G. C. Denault,[1] B.S.; H. H. Takimoto,[1] Ph.D.;
Q. Y. Kwan,[1] D.Crim.; and Andrew Pallos,[1] B.S.

Detectability of Selected Genetic Markers in Dried Blood on Aging

REFERENCE: Denault, G. C., Takimoto, H. H., Kwan, Q. Y., and Pallos, A., "**Detectability of Selected Genetic Markers in Dried Blood on Aging,**" *Journal of Forensic Sciences,* JFSCA, Vol. 25, No. 3, July 1980, pp. 479-498.

ABSTRACT: The detectability of selected blood genetic markers aged up to six months deposited on six substrata, glass, wool, nylon, and three types of cotton (plain cotton, permanent press, and denim), was investigated. 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 1-, 2-, 4-, 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 various lengths of time ranging from 2 to 26 weeks. In particular, Rh factors C, c, and E were affected adversely by high moisture environments. The four enzyme systems selected were adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM), and erythrocyte acid phosphatase (EAP). AK and PGM isoenzymes were still identifiable at 26 weeks, 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 permanent press and denim, which appeared to shorten the detectability time of PGM. Storage of the specimens at −20°C generally preserved the antigens and the enzymes better than storage at room temperature. The discrimination probability was calculated on the basis of the genetic markers 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.

KEY WORDS: pathology and biology, genetic typing, blood, human identification

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. This is one of the few com-

This project was supported by Contract Number J-LEAA-025-73 awarded by the National Institute of Law Enforcement and Criminal Justice, Law Enforcement Assistance Administration, U.S. Department of Justice, under the Omnibus Crime Control and Safe Streets Act of 1968, as amended. Points of view or opinions stated in this document are those of the authors and do not necessarily represent the official position or policies of the U.S. Department of Justice. Received for publication 28 Feb. 1979; revised manuscript received 23 Nov. 1979; accepted for publication 24 Nov. 1979.

[1]Member of the technical staff, senior staff scientist, member of the technical staff, and former research assistant, respectively, Ivan A. Getting Laboratories, The Aerospace Corporation, Los Angeles, Calif.

Copyright © 1980 by ASTM International

prehensive detectability studies that takes into account, for a large number of genetic marker systems, the factors of bloodstain history, that is, the material on which the blood was deposited and the humidity at which it was stored.

Detectability of variants of ABO, MN, Rh, Kidd, Duffy, and Kell red cell antigen systems, and of adenylate kinase (AK), adenosine deaminase (ADA), phosphoglucomutase (PGM_1), and erythrocyte acid phosphatase (EAP) enzyme systems was studied over a six-month period. Blood drawn from twelve volunteer donors was deposited on six materials: glass, wool, nylon, and three types of cotton (plain cotton, permanent press, and denim). Emphasis was placed on the analyses of stains on fabrics because more than 90% of serological clue materials are found on textiles [1].

Information in the literature regarding detectability of genetic markers in dried blood with age for the systems included in this study is given in Table 1. 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, therefore, was initiated because of the large discrepancies in available data and because of the paucity of information on the effects of materials and humidity on the survival of genetic markers in bloodstains.

Experimental Procedure

Whole Blood Analyses

Fresh blood drawn from twelve 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 enzyme polymorphs were phenotyped by electrophoresis on starch gel. The blood specimens provided 15 different red cell antigens and eight different isoenzymes for detectability studies.

Preparation of Aging Test Specimens

Blood drawn with 20-cm^3 syringes from the same twelve donors was directly deposited from the syringes onto six different substrata: glass, wool, nylon, and three types of cotton (plain, permanent press, and denim). Before the deposition of blood, the cloth samples were washed in a phosphate-type detergent (Tide), thoroughly rinsed, dried for 24 h, and cut into 25-mm (1-in.) squares. The nylon, wool, permanent 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 surface. 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 h in a fume hood. They consisted of duplicate sets of twelve specimens each, plus two (stained cotton specimens) reserved for freezing. The duplicate sets were separated into two constant-humidity chambers for aging

TABLE 1—*Detectability, reported in the literature, of genetic markers in dried blood.*

System	Variants	Age of Dried Blood	Reference
ABO	A, B	2-34 years	*2*
MN	M, N	29-39 weeks	*2-4*
	S	26 weeks	*3*
Rh	C, c, D	4-26 weeks	*3, 5, 6*
	E	5-6 weeks	*3, 5*
	e	4-5 weeks	*3, 5*
Duffy	Fy^a	6 days	*7*
Kell	K	3-19 days	*5, 8*
AK	...	4-48 weeks	*9-13*
ADA	...	2-22 weeks	*9, 10*
PGM_1	...	4-22 weeks	*9, 11, 14-16*
EAP	...	0.5-9 weeks	*17-19*

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. The humidity chambers consisted of glass desiccators 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.

To reduce the total number of analyses, specimens aged for four weeks were evaluated first. Antigens and enzyme polymorphisms 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 again repeated for undetectable variants, and the tests performed at one week.

Antigen Typing of Dried Blood

Of the several methods available for the determination of antigens in dried bloodstains, absorption-elution is the most sensitive and the most widely employed. Described by Kind [*20,21*], Nickolls and Pereira [*22*], Outteridge [*23*], and Fiori et al [*2*], absorption-elution has proved to be markedly more sensitive than the absorption-inhibition method [*2*]; it has also been reported to be more successful than mixed agglutination for certain antigens [*4*].

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 2. Although treatments such as the use of papain-, ficin-, or trypsin-treated indicator cells were attempted, insufficient testing was done to draw conclusions as to consistent enhancement of detectability with such treatments.

All antisera were obtained from commercial sources and because significant variability in their titers was observed, they 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 textiles, subjected to procedures identical to those of the bloodstained substrates, were run under both humidity conditions as well as in the freezer. Some of 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.

