysis of the four enzyme systems containing the eight isoenzymes under study. The experimental procedures followed by the Human Genetics Laboratory are detailed in Table 4.

For ADA and EAP (also GPT), preparation for analysis involved the soaking of samples of the dried blood specimens for one hour in two drops of a 1%, β - mercaptoethanol solution in the gel buffer appropriate for each enzyme (see Table 4);

Table 4. 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[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.

for PGM and AK, treatment with the sulfhydryl reagent (β -mercaptoethanol) was generally omitted. The B-mercaptoethanol serves to reduce the S-S disulfide bridges in aged molecules which result in the distortion of electrophoretic patterns absorbed on a Whatman No. 3 filter paper (8 x 5 mm) that was inserted directly into the starch gel for the respective electrophoretic runs.

The isoenzyme separations were routinely carried out with 10.5% Electrostarch (Otto Hiller) gel in a Grafar electrophoresis chamber (13 x 30 x 0.6 cm) equipped with a Buchler power supply (Model No. 3-1014A), 0 to 1000 V, 0 to 250 ma. The biochemicals were obtained from Sigma Chemical Company, St. Louis.

1. Adenosine deaminase (ADA). The determination of ADA in the aged bloodstains was performed with a cooling plate in accordance with the method described by Spencer et al.[14]

2. Adenylate kinase (AK). Starch gel electrophoresis followed the procedure of Fildes and Harris [15] in which a cooling plate in the refrigerator at 180 V (4 V/cm) was used, but without addition of magnesium chloride to the staining agar overlay. (Control runs showed the addition of $MgCl_2$ to be nonessential for good results.)

3. Erythrocyte acid phosphatase (EAP). Starch gel electrophoresis was carried out at 4°C according to the method of Swallow and Harris [16] with the following modification in the staining procedure of Sparkes et al.[17] A solution

containing 0.05 gm of phenolphthalein monophosphate* in 125 ml 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.[18]

After development with a staining agent, the electrophoretic plate was read by qualitatively characterizing the resultant band pattern into five categories:

0	-	No detectable enzymatic activity
1	-	Faintly detectable but not identifiable
2	-	Faintly detectable and identifiable
3	-	Identifiable
4	-	Clearly identifiable

Readings of 2 or more were interpreted as positive results. Although such readings, being based on the judgment of the analyst, are subjective, the relative values can indicate the effects of storage conditions. Further details of the electrophoresis results including these reading values are presented in Appendix B.

*Hydrolysis by 4-methylumbelliferyl phosphate, used by most crime laboratories to visualize EAP, was found by Sparkes et al.[17] to be less satisfactory than phenolphthalein monophosphate.

E. Blind Testing

For the purpose of simulating actual crime laboratory conditions where the identity of bloodstain evidence samples is unknown, as well as to ensure investigator objectivity, all samples were tested blind. That is, testing for a specific antigen or isoenzyme was not limited to those samples known originally to contain that antigen or isoenzyme, but all samples were tested according to the design shown in Figure 1, their identity being unknown to the investigators at the time of testing.

CHAPTER III. RESULTS AND DISCUSSION

The results of the fresh blood analyses for the 12 volunteer donors, performed by the Human Genetics Laboratory, University of California, Los Angeles, are presented in Table 5 for the red cell antigens and in Table 6 for the enzymes. From these results 15 antigens and eight isoenzymes (comprising four enzyme systems) were selected for the detectability study of up to six months. Antigens A_1 and A_2 were not differentiated during the study, i.e., typing was performed only for antigen A in general, and the enzyme GPT and protein Hp were excluded.

The distribution of the 15 antigens and eight isoenzymes on the various substrates is included in Figures 2 through 9. Also included is the number of samples of each genetic marker tested under both low (20%) and high (66%) relative humidity conditions (at 23°C) on each substrate. For example, the antigen A of the ABO system was tested on nylon and on perma press cotton, and on each of these substrates one sample of the antigen was tested for each humidity condition (see Figure 2).

A. Red Cell Antigen Detectability

Results of the detectability study of the 15 red cell antigens of the ABO, MN Rh, Kidd, Duffy, and Kell systems are schematically presented in Figures 2 through 5.

1. ABO antigens (Figure 2). The ABO antigens in dried blood were detected after 26 weeks of aging. The H antigen, in particular, was unaffected by the six types of substrate on which it was deposited and by the two humidity conditions under which it was stored; moreover, on plain cotton it was unaffected by freezing temperature. Antigens A and B, studied less completely, exhibit similar characteristics.

Table 5. Blood Group Phenotype[a] of Donors

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

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

Table 6. 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.

