J Forensic Sci, Jul. 1976, Vol. 21, No. 3

B. W. Grunbaum,[1] *Ph.D., M.Crim.*

Some New Approaches to the Individualization of Fresh and Dried Bloodstains

Human blood theoretically contains sufficient variables to enable the invididualization of one person from any other. The basis for blood individuality is mainly the multitude of polymorphic enzymes and proteins, most of which are products of genetic variation [*1*]. These genetic factors are constant throughout life. Both transient and comparatively permanent environmental factors, such as acquired immune antibodies, also contribute to the differentiation of blood between individuals. Any single factor which is decisively different in two specimens at a given time will discriminate between the two and thus eliminate a common source.

Geneticists, using zone electrophoresis, provided the fundamental knowledge of the variation of hereditary factors among individuals. Use of these methods in forensic blood analysis has been pioneered by B. J. Culliford [2], and his methods are used at the Metropolitan Police Laboratory of London, England.

Potentially, fresh blood and dried bloodstains found at the scene of a crime could be used as routinely as fingerprints for identification. However, a recent survey [*3*] of selected criminalistics laboratories within the United States has shown that only limited use is currently being made of blood analysis results. Several reasons were given: (1) lack of simple, rapid, and inexpensive analytical methods for the detection of genetically derived blood constituents; (2) unavailability to the criminalistics laboratory in readily retrievable form of data on the frequency of occurrence of genetic blood variants in the U.S. population; and (3) such factors as lack of trained personnel, lack of laboratory space, funds, and time.

This paper explores new techniques of physicochemical and immunologic analyses that I have initiated in my laboratory; I hope these methods will provide powerful and sensitive tools to unravel and determine the potential information of individuality in bloodstains and will also be of sufficient simplicity, accuracy, and economy for routine use in criminalistics laboratories. The following topics will be discussed:

(1) simultaneous phenotyping by electrophoresis of genetically controlled polymorphic enzymes and proteins;

(2) isoelectric focusing of bloodstain extracts in a continuous stable and linear pH gradient;

(3) analytical isotachophoresis in a discontinuous electrolyte system;

(4) immunofixation of polymorphic proteins or isoenzymes for which monospecific antiserums are available; and

(5) crossed immunoelectrophoresis of either dissolved or extracted bloodstains.

Presented at the 7th International Meeting of Forensic Sciences, 8–12 Sept. 1975, Zurich, Switzerland. Received for publication 25 Sept. 1975; accepted for publication 10 Nov. 1975.

[1] Research biochemist, White Mountain Research Station, University of California, Berkeley, Calif. 94720.

The typing of dried bloodstains is of utmost importance to the forensic investigator since most of the specimens he receives are in this condition. However, analysis of dried bloodstains is complicated by the degraded and contaminated condition of the biological material. For this reason, studies designed to individualize blood for the purpose of identification must necessarily begin with fresh blood. A later phase of the research will apply these analytical methods to the study of dried bloodstains which will be aged under a variety of conditions. The objective will be to establish standards for comparison for use in crime laboratories.

Simultaneous Phenotyping by Electrophoresis

At present, polymorphic enzymes and proteins are determined by the use of conventional electrophoretic fractionation in stabilized media followed by specific affinity staining. However, concurrent determinations described below offer the advantage of reliability and economy in both time and labor.

Most forensic laboratories use starch gel as the supporting medium. In this laboratory, cellulose acetate is routinely used as an electrophoretic supporting medium. In a study of seven supporting media (see Table 1), I found that cellulose acetate is superior over starch gel in handling properties, completion time, and sample size. It appears to be a useful, all-purpose, average-resolution, supporting medium. We now use cellulose acetate in the determination of the polymorphic enzymes of phosphoglucomutase, glucose-6-phosphate dehydrogenase, erythrocyte acid phosphatase (EAP), alkaline phosphatase, creatine phosphokinase, lactic acid dehydrogenase, and the polymorphic proteins of hemoglobin and group specific component.

Recently designed equipment [*4*] permits the simultaneous application of multiple samples to a supporting medium and the concurrent determination of the enzyme variant systems mentioned above. This technique offers advantages in terms of reliability, accuracy, speed, ease of performance, and economy.