TABLE 2—*Experimental procedures for antigen typing.*

Antigen	Antiserum[a] Added, drops	Incubation: Temperature, °C	Incubation: Time, h	Washing[b] Medium	Diluent for Elution[c]	Indicator Cells: Suspension, %	Indicator Cells: Medium	Reabsorption[d] Temperature, °C	Antihuman Serum Added,[e] drops
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,f] saline	saline	0.5	saline	37	1
Rh (C, c, D, E, e)	2	37	12-18	RT[f] saline	BSA[g]	0.5-1.0	BSA[g]	37	0
Fy^a, Jk^a, K	1	37	32-48	RT[f] saline	saline	0.5	saline	37	1-2[h]

[a] Antisera supplier was Hyland; exceptions were Fy^a (Spectra Biological) and anti-Jk^a and antihuman globulin (Dade).
[b] Washed six times with a 15- to 20-min standing between each wash.
[c] Elution was carried out at 56°C for 15 min in a circulating water bath.
[d] Reabsorption was allowed to occur for approximately 1 h.
[e] Subsequent centrifugation at 1500 rpm for 3 min followed by reading for agglutination with concave mirror and light.
[f] Room temperature saline solution.
[g] Bovine serum albumin in 0.3% concentration.
[h] Dependent on specific instructions accompanying the batch of antiserum used.

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.

ABO (A, B, H) and MN (M, N, s) Antigens—Fifteen 3- by 3-mm squares were cut from each of the bloodstained specimens tested at every aging period and placed in 10- by 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 2). 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 pipet 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 min 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 min. The tubes were then removed from the water bath, the cloth specimens were 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 1 h in the refrigerator (4°C), one drop of antihuman serum was added (Coombs technique) when necessary (see Table 2). The tubes were centrifuged for 3 min at 1500 rpm, and their contents examined for agglutination macroscopically with a concave mirror (producing 1.2× magnification) and a light; microscopic examinations were made for confirmation when necessary.

MN (S), Rh (C, c, D, E, e), 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 2. For Kidd, Duffy, and Kell systems, the method was adapted from Ruffie and Ducos [8].

Enzyme Analysis of Dried Blood by Electrophoresis

Twenty-five-millimetre (1-in.) square cuttings of all bloodstain specimens tested at every aging period were submitted to electrophoretic analysis of the four enzyme systems containing the eight isoenzymes under study. The experimental procedures are detailed in Table 3.

For ADA and EAP, preparation for analysis involved soaking samples of the dried blood specimens for 1 h in two drops of a 1% β-mercaptoethanol ($HSCH_2CH_2OH$) solution in the gel buffer appropriate for each enzyme (see Table 3). Mercaptoethanol serves to cleave and reduce the -S-S- groups in molecules, which are known to cause aging artifacts [17]. Each specimen extract was then absorbed on a Whatman No. 3 filter paper (8 by 5 mm) that was inserted directly into the starch gel for the electrophoretic runs.

The isoenzyme separations were routinely carried out with 10.05% Electrostarch® (Otto Hiller) gel in a Grafar electrophoresis chamber (13 by 30 by 0.6 cm) equipped with a Buchler power supply (Model 3-1014A), 0 to 1000 V, 0 to 250 mA. The biochemicals were obtained from Sigma Chemical Co.

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,

TABLE 3—*Experimental procedures for blood enzyme analyses.*

Enzyme System	Procedures							
	Electrophoresis[a]				Staining			
	Bridge Buffer		Gel Buffer/ Dilution Factor of Bridge Buffer	Constant Voltage Setting, V/cm				
	System	pH			Stain Mixture Ingredients[b]	Overlay Material	Time,[c] min	
ADA	citric acid	5.0	1/20	5	adenosine-MTT, xanthanine oxidase, nucleoside phosphorylase	agar	60	
AK	citric acid/ sodium hydroxide	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, ammonium hydroxide	filter paper	120	
PGM_1	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 h 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 methosulfate."
G-6-PD = glucose-6-phosphate dehydrogenase.
TRIS = Tris(hydroxymethyl)aminomethane.
G-1-P = glucose-1-phosphate.
[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.

2—faintly detectable and identifiable,
3—identifiable, and
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.

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 stated earlier, their identity being unknown to the investigators at the time of testing. Furthermore, the investigators did not know which particular phenotypes were represented in the group of samples to be tested, thus precluding any bias or anticipation of what specific phenotypes to look for.

Results and Discussion

The results of the fresh blood analyses are presented in Table 4 for the red cell antigens and for the enzymes. From these results, 15 antigens and eight isoenzymes (composing 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, that is, typing was performed only for Antigen A in general, and the enzyme glutamate pyruvate transaminase (GPT) and protein haptoglobin (Hp) were excluded from the aged stain testing.

The results of the survival studies are summarized in Figs. 1 to 8. Each bar represents the detectable survival of the indicated marker preserved under the indicated conditions. For example, Antigen A of the ABO system was tested on nylon and on permanent press cotton and on each of these materials one sample of the antigen was stored under each humidity condition. These results are discussed in greater detail in the following paragraphs.

Red Cell Antigen Detectability

ABO Antigens—The ABO antigens in dried blood were detected after 26 weeks of aging (Fig. 1). The H antigen, in particular, was unaffected by the six types of substrata 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.

Hence, the evidence implies no significant substratum, humidity, or temperature effects on the persistence of the ABO antigens, at least up to 26 weeks. Fiori et al [*2*] have reported A and B antigens to be detectable on bloodstained fabric at ambient conditions after two years. They also successfully typed a 34-year-old Type A stain. Their analyses were also conducted with the absorption-elution method on stains prepared on different fabric substrata 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.

MN Antigens—The M, N, and s antigens behave similarly to the ABO antigens, being insensitive to substratum, cold temperature, and humidity (Fig. 2). 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 substrata or humidity conditions were noted; the short detectability time appears

TABLE 4—*Blood phenotype*[a] of donors.