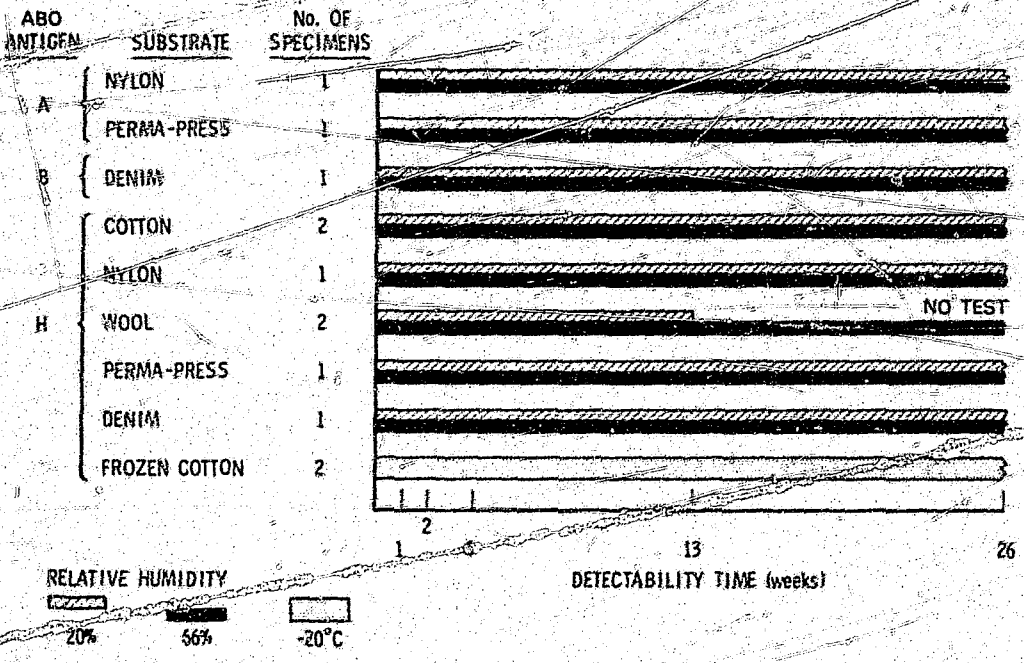

Figure 2. Detectability of ABO Antigens

Hence, the evidence implies no significant substrate, humidity or temperature effects on the persistence of the ABO antigens, at least up to 26 weeks. Fiori[10] has reported A and B antigens to be detectable on bloodstained fabric at ambient conditions after two years. He also successfully typed a 34-year old type A stain. Fiori's analyses were also 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.

2. MN Antigens (Figure 3). The M, N, and s antigens behave similarly to the ABO antigens, being insensitive to substrate, cold temperature, and humidity. They were generally identifiable for up to 26 weeks of aging.

The S antigen, least stable of the MN system, was detectable for four weeks. No specific effects of substrate or humidity conditions were noted; the short detectability time appears to result from deficiencies in the analytical technique as Lincoln and Dodd[11] were able to type S antigen in stains for 26 weeks.

M and N antigens in stained cloth have been reported by Fiori[10] to be detectable after 29 weeks of aging under ambient conditions. In a study conducted by Pereira,[12] M and N were detectable after 39 weeks. It appears 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 variations in the detectability of a specific antigen. These considerations may explain some of the discrepancies in the reported detectabilities.

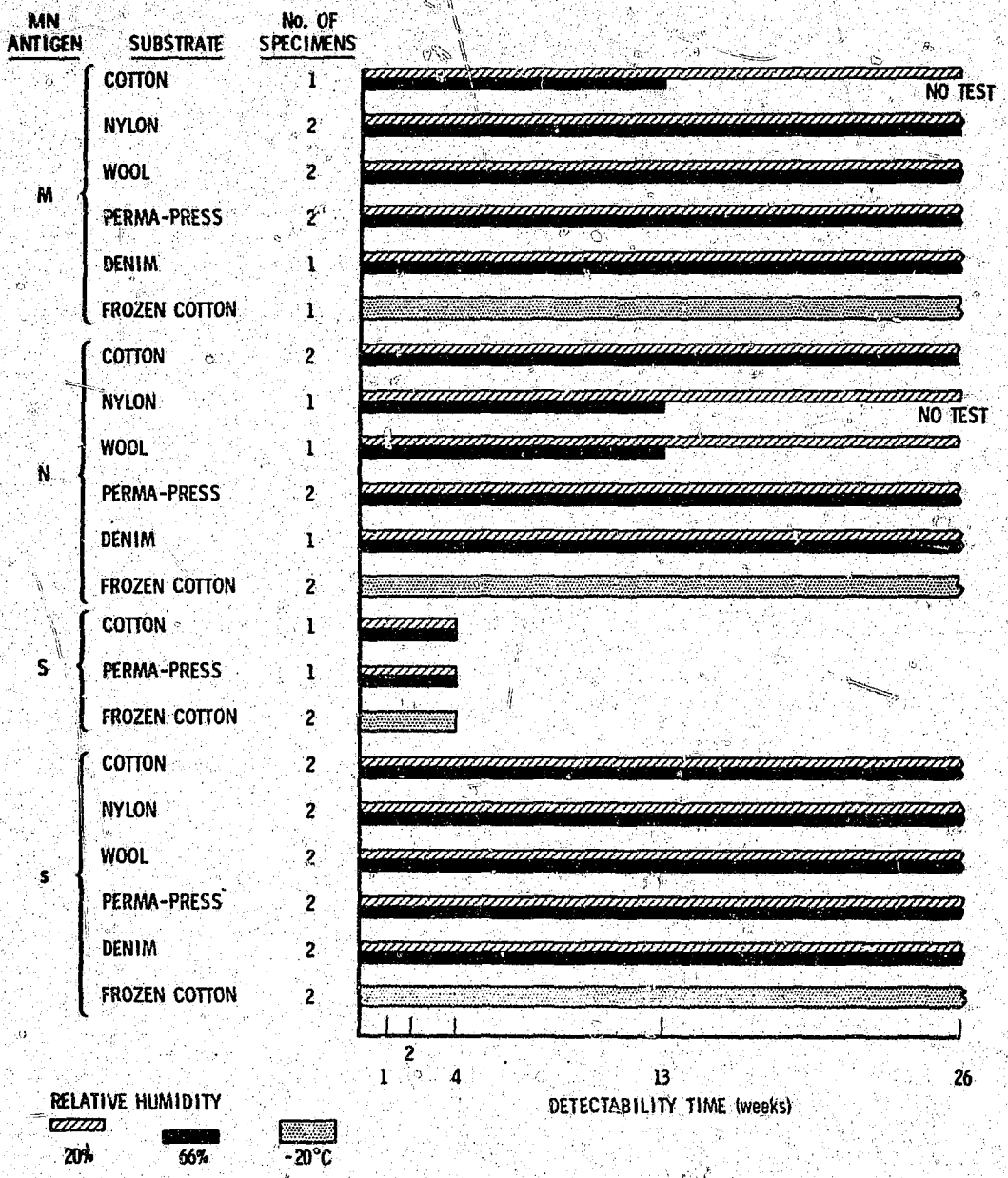

Figure 3. Detectability of M and N Antigens

3. Rh Antigens (Figure 4). The D antigen was detectable after 26 weeks in every specimen tested. Antigen e, on the other hand, was detectable for only about two weeks. Both D and e detectabilities appear to be independent of substrate, humidity, and temperature.