In an investigation now under way, five genetically controlled variants were selected as model systems for concurrent phenotyping. These were selected because they are pertinent to forensic applications and afford the opportunity to demonstrate the feasibility of the selected approaches without undue concern regarding the stability and availability of reagents and the potential requirement for special handling procedures.

The proposed approach is based on the sampling system developed and tested by Grunbaum [*4*]. This sampling system has three distinctive features: (a) a multiple sample applicator, (b) a holder for 40 samples, and (c) a uniquely indexed cell cover. The eight-sample applicator can deliver up to eight different specimens simultaneously, or each sample can be applied individually. However, simultaneous application of specimens has many advantages over sequential application. It is time-consuming to apply one sample at a time; consequently, the samples applied first are subject to local diffusion and mobility (due to convection currents) while the subsequent samples are being applied. This becomes serious when high resolution is required in comparative electrophoresis of unknown specimens.

The instruments and methods described also make feasible for the first time the concurrent determination of several genetic variants. It is thus well suited for use in bloodstain analysis in the forensic laboratory.

To accomplish the analysis, the sample applicator is used in combination with five separate electrophoretic cells. Each of the cells is filled with a buffer appropriate for the analysis of a separate enzyme system: phosphoglucomutase (PGM), glucose-6-phosphate dehydrogenase (G-6-PD), adenylate kinase (AK), adenosine deaminase (ADA), or glutamate pyruvate transaminase (GPT). The cells are arranged as shown in Fig. 1, and the five enzyme systems from a single blood sample are resolved concurrently and compared with three major known phenotypes of each variant.

TABLE 1—*Gross comparison of electrophoretic supporting media in the fractionation of plasma proteins.*

Supporting Media	Visible Plasma Bands, no.	Completion Time, h	Resolution	Sample Size, μl	Handling Properties [a]	Clinical Use	Clear Optical Properties	Other Applications
Filter paper	5	12 –24	poor	10	good	poor	no	very limited
Cellulose acetate	10+	0.5	very good	0.25	excellent	excellent	yes [b]	IE1, DID, COE1, EID, IF, Hb, Hp, Lp, Gp, LDH, AP, CPK, genetic enzyme variants [d]
Cellogel	8	2 – 4	good	2 – 5	poor	poor	yes [c]	limited
Starch gel	12	6 –12	good	1 –10	poor	poor	no	El of genetic enzyme variants [d]
Agar gel	4	6 –10	poor	1 – 5	poor	poor	yes	IE1, DID, SRID, COE1
Agarose (wet and rehydratable)	7	2 – 3	good	1	poor to fair	fair	yes	IE1, COE1, EID, IF (excellent for lipoprotein E1)
Acrylamide gel	25	5 – 6	excellent	1 –15	good	poor	yes	best for Hp typing, electro-focusing, and research

El—electrophoresis
IE1—immunoelectrophoresis
DID—double immunodiffusion
SRID—single radial immunodiffusion
COE1—cross-over electrophoresis
EID—electro-immunodiffusion (Laurell rocket immunoelectrophoresis and antigen/antibody crossed E1)
IF—immunofixation

Hb—hemoglobin
Hp—haptoglobin
Lp—lipoprotein
Gp—glycoprotein
LDH—lactic acid dehydrogenase isoenzymes
AP—alkaline phosphatase isoenzymes
CPK—creatine phosphokinase isoenzymes

[a] Including preparation and preservation as a record.
[b] After simple chemical treatment.
[c] After drying.
[d] PGM, AK, ADA, G-6-PD, 6-PGD, GPT, and so forth.