	Red Cell Antigen System						Enzyme-Protein System					
Donor	ABO	MNSs	Rh	Jk^a	Fy^a	K	AK	ADA	PGM_1	EAP	GPT[b]	Hp[b]
I	O	MNSs	cde	−	a−b+	−	1-1	1-1	2-2	B	2-2	2-2
II	B	Ms	CdDEe	+	a−b+	−	1-1	2-1	1-1	BA	1-1	2-2
III	O	Ns	cDEe	−	a−b+	+	1-1	1-1	1-1	B	2-2	1-1
IV	O	Ms	CDe	−	a+b−	−	1-1	2-1	2-2	B	1-1	2-1
V	O	Ns	CcDe	+	a−b+	−	1-1	2-1	2-1	B	2-1	1-1
VI	A_1	MNs	CcDe	+	a−b+	−	1-1	1-1	2-1	B	2-1	1-1
VII	O	Ns	CcDEe	−	a+b−	−	1-1	1-1	1-1	BA	2-1	2-1
VIII	O	MNSs	CcDe	−	a+b−	−	1-1	1-1	1-1	B	2-1	2-1
IX	O	MNSs	CcDe	−	a+b−	−	1-1	1-1	1-1	B	3-2	2-2
X	A_2	Ms	CcDEe	−	a+b+	−	1-1	1-1	1-1	B	2-1	2-2
XI	O	MNs	cDEe	−	a−b+	−	1-1	1-1	1-1	BA	1-1	1-1
XII	O	MNSs	CcDe	+	a+b−	−	1-1	1-1	1-1	BA	2-1	2-1

[a] Analyses performed by the Human Genetics Laboratory, University of California, Los Angeles.
[b] Aging analysis not performed because of difficulties encountered.

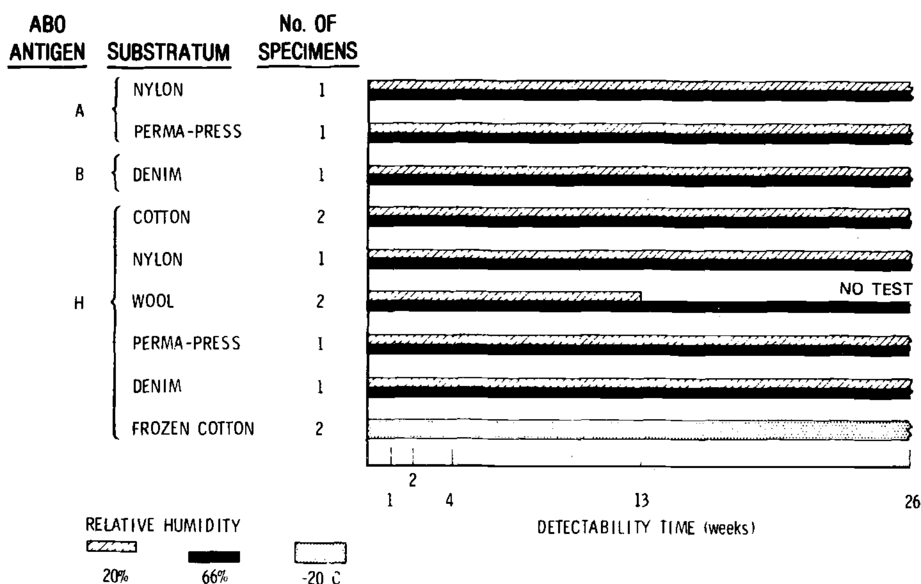

FIG. 1—*Detectability of ABO antigens.*

to result from deficiencies in the analytical technique because Lincoln and Dodd [*3*] were able to type S antigen in stains for 26 weeks.

M and N antigens in stained cloth have been reported by Fiori et al [*2*] to be detectable after 29 weeks of aging under ambient conditions. In a study conducted by Pereira [*4*], 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 in titer were found. (Screening had to be conducted on each batch of antisera purchased in order to arrive at the appropriate dilution for use in testing; the appropriate dilution was the one that would not yield positive results with clean unstained cloth controls.) These considerations may explain some of the discrepancies in the reported detectabilities.

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

Detectability of Antigens C, c, and E is affected by humidity but not by substratum or temperature. Upon storage of the specimens at 20% relative humidity at ambient laboratory temperature (23 ± 3°C), C was generally detectable at 26 weeks, and c and E were 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 [*3*], and they have been identified in specimens aged from four to eight weeks by other investigators [*4-6*]. Antigens E and e have been reported to persist from four to six weeks [*3-5,8,24-28*]; however, failure to detect e even in fresh stains because of poor quality antiserum has also been reported [*7*].

Kidd, Duffy, and Kell Antigens—The antigens of the Kidd, Duffy, and Kell systems are extremely difficult to type because of weak agglutination between the antigens and their an-