Detectability of antigen C, c, and E is affected by humidity, but not by substrate or temperature. Upon storage of the specimens at 20% relative humidity at ambient laboratory temperature (23 $\pm$ 3°C), C was generally detectable at 26 weeks, and c and E at 13 weeks. Storage at 66% relative humidity, however, shortened detectability time to two weeks for C and E, and to between two and four weeks for c. Moisture, therefore, has an adverse effect on the persistence of these Rh antigens, which suggests that blood in general should be preserved in a dry environment. This conclusion confirms the experience of criminalists, although no study attempting to verify this has been reported.

Antigens C, c, and D have been successfully typed on 26 week old stains by Lincoln and Dodd,[11] and they have been identified in specimens aged from four to eight weeks by other investigators.[19-21] Antigens E and e have been reported to persist from four to six weeks;[11-19] however, failure to detect e even in fresh stains because of poor quality antiserum has also been reported.[21]

4. Kidd, Duffy, and Kell Antigens (Figure 5). The antigens of the Kidd, Duffy, and Kell systems are extremely difficult to type because of weak agglutination between the antigens and their antibodies. The antibodies will not directly agglutinate cells containing the appropriate antigens because the antigens, being univalent, lack a second binding site. The cells must, therefore, first be sensitized

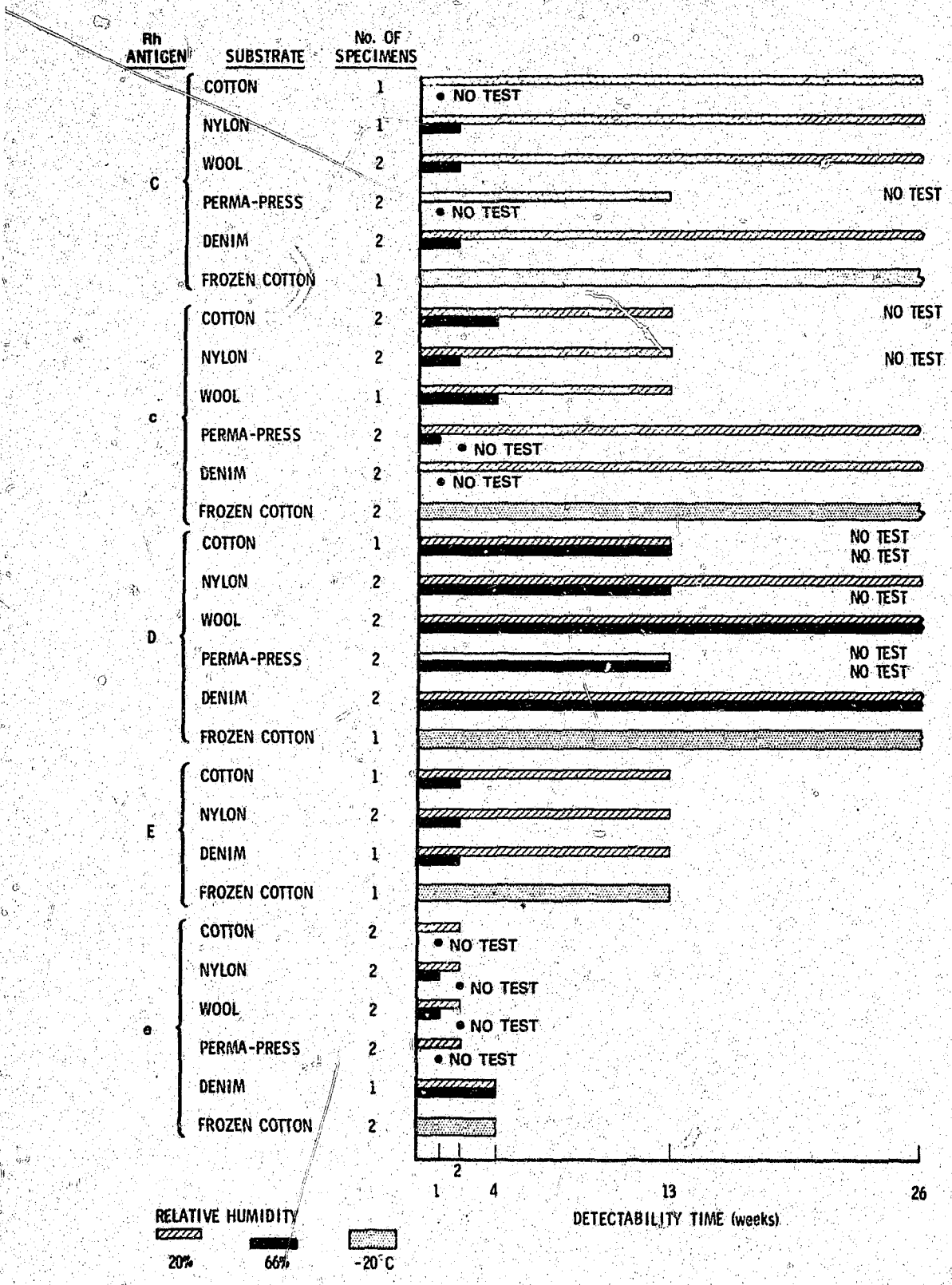

Figure 4. Detectability of Rh Antigens

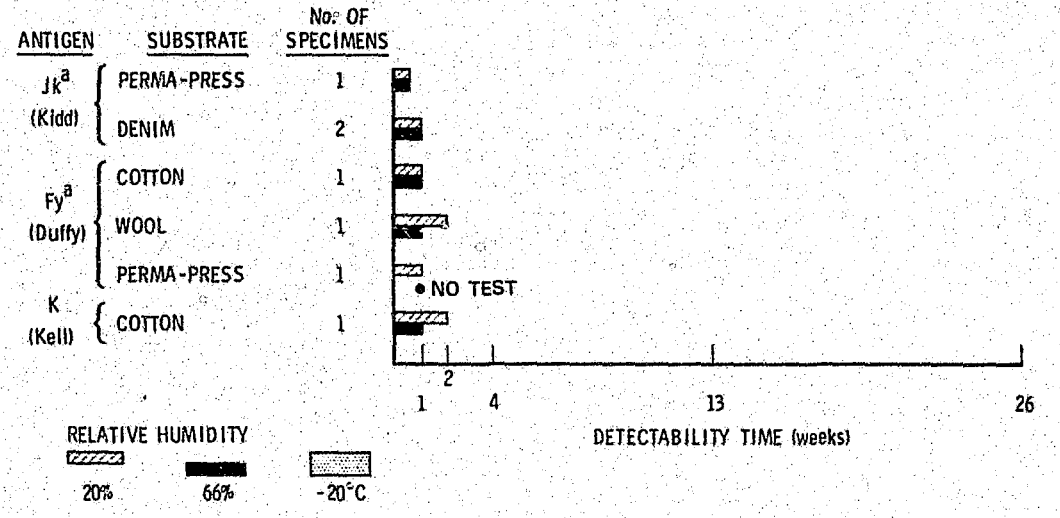

Figure 5. Detectability of Kidd, Duffy, and Kell Antigens

with the antibody, and then agglutination brought about by the addition of Coombs (antihuman globulin) serum. Often, no clear-cut results were obtained. Jk^a of Kidd (on denim at both humidities), Fy^a of Duffy (on cotton, wool, and perma press, at both humidities) and K of Kelly (on cotton, at 66% humidity) were identified after repeated analyses of specimens aged one week. Fy^a and K were detected in specimens stored two weeks at the 20% humidity level.

Stains on cotton, wool, perma press, and nylon containing no Kidd Jk^a antigen were tested for Jk^a antigen, to determine if false positives would develop. No false positives were obtained, indicating that, in general, substrate-blood interactions produce no materials that react with Jk^a antisera.

According to the literature, the detectability of Kidd, Duffy, and Kell antigens has not been studied in detail. It has been reported, however, that Fy^a can be detected after six days and K after 19 days.[19]

B. Erroneous Antigenic Test Resutls

This study shows that erroneous tests results, either positive or negative, can be obtained even when using the most dependable techniques available. In some instances, negative results were obtained for antigens detected at later times and shown to persist for longer periods in subsequent tests (see Table 7). For example, K was detected after two weeks of incubation at 20% humidity, but not after one week. At other times, positive results were obtained for antigens known to be absent from the sample (see Table 8). The false negatives are probably caused by overwashing of the samples, whereas the false positives may be caused by sample underwashing, or by some substrate effect. Bloodstains deposited on denim, nylon, and wool produced most