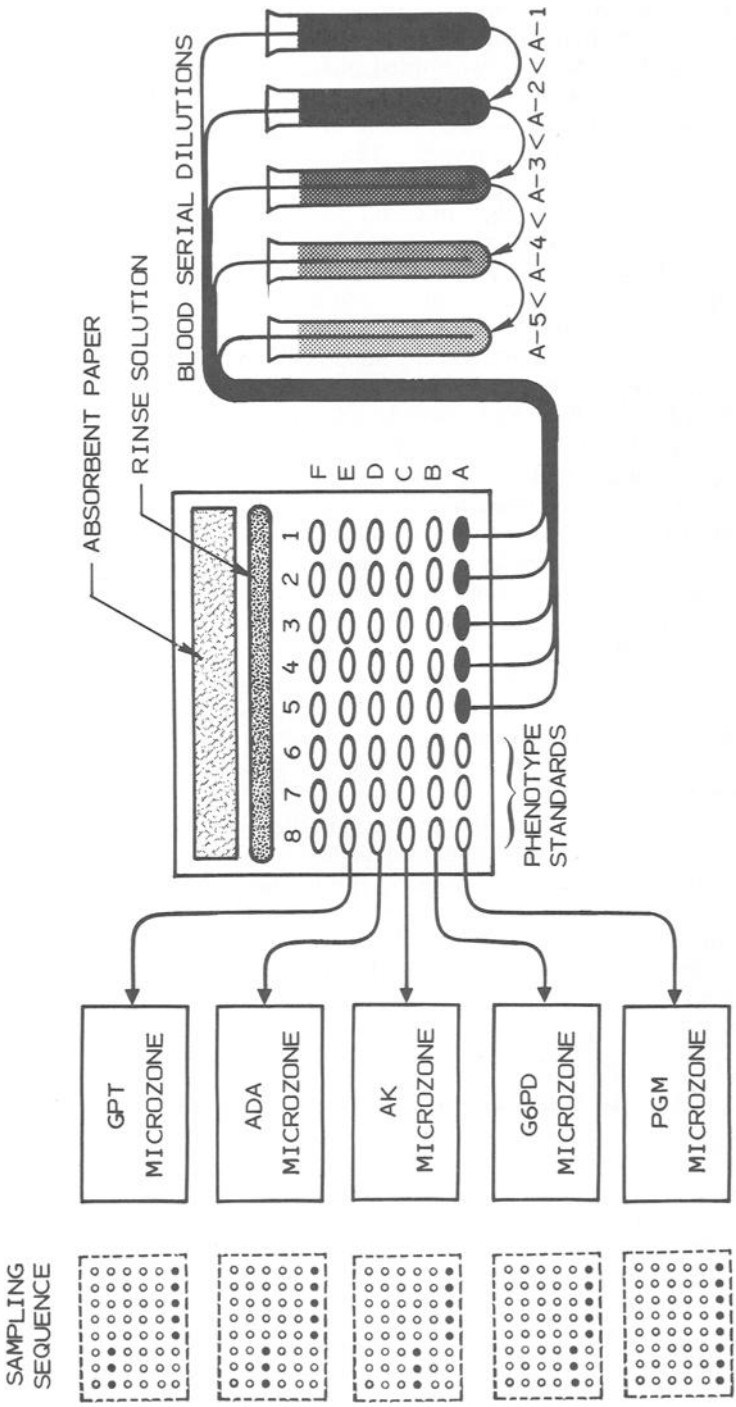

FIG. 1—*Sampling/analysis scheme for concurrent multiple isoenzyme analyses.*

The first step in the analysis is the preparation of the sample holder. This holder has six rows (however, only five are used at this time). Each row has eight wells. Each well is indexed by letter and number.

The blood sample to be analyzed is serially diluted to five concentrations to develop a technique to be used later with dried blood. (The concentration of the soluble components in dried blood extracts is unknown; therefore, it is necessary to go from high concentration to low concentration to attain one or two samples that have the optimum concentration of isoenzymes.) The five concentrations of the blood sample are placed in Wells A-1 through A-5.

Each of the wells, six to eight in each row, contains a different, known, specific, enzyme phenotype. For instance, Well A-6 contains phenotype PGM 1–1, Well A-7 contains phenotype 2–2, and Well A-8 contains phenotype 1–2. Once the holder has been set up, electrophoresis can begin within a few minutes.

The eight-sample applicator picks up a fixed amount from each of the samples from Row A and deposits them onto the electrophoretic unit charged with the appropriate buffers for that enzyme system. Electrophoresis starts at once.