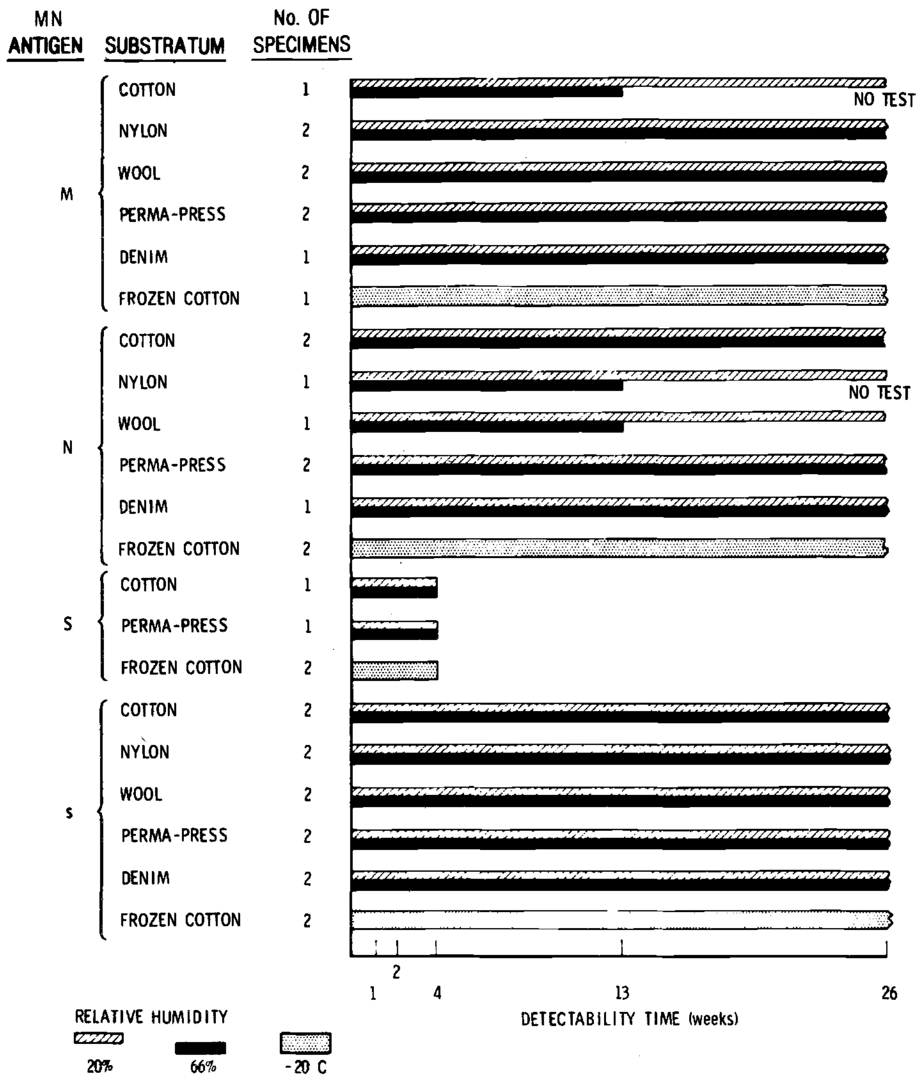

FIG. 2—*Detectability of M and N antigens*

tibodies. 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 with the antibody, and then agglutination brought about by the addition of Coombs (antihuman globulin) serum. Often, no clear-cut results were obtained (Fig. 4). Jk^a of Kidd (on denim at both humidities), Fy^a of Duffy (on cotton, wool, and permanent press, at both humidities), and K of Kell (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, permanent press, and nylon containing no Kidd Jk^a antigen were tested for that antigen to determine whether false positives would develop. No false positives were obtained, indicating that, in general, substratum-blood interactions produce no materials that react with Jk^a antisera.