Table 7. False Negative Test Results

Substrate	System	Antigen	Weeks	Relative Humidity (%)
Denim	MN	M	2	66
Denim	Rh	E	2	20
Denim	Rh	E	4	20
Denim	Rh	e	1	66
Cotton	ABO	H	4	66
Cotton	MN	N	2	66
Cotton	MN	s	2	66
Cotton	Kell	K	1	20
Wool	MN	s	2	20
Wool	MN	s	13	20
Wool	Rh	e	1	20
Perma press	MN	S	2	20
Perma press	MN	s	2	20
Perma press	Rh	e	1	20
Nylon	MN	s	2	66

Table 8. False Positive Test Results

Substrate	System	False Antigen	Weeks	Relative Humidity (%)
Denim	ABO	A	26	66
Denim	ABO	B	26	20
Denim	ABO	B	26	66
Denim	MN	M	13	20
Denim	MN	N	2	66
Denim	MN	S	2	66
Nylon	ABO	A	26	20
Nylon	MN	N	26	66
Nylon	Rh	C	2	66
Wool	MN	N	2	20
Wool	Rh	c	26	20
Wool	Rh	E	2	20
Cotton	MN	M	4	20
Perma press	Rh	E	2	20

of the false positives. Denim regularly came apart upon washing, and the attempt to avoid this may have induced insufficient washing. Nylon, being a polyamide, contains peptide-like linkages (-CO·NH-), and wool contains the proteinaceous substance keratin; both of these may conceivably resemble antigenic sites and thus interfere. Difficulties in typing on nylon have been reported by de Ren et al.[22]

The large number of analyses conducted at each aging test period precluded the repetition of many tests. In the actual practice of serological evidence analysis, however, the importance of conducting tests at least in triplicate, and with proper controls, cannot be overemphasized. Reproducibility is necessary if the results are to be used in court testimony and if the validity of the ensuing conclusions is to go unchallenged.

C. Detectability of Isoenzymes

The electrophoretic separation and identification of isoenzymes 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 determination of antigens. Although the interpretation of the electrophoretic band patterns is subjective, being based on the judgment of the analyst, the results may indicate some effects of storage conditions.

1. AK Isoenzyme (Figure 6). For the AK system, all 12 donors participating in the study were of phenotype 1. The isoenzyme was faintly detectable and identifiable at 26 weeks for both the 20 and 66% relative humidity conditions on all six substrates, as well as for the frozen samples. At the 13 week period, the band patterns were clearly defined for all storage conditions. No differences among aging conditions or substrates were discerned.

The activity of AK enzymes was reported by Welch [23] to decrease somewhat on aging of the stain from 15 to 30 days, although at 30 days the pattern was still clear and readily discernible. Brinkmann et al.[24] and Rothwell [25] were able to analyze the isoenzymes after 11 months of storage at room temperature. Brinkmann et al. also reported that although the band patterns were indiscernible, the activity could still be detected on six year old stains. Rothwell was able to identify the AK isoenzymes after storing the samples at -20°C for two years.