Next, the applicator picks up at one time a fixed amount of Samples A-1 through A-5 and B-6 through B-8. These in turn are placed on a second electrophoretic unit with its appropriately charged buffer for the enzyme system in Row B. Enzymes in the remaining rows are then applied in turn in a similar manner.

Each electrophoretic cell is designed so that it may be set to turn itself off after the required electrophoresis time for the particular enzyme variants being tested. A light or buzzer signals when the run is finished. When the run is completed the cellulose acetate membrane is removed and placed on a previously prepared substrate appropriate for that enzyme system.

To date, the method has been tested for three enzyme systems: PGM [*5*], G-6-PD, and EAP [*6*]. Now I propose to develop the method and instrumentation for five enzyme systems to be run concurrently. It is obvious that this approach can later be expanded to include even more enzyme systems, once the basic multiple approach has been demonstrated.

This research is designed to fill a real need in the forensic science laboratory. Other methods are very complex by comparison, and it is difficult for criminalists to learn the procedures and obtain consistently reliable results. While the proposed procedures can be completed in 2 to 4 h by one person, other commonly used procedures require 20 to 30 h. Because of their complexity, these other methods are usually done in succession, producing potential artifacts. In addition, the traditional methods require a much larger sample, often a critical factor in a forensic laboratory.

Isoelectric Focusing

Electrophoresis has been used for many years and much valuable information in protein chemistry has been obtained with this technique. Conventional electrophoresis is performed in a continuous buffer solution with a constant pH and ionic strength. As in most other chromatographic processes, there is, during the separation, a continuous diffusion of the protein zone which affects the achievable resolution.

In electrofocusing, however, the separation is performed in a stable, linear pH gradient. The proteins being separated are forced to move towards a pH value equal to the isoelectric point — the pI of the protein. The proteins are concentrated into extremely narrow zones, thus bringing about high resolution of protein bands. One of the greatest advantages with electrofocusing is the constant counteraction of diffusion. The proteins are separated solely with respect to the pI, at which the molecule has zero charge.

In repeated experiments I find about 60 bands after isoelectric focusing of reconsti-

tuted whole dried bloodstains and 20 to 40 bands when electrofocusing dried red blood cell hemolysates. The number of bands tends to vary with the condition of the bloodstain.

The isoelectric focusing was carried out using an LKB 2117–010 Multiphor apparatus and prepared thin-layer polyacrylamide gels called "Ampholine® PAGplates." (Both items are available from LKB Produkter, Bromma, Sweden.) The ampholine concentration in the PAGplates is 2.4%, with a pH span of 3.5 to 9.5. The PAGplate dimensions are 245 by 110 by 1 mm. When it is known that successive thin gels are uniform in both their physical and chemical makeup, experiments can be performed both economically and quickly.

A study is underway to explore isoelectric focusing for the differentiation of two or more blood specimens, first by simple comparison of the many resulting protein bands visualized by using a general protein stain, and second by specific staining techniques which should permit visualization of the genetically controlled isoenzyme variants. It is expected that a much better definition of the genetically controlled isoenzymes will be obtained because of the superior resolution inherent in the electrofocusing method. The whole procedure can be completed in about 3 h. Micromodifications are also possible, permitting perhaps as many as ten enzyme systems to be determined on the same supporting medium and at the same time interval.

A model system of simultaneous phenotyping of five enzyme variants is currently being tested (Fig. 2). The polymorphic enzymes are the same as shown in Fig. 1, where conventional electrophoresis is used.