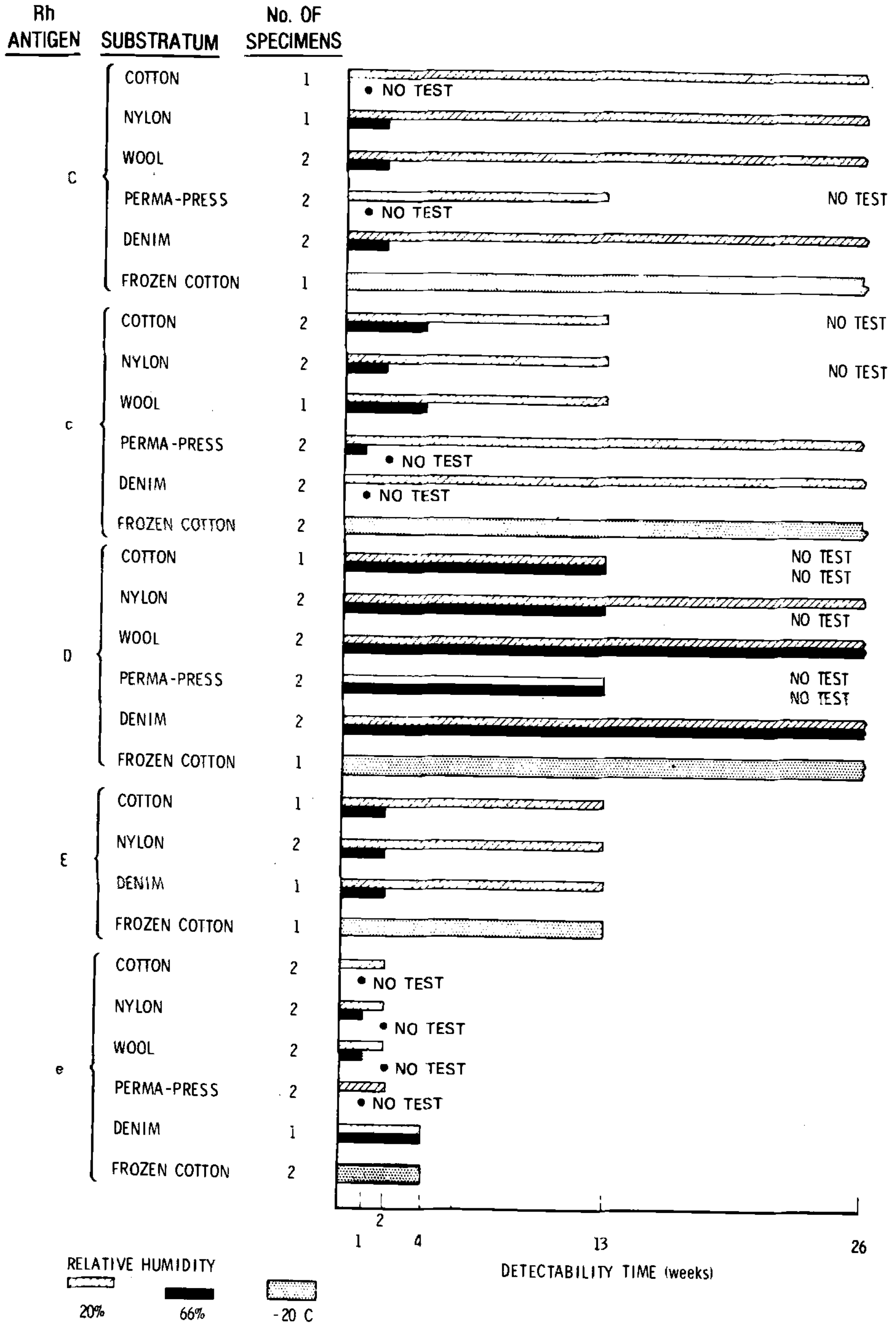

FIG. 3—*Detectability of Rh antigens.*

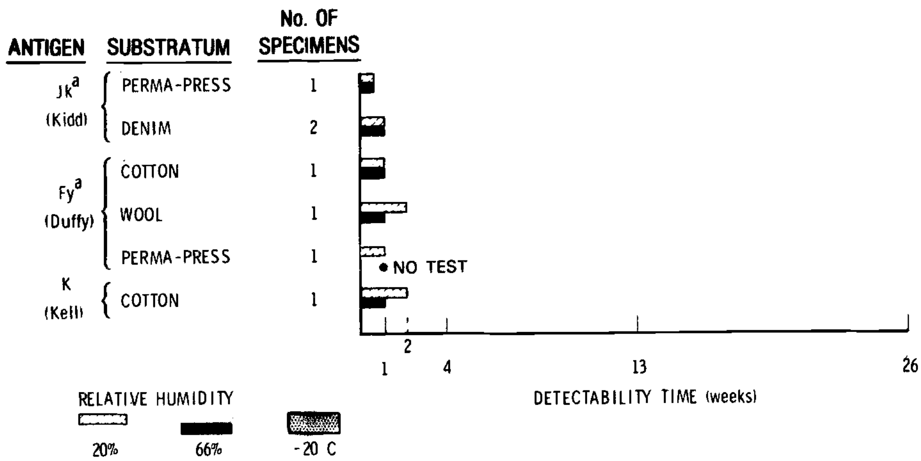

FIG. 4—*Detectability of Kidd, Duffy, and Kell antigens.*

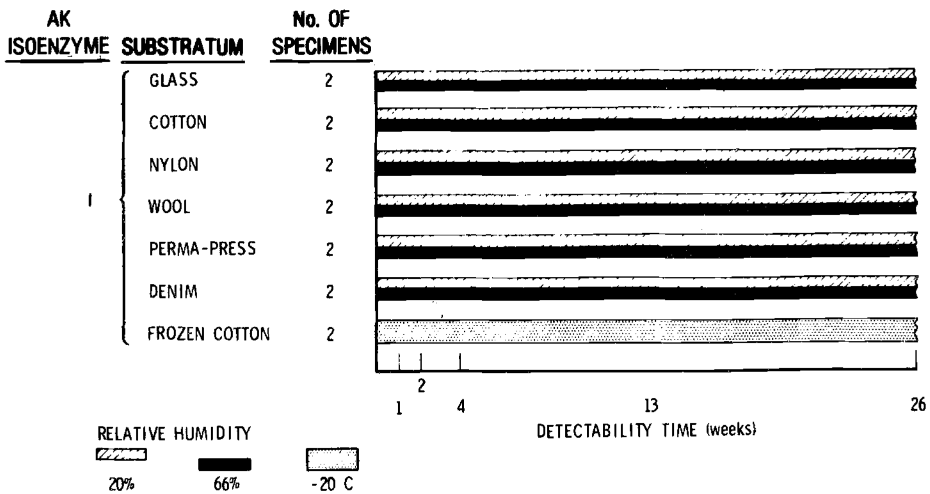

FIG. 5—*Detectability of AK isoenzyme.*

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 [5].

Erroneous Antigenic Test Results

This study shows that erroneous test results, either positive or negative, can be obtained even when the most dependable techniques available are used. In some instances, negative results were obtained for 12% of the antigens, which were detected at later times and shown to persist for longer periods in subsequent tests (see Table 5). 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 6). The false negatives are probably caused by overwashing of the samples, whereas the false

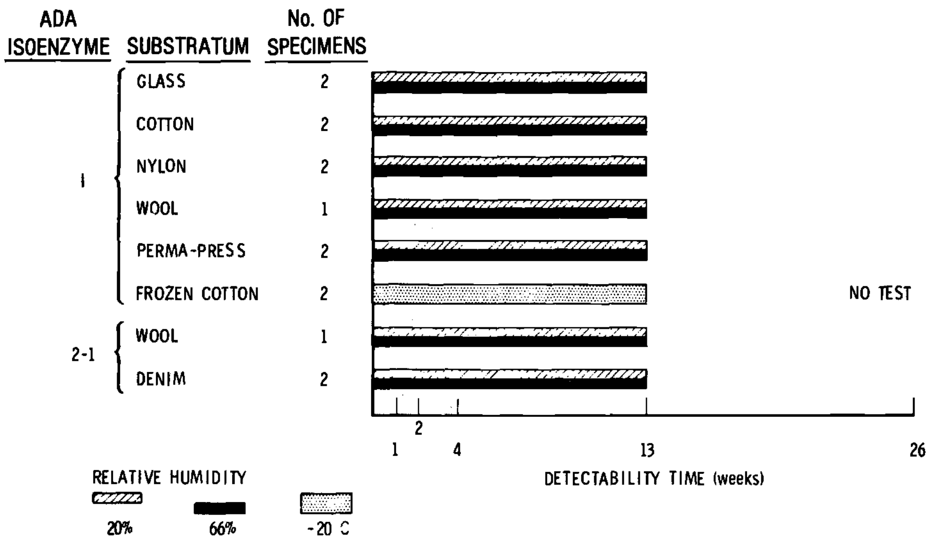

FIG. 6—*Detectability of ADA isoenzymes.*

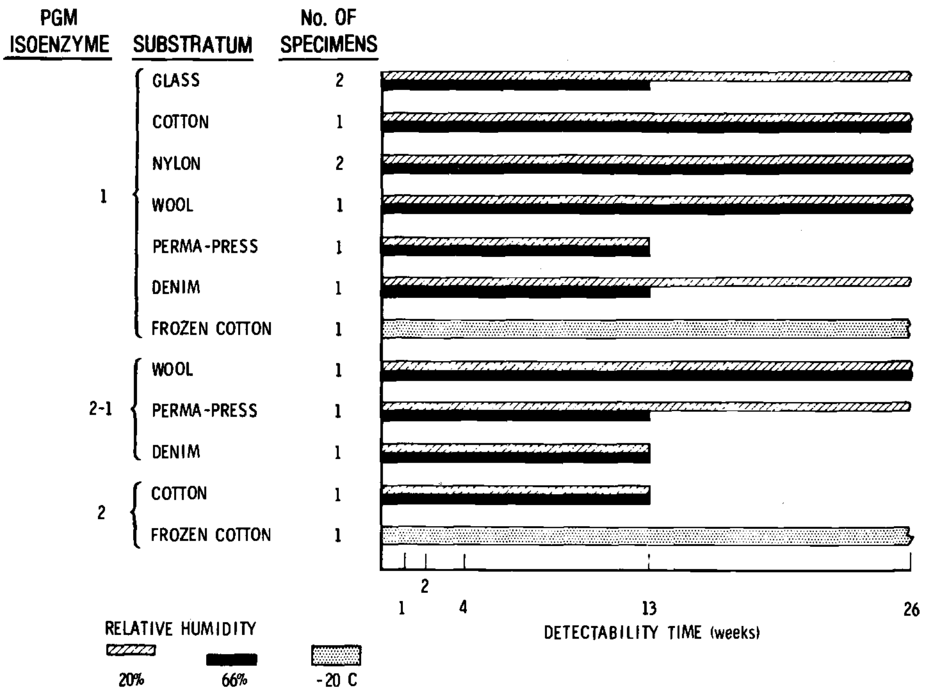

FIG. 7—*Detectability of* PGM_1 *isoenzymes.*

positives may be caused by sample underwashing or by some substratum effect. Bloodstains deposited on denim, nylon, and wool produced most of the false positives. Denim regularly unraveled upon being washed, and the attempt to avoid this may have induced insufficient washing. Nylon, being a polyamide, contains peptide-like linkages (-CONH-), and wool