2. ADA Isoenzymes (Figure 7). At the 26 week analysis period, only one out of the 14 samples of the ADA isoenzymes tested (one of two samples of phenotype 1 run on frozen cotton) was clearly identifiable. All others exhibited only faintly detectable enzymatic activity. At 13 weeks, all ADA isoenzymes were identifiable; however, specimens stored at high humidity (66%) produced better defined ADA band patterns than those stored at low humidity (20%). No difference in persistence was found between phenotypes 1 and 2-1 nor was there any effect of the various fabric substrates. The specimens stored in the freezer fared best, confirming that blood samples in general should be preserved at low temperature, where the humidity is also low (see Appendix A, Table A-3).

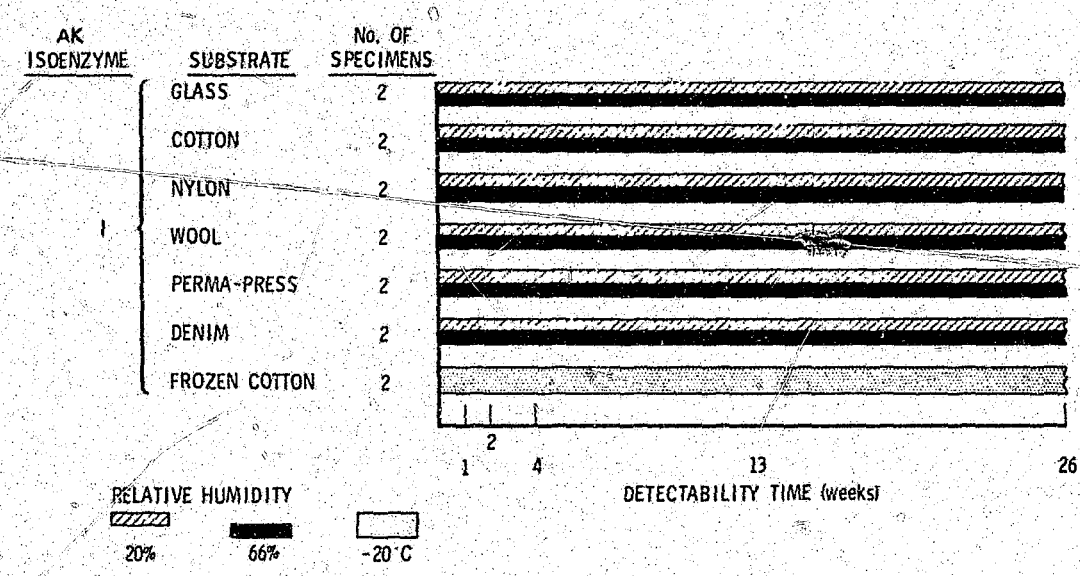

Figure 6. Detectability of AK Isoenzyme

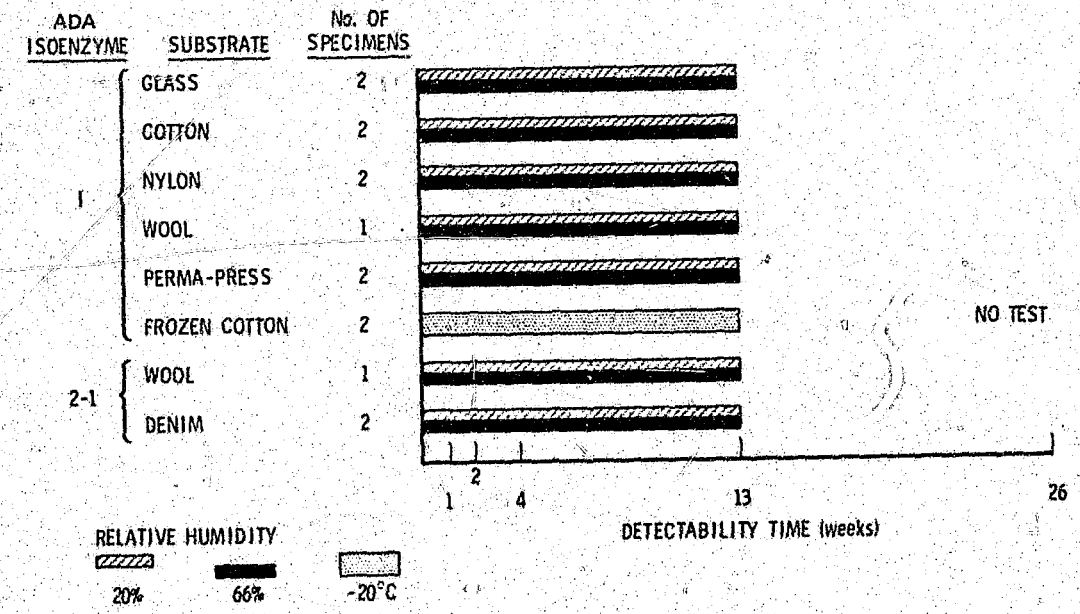

Figure 7. Detectability of ADA Isoenzymes

The results of this study, which indicate that ADA isoenzymes are detectable in dried blood at 13 but not at 26 weeks, are consistent with a 22 week persistence reported by Brinkmann and Dirks.[24] In contrast, Welch [23] found that the ADA isoenzymes in bloodstains on cotton, exposed to air and ambient temperatures, could be identified after 15 but not after 20 days.

3. PGM_1 Isoenzymes (Figure 8). Perma press and denim appear to hinder the detection of PGM_1^{1} and PGM_1^{2-1} at 26 weeks. All three PGM_1 isoenzymes were detected after 13 weeks of aging, although the band patterns were clearer for the specimens stored at the lower level of humidity (in contrast to the ADA isoenzymes).

PGM isoenzymes have been reliably detected in dried blood after four to 13 weeks, [23, 25-28] and occasionally after five months.[25-28]

4. EAP Isoenzymes (Figure 9). Both B and BA isoenzymes of the EAP system were detected after 13 weeks of storage in all cases but one (B on cotton). Detection after 26 weeks occurred twice (B on denim, and BA on perma press, the two substrates being those appearing to have an adverse effect on PGM) under high humidity (66%) storage, and once on frozen cotton (B). At 13 weeks, greater enzymatic activity was consistently observed for the specimens aged under the higher humidity level (66%) than for those stored at the low level (20%).