An unknown blood specimen is placed on an Ampholine® PAGplate supporting medium for electrofocusing in five positions parallel to the electrodes. Known isoenzyme standards (phenotypes) are placed beside each unknown. A single PAGplate readily accommodates 25 individual samples. Thus, five enzyme systems of a given, unknown specimen of fresh or dried blood can be determined in a single run. At the end of focusing, which can be determined by use of colored markers, each enzyme system

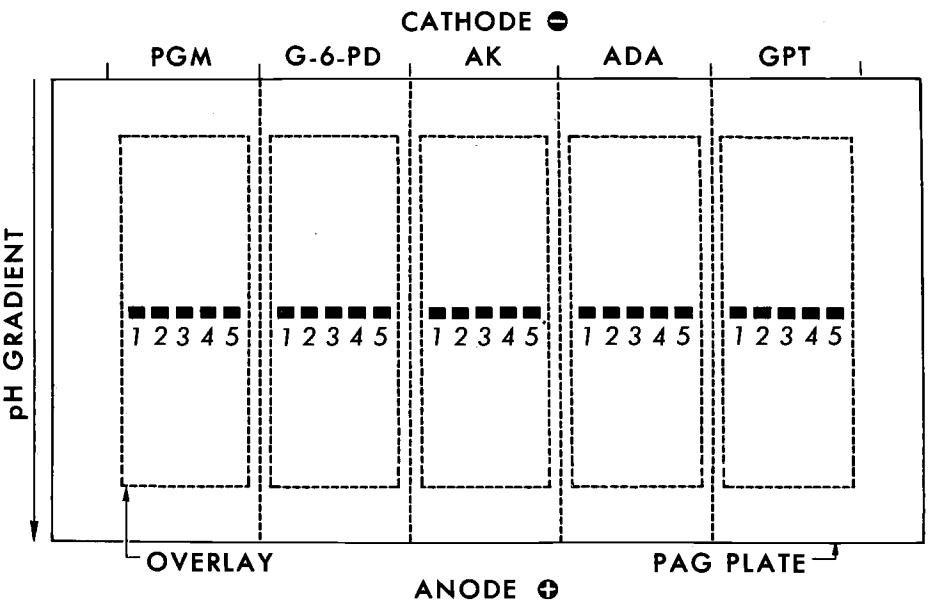

FIG. 2—*Simultaneous phenotyping of five enzyme variants by isoelectric focusing. For each enzyme system, Positions 1 and 2 are for the unknown blood or bloodstain and Positions 3, 4, and 5 are for the respective phenotype standards.*

in the diagram is overlaid with an appropriate substrate, stain, and buffer system. This is done by soaking (saturation without excess fluid) of a "wafer" of cellulose acetate membrane or other material suitable to the visualization of a particular enzyme system and placing it, without trapping air, over the corresponding known and unknown phenotypes. Since the wafer contains the necessary visualizing stain, it therefore also contains the zymogram impression and thus serves as the permanent record of the phenotype for a given genetically determined isoenzyme pattern.

Isotachophoresis

With isoelectric focusing the protein molecules concentrate in sharp zones according to their pI; whereas in isotachophoresis, fractionation is the result of net mobility of the respective molecules in a discontinuous electrolyte system. While both fractionating techniques have a high resolving power, isotachophoresis produces zone boundaries that remain sharp and continue to restore themselves after the steady state has been reached, for as long as the current remains on. Since all zones move at the same velocity (isotachophoresis) following the steady state, each fraction can be eluted without destruction of the sharp zone boundaries. (For the principle of the isotachophoresis method, see Application Note Number 146, LKB Produkter, Bromma, Sweden.)

Isotachophoresis is an advanced electrophoretic technique and holds considerable promise of becoming a most important tool in the purification, characterization, and analysis of single molecular species proteins. LKB Produkter AB in Sweden manufactures two instruments for isotachophoresis. The first is designed for fractionation of relatively large samples resulting in highly purified proteins. In forensic application, this instrument would provide a valuable source of purified antigens. These antigens can then be used for the production of monospecific antiserums needed in the highly specific and sensitive immunochemical reaction for identification and quantification of single molecular species proteins.

The second LKB instrument is designed for analytical microscale fractionations. The major characteristics of this apparatus are as follows.

1. An effective and active sharpening of zone boundaries results in very high resolution.
2. A concentrating effect makes it possible to use small amounts of sample in dilute form.
3. Direct detection of fraction and estimation of concentration is possible by use of both ultraviolet and thermal detectors.
4. Fractionation takes place in a 0.5-mm inside diameter Teflon® capillary; no stabilizing medium is required.
5. Counterflow can be applied to increase the effective separation capacity.
6. Analysis time is complete between 15 and 50 min.
7. Sample volume is in the order of 0.1 to 50 μl. This permits estimation of in the nanomole range.
8. Pretreatment of the test specimen is not required.
9. Nonaqueous solvents can be used, which may be advantageous in some circumstances.