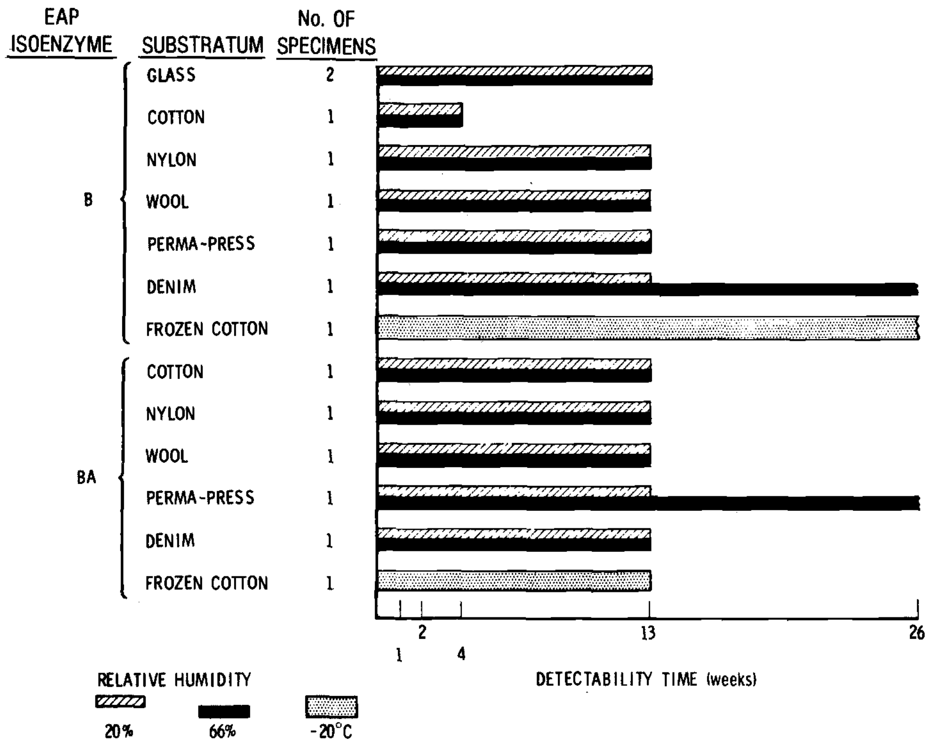

FIG. 8—*Detectability of EAP isoenzymes.*

TABLE 5—*False negative test results.*

Marker System	Antigen Missed	Test Conditions: Time, weeks	Relative Humidity, %	Substratum
MN	M	2	66	denim
Rh	E	2	20	denim
Rh	E	4	20	denim
Rh	e	1	66	denim
ABO	H	4	66	cotton
MN	N	2	66	cotton
MN	s	2	66	cotton
Kell	K	1	20	cotton
MN	s	2	20	wool
MN	s	13	20	wool
Rh	e	1	20	wool
MN	S	2	20	permanent press
MN	s	2	20	permanent press
Rh	e	1	20	permanent press
MN	s	2	66	nylon

TABLE 6—*False positive test results.*

Marker System	Antigen Falsely Detected	Test Conditions: Time, weeks	Relative Humidity, %	Substratum
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	denim
ABO	A	26	20	nylon
MN	N	26	66	nylon
Rh	C	2	66	nylon
MN	N	2	20	wool
Rh	c	26	20	wool
Rh	E	2	20	wool
MN	M	4	20	cotton
Rh	E	2	20	permanent press

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 [*29*].

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 duplicate and sometimes 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.

Detectability of Isoenzymes

The electrophoretic separation and identification of isoenzymes of AK, ADA, PGM, and EAP systems in dried blood aged under conditions of 20 and 66% relative humidity were conducted. 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.

AK Isoenzyme—For the AK system, all twelve donors participating in the study were of Phenotype 1 (Fig. 5). The isoenzyme was faintly detectable and identifiable at 26 weeks for both the 20 and 66% relative humidity conditions on all six substrata, 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 substrata were discerned.

The activity of AK enzymes was reported by Welch [*9*] to decrease somewhat in stains aged from 15 to 30 days, although at 30 days the pattern was still clear and readily discernible. Brinkmann and Dirks [*10*] and Rothwell [*11*] were able to analyze the isoenzymes after eleven months of storage at room temperature. Brinkmann and Dirks 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.

ADA Isoenzymes—At the 26-week analysis period, only 1 of the 14 samples of the ADA isoenzymes tested (one of two samples of Phenotype 1 run on frozen cotton) was clearly iden-

tifiable. All others exhibited only faintly detectable enzymatic activity. At 13 weeks, all ADA isoenzymes were identifiable (Fig. 6); however, specimens stored at high humidity (66%) produced ADA band patterns better defined 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 substrata. 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.

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 [*10*]. In contrast, Welch [*9*] 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.

PGM_1 Isoenzymes—Permanent press and denim appear to hinder the detection of $PGM_1{}^1$ and $PGM_1{}^{2\text{-}1}$ at 26 weeks (Fig. 7). No enzyme activity was detectable on those bloodstained substrata at high relative humidity for 26 weeks while those bloodstained substrata stored at low relative humidity for 26 weeks exhibited only faintly detectable but identifiable band patterns. 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_1 isoenzymes have been reliably detected in dried blood after four to 13 weeks [*9,11,14-16*], and occasionally after five months [*11,14-16*].