In a study conducted by Brinkmann et al., [29] B and BA phenotypes were identified in stains up to nine and six weeks, respectively. In the present study no significant difference in the detectability of these two phenotypes was observed, both phenotypes being generally detectable at 13 weeks. In other literature, a large discrepancy exists in the detectability of the EAP isoenzymes in stains. Reported time

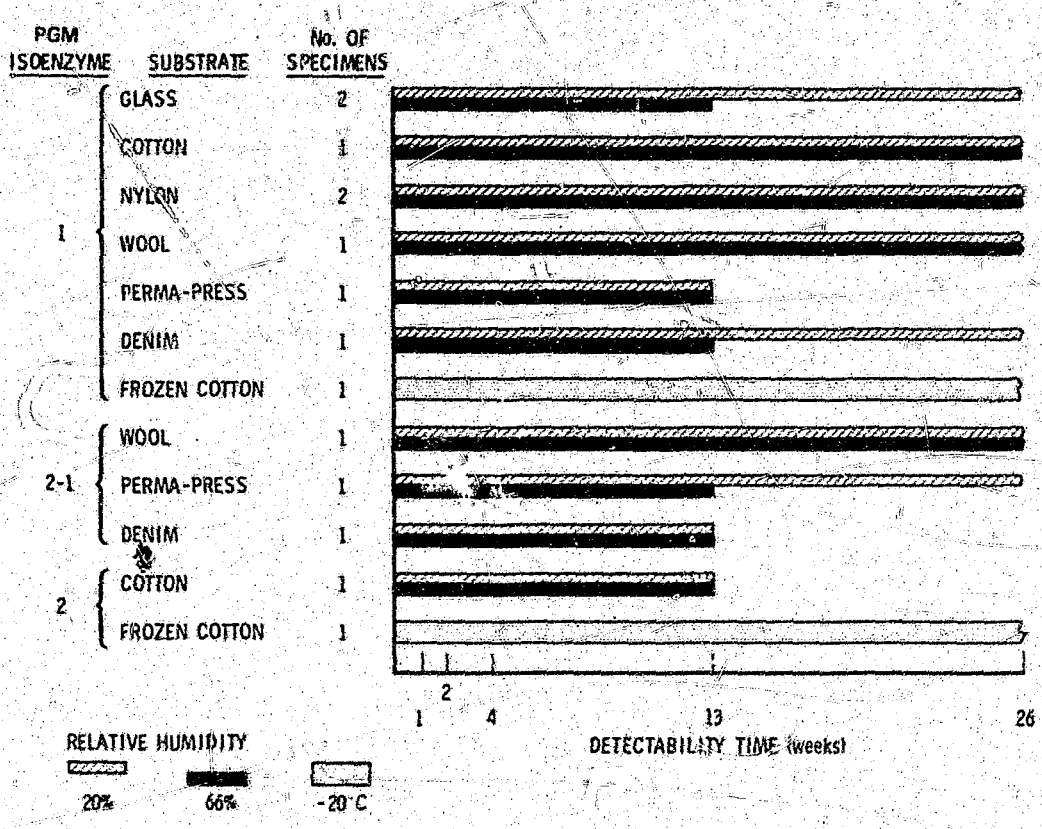

Figure 8. Detectability of PGM_1 Isoenzymes

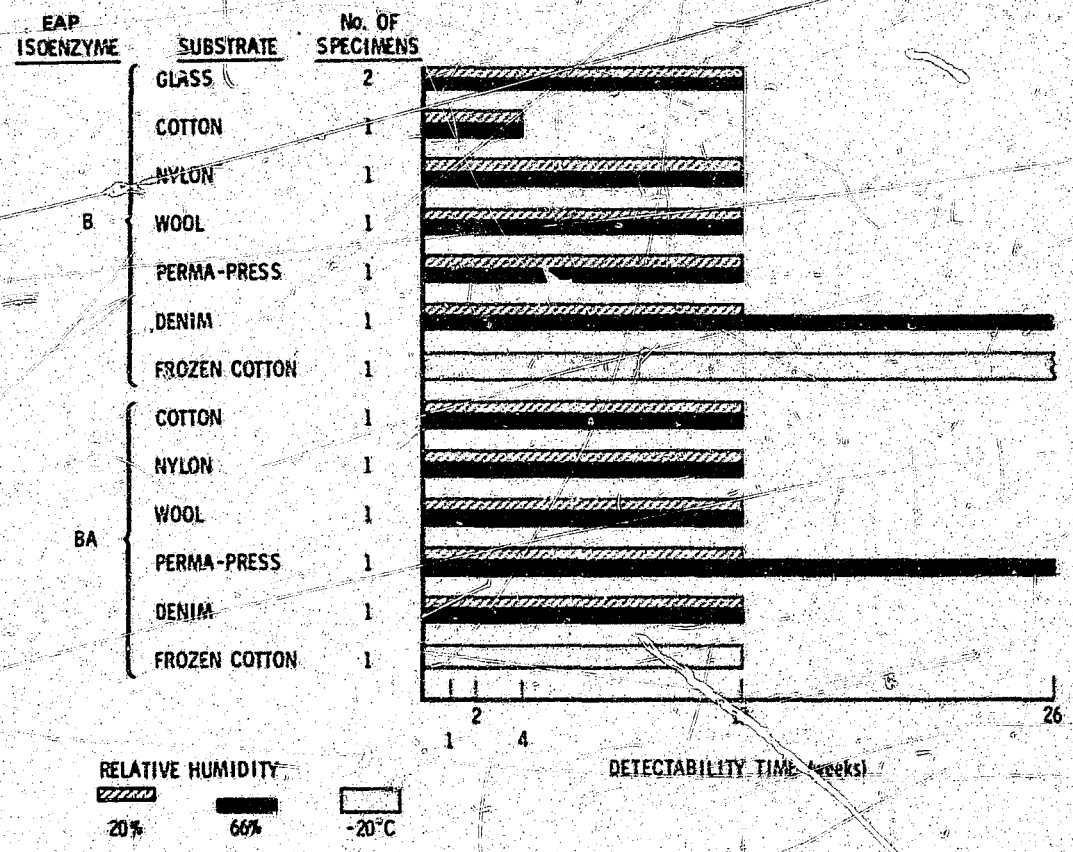

Figure 9. Detectability of EAP Isoenzymes

limits for their identification vary from a few days,[30] 22 days,[23] 30 days,[31] to eight or nine weeks. This study has shown that EAP isoenzymes can be detected after 13 weeks of storage under a variety of conditions.