Simultaneous fractionation of two or more specimens is obviously impossible by this electrophoretic method. However, since individual specimens can be fractionated in rapid succession, the respective fractions from a questioned forensic sample can be analyzed simultaneously against standards treated in a similar manner. This can be accomplished by the application of modern immunochemical techniques. Such tech-

niques as the tandem crossed immunoelectrophoresis described by Kroll [7] is a fast and extremely sensitive procedure. In this method, two to four individual fractions from different specimens are placed in adjacent wells, formed in agarose, and electrophoresed along the axis on which the wells lie. Subsequently, the gel segment is placed in an antibody-containing gel and again electrophoresed perpendicularly to the first electrophoretic axis. In this procedure, precipitin peaks form from the respective samples. The samples that are related fuse into double peaks. Those not related (not identical) do not. This procedure therefore permits a direct comparison between two, three, or four specimens. For example, a questioned specimen could be tested against three known standards.

Another useful technique for the analysis of isotachophoretic fractions is the fused rocket immunoelectrophoresis [8]. Samples from each fraction are placed into wells formed in agarose gel. They are allowed to diffuse radially for about 30 min. The whole agarose gel segment containing the wells is placed against another agarose gel. The second agarose gel contains a polyvalent antiserum of known antibodies to all possible components emerging from the isotachophoretically fractionated specimen. Subsequently the radially diffused proteins are forced to move anodically by electrophoresis. A number of rocket-shaped immunoprecipitins are formed simultaneously. The appearance of a precipitin is a positive indication of the presence of a specific antigen in the sample analyzed, and the height of the rocket is a measure of its quantity.

Using a fused rocket immunoelectrophoretic precipitation of isotachophoretically eluted fractions of nonionic, detergent-solubilized, human red blood cell membranes, Bog-Hansen et al [9] were able to detect 16 proteins (antigens).

It is clear that isotachophoresis in combination with the other techniques described offers considerable promise in exposing and identifying hitherto hidden antigens, especially in the dried blood specimen.

Immunofixation

Immunofixation is an immunoprecipitation technique in which antigen-antibody systems interact in a highly specific and sensitive manner due to structural affinities. Provided an antibody is available in an absolutely pure form, an antigen (a protein) of a single molecular species can be isolated for visualization. Also, provided that the concentration of the antigen and the antibody is sufficiently high, the resulting precipitin can be seen directly when using clear transparent gels as the supporting medium. In weaker concentrations the invisible precipitin can be made visible by the use of protein stains.

Thus, any kind of protein fractionation (conventional electrophoresis, isoelectric focusing, or isotachophoresis) which is subsequently subjected to immunofixation with known truly monospecific antiserums will result in positive identification in any number of conditions.

The phenotypes 1–1, 1–2, and 2–2 of the genetically controlled polymorphic protein called the group specific component (Gc) have been identified by this relatively simple technique of immunofixation. In theory the method appears to have extensive application in the crime and forensic laboratories, specifically in the phenotyping of the genetic variants of enzymes and proteins.

Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis [10] is a powerful technique that permits both qualitative and quantitative determination of multiple antigens (proteins) in a single run and in a relatively short period.

In this technique a protein solution is initially subjected to electrophoresis. Follow-

ing electrophoresis, the entire strip with the antigenic proteins is placed perpendicularly onto the cathodic end of an agarose gel plate containing antibodies and again electrophoresed. Rocket-shaped precipitates are obtained. The height of the rockets is directly proportional to the antigen-antibody ratio of the system.

Positive identification of individual precipitates in a crossed immunoelectrophoretogram is carried out immunochemically by the addition of known antigens or antibodies. Reference patterns can be obtained through use of a pooled serum from a large number of donors, a reference of antiserum, and standardized technique.