EAP Isoenzymes—Both B and BA isoenzymes of the EAP system were detected after 13 weeks of storage in all cases but one (B on cotton) (Fig. 8). Detection after 26 weeks occurred twice (B on denim and BA on permanent press, the two substrata being those appearing to have an adverse effect on PGM_1) 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%). It should be noted that bias on the part of the analyst was avoided by his being kept unaware of what phenotypes to expect.

In a study conducted by Brinkmann et al [*17*], 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 limits for their identification vary from a few days [*18*], 22 days [*9*], 30 days [*19*], 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.

Discrimination Probability

It is clear that the longer a sample is stored, the more genetic information is lost. This is illustrated in Fig. 9, which is a plot of the discrimination index as a function of the time of storage. The discrimination index is defined as the probability that two randomly selected individuals will have an identical combination of detectable genetic markers. The frequency of occurrence data for each genetic marker were taken from the literature.

A discrimination of 1 out of approximately 2500 is possible after one week of storage under both 20 and 66% relative humidity conditions; that is, 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 1 in 65 and 1 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.

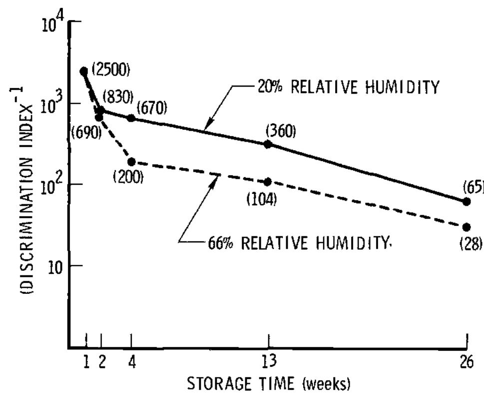

FIG. 9—*Discrimination index with age of dried blood.*

Many factors affect the detectability of genetic markers in dried blood. The loss of antigen detection can be partially attributed to degradation and inaccessibility of the antigenic sites with time, the latter caused by cell shrinkage through dehydration [*30*]. Thus the number of antigenic sites per cell surface for each antigen type becomes an important factor in the blood group determination of old stains and may explain the erratic differences in detectability found in the literature. The number of antigenic sites per cell has been determined for some antigens, and the trend indicates that the more sites per cell the longer that specific antigen is detectable. In the ABO system, which has been detectable for the longest period, the A and B cells have approximately 1 000 000 and 700 000 sites, respectively [*31*]. In the Rh system, D (R_1R_2 type) cells have 30 000 [*32*], C cells 79 000, and e cells 21 000 [*33*]. In addition, other factors that have a significant effect on variant detectability include the specificity of antisera and the strength and "hemagglutinability"; these requirements may also change with the age of the stains.

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.

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 systems greatly enhancing discrimination probability (because of their favorable frequency distribution in the population) are the gamma globulin (Gm) and human leukocyte antigens, loci A, B, and C (HLA A, B, or C)[2] antigens, the GPT isoenzymes, and the group-specific component (Gc) and Hp polymorphic serum proteins. Methods of grouping these systems in bloodstains, though available, have not been routinely carried out in U.S. crime laboratories as of the time this study was conducted.

[2]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 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. The study, "Identification of Dried Human Blood Samples by HLA Antigens," by M. Tagasugi, D. Akira, and P. I. Terasaki, was presented at the 43rd Semiannual Seminar of The California Association of Criminalists in Long Beach, Calif., 11 May 1974.

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 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.

Conclusions

This effort is the first comprehensive study in the United States of the detectability of selected genetic markers (15 antigens and eight isoenzymes) that also takes into account the history of serological evidence (including the factors of age, surface on which it is deposited, 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. For example, on a bloodstain three months old, to perform an analysis for the e antigen of the Rh system may be unreasonable; testing for the PGM_1 Isoenzymes 1, 2-1, and 2, however, could be done with confidence.

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 substrata, 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 in June 1974, when the project was initiated [*1*]. Additionally, investigation of these markers yields discrimination probabilities considerably greater than those 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 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.

Acknowledgments

The authors express their appreciation for the valuable consulting assistance provided by Dr. Robert S. Sparkes, associate professor and chief, Department of Medicine and Pediatrics, UCLA School of Medicine, and to his laboratory personnel, especially Ms. Mary Ellen Sparkes, for performing the whole blood analyses and the enzyme determinations on dried blood. Our gratitude is also extended to Mr. David J. Carre and Ms. Juanita Ingram for laboratory assistance during the early and the late phases of this study, respectively. We are also grateful to all the volunteer donors, without whose cooperation this study would not have been possible. Appreciation is also expressed to Mr. E. J. Rattin, Drs. Peter F. Jones, Gerard M. Wolten, and George F. Sensabaugh for many helpful discussions. The support and encouragement of the National Institute of Law Enforcement and Criminal Justice, particularly Messrs. J. O. Sullivan, J. Kochanski, G. Schollenberger, and L. Shubin, are acknowledged with special thanks.