Further details of the electrophoretic results including band characterizations are presented in Appendix A.

D. Discrimination Probability

On the basis of the results of this aging study and of the frequencies of occurrence of the different variants (see Appendix B, Table B-1), it is possible to calculate the discrimination probability achievable by the analyses of the genetic markers persisting at each test period.* Discrimination probability as applied in this report is defined as the probability that two randomly selected individuals will have the same combination of the detectable blood variants included in this study. Whenever a variant becomes unidentifiable, the degree of individualization resulting from the bloodstain analysis decreases. The degree of individualization determines the

*Admittedly, this aging study is incomplete, since it covers a limited number of genetic marker storage conditions, analytical techniques, and investigative skills. The frequencies of occurrence of the genetic markers are for the British population (being unknown for most of the U. S. population) and thus do not strictly apply to the U. S. Nevertheless, calculation of the discrimination probabilities is considered useful as a guide for showing the approximate degree of individualization that can be obtained based on the detectability of the factors in aged bloodstains.

extent to which a suspect can be excluded from involvement in a crime, or, alternatively, identified as the criminal from a closed group of suspects. The overall discrimination probability for each aging test period is obtained by multiplying the discrimination probabilities of all the variants detectable at that period. Figure 10 shows the calculated discrimination probabilities with time for the two relative humidity conditions. The method of calculation and the discrimination probabilities of individual systems for the different test periods are presented in Appendix B.

A discrimination probability of one out of approximately 2500 is possible after one week of storage under both 20 and 66% relative humidity conditions; i.e., out of 2500 people, two are expected to have the same combination of the variants included in this study. Since most serological evidence reaches the local crime laboratories within one week, this value applies in many cases.[1] At 26 weeks, however, the values are one in 65 and one in 28 for aging under low and high moisture levels, respectively. Thus, the degree of individualization decreases rapidly with increasing age of dried blood, especially when exposed to a humid environment.

Development of more sensitive analysis techniques may extend the detectability times of the genetic markers and thereby enhance the degree of individualization of dried blood upon aging. Moreover, to achieve still greater discrimination of bloodstains, analysis of additional genetic markers can be performed. Among the

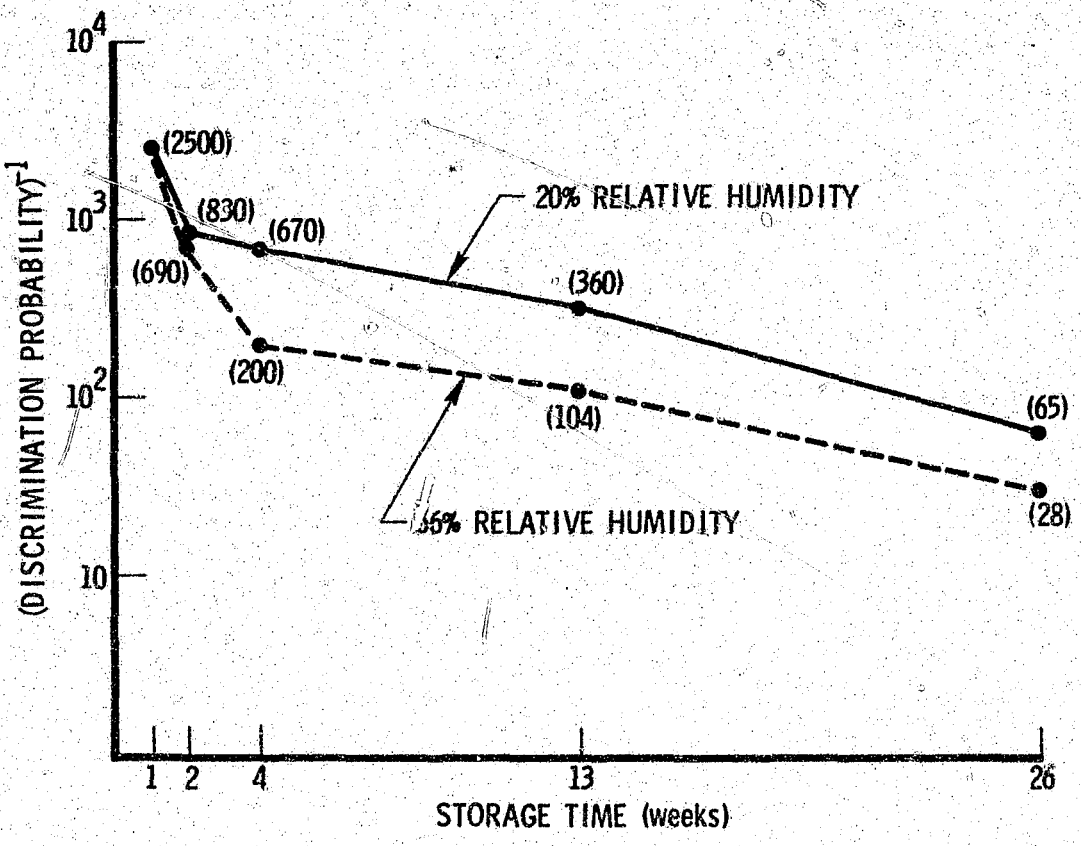

Figure 10. Discrimination Probability with Age of Dried Blood

systems greatly enhancing discrimination probability (because of their favorable frequency distribution in the population) are the gamma globulin (Gm) and human leucocyte antigens, loci A, B, and C (HLA A, B, or C)* antigens, the glutamate-pyruvate transaminase (GPT, also known as alanine aminotransferase) isoenzymes, and the group specific component (Gc) and haptoglobin (Hp) polymorphic serum proteins. Methods of grouping these systems in bloodstains, though available, have not been routinely carried out in U. S. crime laboratories.

Additional systems, having less favorable frequencies of occurrence, include hemoglobin (Hb), glucose-6-phosphate dehydrogenase (G6PD), 6-phosphogluconate dehydrogenase (6 PGD), esterase D (EsD), and pseudocholinesterase (PCE). Use of these systems by U. S. criminalists is rare because the analytical techniques are complex and validity of the results is uncertain. Improved techniques and additional studies on the persistence of these genetic markers are therefore desirable.

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*The Aerospace Corporation sponsored a persistence study at the Department of Surgery, University of California, Los Angeles, which showed that HLA loci A1, A3, A9, and locus B7 persist at least one month. The preliminary study also demonstrated the feasibility of the application of the HLA system to the individualization of blood evidence in the law enforcement field. A report, "Identification of Dried Human Blood Samples by HLA Antigens," M. Tagasugi, D. Akira, and P. I. Terasaki, was presented at the 43rd Semi-annual Seminar of The California Association of Criminalists in Long Beach, California, 9-11 May 1974.

E. Limitations

The information in this report will be useful to the forensic serologist, who must decide for each bloodstain problem the genetic markers to test. However, emphasis must be placed on the limitations of this study. It is intended as a beginning, as a starting point for future research. It covers a limited number of genetic markers and marker systems, aging conditions, analytical techniques, and investigative skills. Moreover, the tests were conducted on clean specimens free of impurities. It is realized that in actual practice serological evidence preserved under known and constant conditions is rare, and the specimens may be contaminated with impurities such as perspiration, urine, soil, and bacteria. These factors limit the application of the results of the study. Nevertheless, this is the first comprehensive study of genetic marker detectability to include the storage factors of substrate and humidity.

CHAPTER IV. CONCLUSIONS

This effort is the first comprehensive study of the detectability of selected genetic markers (15 antigens and 8 isoenzymes) that also takes into account the history of serological evidence (including the factors of age, substrate, humidity, light, and temperature). The results should be of direct translatable benefit to all crime laboratories that perform bloodstain analyses. With some knowledge of the history of a bloodstain, the criminalist can now determine the reasonableness of performing certain analyses (see Figures 2 through 9). For example, on a bloodstain three months old, to perform an analysis for the e antigen of the Rh system may be unreasonable (see Figure 4); testing for the PGM isoenzymes 1, 2-1, and 2, however, could be done with confidence (see Figure 8).

The study confirmed literature reports that there are large differences in the detectability of different genetic markers in dried blood. For the variants investigated on various substrates, the aging time at which detection was successful ranged from zero to 26 weeks.

Genetic markers tested in this study include many more than were routinely tested in U. S. crime laboratories at the time the project was initiated (June 1974).[1] Additionally, investigation of these markers yields discrimination probabilities considerably greater than are commonly obtained by current routine bloodstain analysis.

With the development of more sensitive analysis techniques, it may be possible to extend the time limits for genetic marker detectability in older dried blood. Such techniques will be especially beneficial to crime laboratories in cases where serological evidence is obtained some time after the commission of the crime.

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

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APPENDIX A. ISOENZYME ANALYSES

The results of electrophoretic separation and identification of AK, ADA, PGM, and EAP isoenzymes in dried blood aged under 20 and 66% relative humidities at ambient temperature, and also for the specimens stored at -20°C, are summarized in Table A-1. The fractional values represent the number of specimens identifiable over the total number analyzed. AK and PGM isoenzymes persisted 26 weeks and, consequently, are more stable than the ADA and EAP isoenzymes, which persisted only 13 weeks. No differences in isoenzyme persistence were found for the enzyme systems tested.

The band patterns were categorized 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 readings, together with the substrates and phenotypes, are given in Tables A-2 through A-5. All the AK and the majority of the PGM analyses of specimens stored for 26 weeks yielded identifiable band patterns. The 13 week reading values for the PGM system reflect clearer band patterns for specimens exposed to the lower humidity. In contrast, exposure to the higher humidity appears to be beneficial for the preservation of ADA and EAP isoenzymes (Tables A-3 and A-5, reading values at 13 weeks). The persistences of the isoenzymes stored at -20°C were generally better than those of the isoenzymes stored at room temperature under either relative humidity level. No significant differences in persistence resulting from substrate effect were evident.

Table A-1. Summary of Enzyme Detectability 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	5/12	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 A-2. Detectability of AK Isoenzymes 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 A-3. Detectability of ADA Isoenzymes 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 A-4. Detectability of PGM Isoenzymes 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	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 A-5. Detectability of EAP Isoenzymes 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 B. CALCULATION OF DISCRIMINATION PROBABILITIES

The discrimination probability, i.e., the probability that two randomly selected individuals will have the same combination of the genetic markers included in this study, was calculated for the variants identified at each aging test period. For each genetic marker system, the value was obtained by summation of the squares of its phenotypic frequencies of occurrence (see Table B-1). The overall discrimination probability was obtained by multiplying the values available for each system.

Table B-1 presents the discrimination probabilities achievable by the analyses of the variants that were detectable at each aging test period. In the MN system, for instance, M, N, S, and s antigens could be typed up to four weeks, yielding a discrimination probability of 0.163. This value increases to 0.309 at 13 weeks because of the failure to type S, which makes the s and Ss phenotypes indistinguishable. Larger numbers represent lower degrees of individualization.

Sample calculations for the MN system (see Figure 3) are given in Table B-2.

Table B-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. $\times 10^4$	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.

Table B-2. Sample 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	

GLOSSARY

ABSORPTION. Removal of antibodies from serum by adsorption on 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 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 product 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 visibly with red cells that contain 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.

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