References

[1] "Survey and Assessment, Blood and Bloodstain Analysis, Volume I: Technical Discussion," ATR-74(7910)-1, The Aerospace Corp., Los Angeles, April 1974.
[2] Fiori, A., Marigo, M., and Benciolini, P., "Modified Absorption-Elution Method of Siracusa for ABO and MN Grouping of Bloodstains," *Journal of Forensic Sciences,* Vol. 8, 1963, pp. 419-445.
[3] Lincoln, P. J. and Dodd, B. E., "The Detection of the Rh Antigens C, C^w, c, D, E, e, and the Antigen S of the MNSs System in Bloodstains," *Medicine, Science and the Law,* Vol. 8, 1968, pp. 288-295.
[4] Pereira, M., "The Identification of MN Groups in Dried Bloodstains," *Medicine, Science and the Law,* Vol. 3, 1963, p. 268.
[5] Douglas, R. and Staveley, J. M., "Rh and Kell Typings of Dried Bloodstains," *Journal of Forensic Sciences,* Vol. 14, 1969, pp. 255-262.
[6] Pereira, M., "Automated Rh Genotyping of Dried Bloodstains," *Technicon Quarterly,* Vol. 3, 1971, pp. 16-18.
[7] Bargagna, M. and Pereira, M., "A Study of Absorption-Elution as a Method of Identification of Rhesus Antigens in Dried Bloodstains," *Journal of the Forensic Science Society,* Vol. 7, 1967, pp. 123-130.
[8] Ruffie, J. and Ducos, J., "Absorption des Anticorps anti-Fy^a par le Sang Desseche," *Comptes Rendus des Seances de la Societe Biologie et de ses Filiales,* Vol. 151, 1957, pp. 393-395.
[9] Welch, S. G., "Glutamatepyruvate Transaminase in Blood Stains," *Journal of the Forensic Science Society,* Vol. 12, 1972, pp. 605-607.
[10] Brinkmann, B. and Dirks, J., "Identification and Demonstration of Three Enzyme Polymorphisms from Bloodstains by Simultaneous Electrophoresis," *Zeitschrift für Rechtsmedizin,* Vol. 69, 1971, pp. 185-190.
[11] Rothwell, T. J., "The Effect of Storage upon the Activity of Phosphoglucomutase and Adenylate Kinase Enzymes in Blood Samples and Bloodstains," *Medicine, Science and the Law,* Vol. 10, 1970, pp. 230-234.
[12] Jones, A. R. and Diamond, L. K., "Identification of the Kell Factor," *Journal of Forensic Science Medicine,* Vol. 2, 1955, pp. 243-246.
[13] Culliford, B. J. and Wraxall, B. G., "Adenylate Kinase (AK) Types in Bloodstains," *Journal of the Forensic Science Society,* Vol. 9, 1968, pp. 79-80.
[14] Culliford, B. J., "The Determination of Phosphoglucomutase (PGM) Types in Bloodstain," *Journal of the Forensic Science Society,* Vol. 7, 1967, pp. 131-133.
[15] Brinkmann, B., "Bestimmung der Phosphoglucomutase-Typen aus Blutspuren," *Deutsche Zeitschrift für die gesamte gerichtliche Medizin,* Vol. 66, 1969, pp. 31-34.
[16] Oepen, I., "Dunnschicht-Starkegel-Electrophorese zur Bestimmung der Phosphoglucomutase-Typen an Blutspuren," *Zeitschrift für Rechtsmedizin,* Vol. 67, 1970, pp. 309-312.
[17] Brinkmann, B., Gunnemann, M., and Koops, E., "Investigations on the Decay of Acid Phosphatase Types in Stored Blood Stains and Blood Samples," *Zeitchrift für Rechtsmedizin,* Vol. 70, 1972, pp. 68-71.
[18] Nagata, T. and Dotzauer, G., "Nachweis and Typenbestimmbarkeit der saueren Erythrocytenphosphatase in Blutspuren," *Zeitschrift für Rechtsmedizin,* Vol. 67, 1970, pp. 359-363.
[19] Heidel, G., "Die spurenkundliche Bedeuting der Typen der saueren Erythrocytenphosphatase," *Deutsche Zeitschrift für die gesamte gerichtliche Medizin,* Vol. 63, 1963, pp. 37-43.
[20] Kind, S. S., "Absorption-Elution Grouping of Dried Blood Smears," *Nature* (London), Vol. 185, 1960, pp. 397-398.
[21] Kind, S. S., "Absorption-Elution of Dried Blood on Fabrics," *Nature* (London), Vol. 187, 1960, pp. 789-790.
[22] Nickolls, L. C. and Pereira, M., "A Study of Modern Methods of Grouping Dried Bloodstains," *Medicine, Science and the Law,* Vol. 2, 1962, pp. 172-179.
[23] Outteridge, R. A., "Recent Advances in the Grouping of Dried Blood and Secretion Stains," in *Methods of Forensic Sciences,* Vol. IV, A. S. Curry, Ed., Interscience, New York, 1965, pp. 299-332.
[24] Spencer, N., Hopkinson, D. H., and Harris, H., "Adenosine Deaminase Polymorphism in Man," *Annals of Human Genetics,* Vol. 32, 1968, p. 9.
[25] Fildes, R. A. and Harris, H., "Genetically Determined Variation of Adenylate Kinase in Man," *Nature* (London), Vol. 209, 1966, pp. 261-263.
[26] Swallow, D. M. and Harris, H., "A New Variant of the Placental Acid Phosphatases: Its Implications Regarding Their Subunit Structures and Genetical Determination," *Annals of Human Genetics,* Vol. 36, 1972, p. 141.
[27] Sparkes, M. E., Kris, M., and Sparkes, R., "High Sensitivity of Phenolphthalein Monophosphate in Detecting Acid Phosphatase Isozymes," *Analytical Biochemistry,* Vol. 1, 1975, pp. 316-318.

[28] Spencer, N., Hopkinson, D. A., and Harris, H., "Phosphoglucomutase Polymorphism in Man," *Nature* (London), Vol. 204, 1964, pp. 742-745.
[29] de Ren, G., Petiet, G., Streiff, F., and Genetet, B., "Difficulties Recontrees dans la Determination des Antigenes de Groupes Sanguins des Taches de Sang Sec," *Medecine Legale et Dommage Corporel*, Vol. 3, 1970, pp. 71-73.
[30] Rosenfield, R. E., Berkman, E. M., Nusbacher, J., Hyams, L., Dabinsky, C., Stux, S., Hirsch, A., and Kochwa, S., "Specific Agglutinability of Erythrocytes from Whole Blood Stored at 4°C," *Transfusion*, Vol. 11, 1971, pp. 177-191.
[31] Economidou, J., Hughes-Jones, N. C., and Gardner, B., "Quantitative Measurements Concerning A and B Antigen Sites," *Vox Sanguinis*, Vol. 12, 1967, pp. 321-328.
[32] Hughes-Jones, N. C., "Reactivity of Anti-D Immunoglobulin G Subunits," *Nature* (London), Vol. 227, 1970, p. 174.
[33] Hughes-Jones, N. C., Gardner, B., and Lincoln, P. J., "Observations on the Number of Available c, D, and E Antigen Sites on Red Cells," *Vox Sanguinis*, Vol. 21, 1971, p. 210.

Address requests for reprints or additional information to
Genevieve C. Denault, A6/1345
The Aerospace Corporation
P.O. Box 92957
Los Angeles, Calif. 90009