Provided high quality monospecific and polyvalent antiserums can be obtained, this technique of crossed immunoelectrophoresis can be very useful in conjunction with conventional electrophoresis, isoelectric focusing, and isotachophoresis.

Modifications of the method have been used effectively in the investigation of proteins (antigens) obtained as a result of the dissolution of the red cell membrane by nonionic detergents. No attempts have yet been described to use this method in the investigation of dried bloodstains. The method should be sensitive to nanogram quantities of protein. Thus, it is proposed to investigate this approach for possible use with dried bloodstains. More extensive and positive identification of antigens residing in the red cell membrane should be possible.

Summary

The individualization of human beings by biochemical "fingerprinting" is yet a goal of the future. However, recent progress in methods and instruments permits the forensic analyst to use the available techniques for partial individualization. For practical purposes, it is sufficient to demonstrate the capability of discriminating one human blood among a thousand others. The combination of some of the methods described makes such a discrimination possible. It is critical that both instruments and methods be standard so that two or more laboratories may obtain the identical results from analyses of the same questioned specimen. Only then can such evidence be presented with a high degree of reliability in medicolegal cases.

The objective of the studies described is to develop an immunohematological and biochemical analysis for individual blood samples from human beings to resolve and define phenotypic components of the blood to the maximum extent in the smallest volume. The result of this work is expected to permit the objective characterization of individuals in terms of their own unique genetic constitution; it will permit comparison of individuals as to degrees of similarity or dissimilarity of their genetically determined blood protein composition.

References

[1] Kirk, P. L., and Grunbaum, B. W., "Individuality of Blood and Its Forensic Significance" in *Legal Medicine Annual 1969*, C. H. Wecht, Ed., Appleton-Century-Crofts, New York, 1969, pp. 287–325.

[2] Culliford, B. J., *The Examination and Typing of Bloodstains in the Crime Laboratory*, U.S. Government Printing Office, Washington, D.C., 1971.

[3] *Survey and Assessment, Blood and Bloodstains Analysis Program*, Vol. I, *Technical Discussion*, The Aerospace Corp., El Segundo, Calif., April 1974.

[4] Grunbaum, B. W., "An Automatic One-to-Eight-Sample Applicator for Fast Qualitative and Quantitative Microelectrophoresis of Plasma Proteins, Hemoglobins, Isoenzymes and Cross-Over Electrophoresis," *Microchemical Journal*, Vol. 20, 1975, pp. 495–510.

[5] Grunbaum, B. W., "A Micro-Procedure for Fast Typing of the Genetic Variants of Phosphoglucomutase," *Journal of the Forensic Science Society*, Vol. 14, No. 2, 1974, pp. 151–157.

[6] Grunbaum, B. W., "Rapid Phenotyping of Erythrocyte Acid Phosphatase and Glucose-6

Phosphodehydrogenase'' in *Abstracts of 45th Semi-annual Seminar of the California Association of Criminalists,* 15–17 May 1975.

[7] Kroll, J., ''Tandem-Crossed Immunoelectrophoresis'' in *A Manual of Quantitative Immunoelectrophoresis,* N. H. Axelsen, J. Kroll, and B. Weeke, Eds., Universitets forlaget, Oslo, Norway, 1973, pp. 57–59.

[8] Svendsen, P. J., ''Fused Rocket Immunoelectrophoresis'' in *A Manual of Quantitative Immunoelectrophoresis,* N. H. Axelsen, J. Kroll, and B. Weeke, Eds., Universitets forlaget, Oslo, Norway, 1973, pp. 69–70.

[9] Bog-Hansen, T. C., Bjerrum, O. J., and Svendsen, P. J., ''Isotachophoresis of Membrane Proteins'' in *Science Tools* (An LKB Instrument Journal), Vol. 21, Nos. 2–3, 1974, pp. 33–34.

[10] Clarke, H. G. M. and Freeman, T., ''A Quantitative Immuno-Electrophoresis Method (Laurell Electrophoresis)'' in *Protides Biological Fluids,* H. Peeters, Ed., Elsevier, Amsterdam, 1967, pp. 503–509.

White Mountain Research Station
Bldg. T-2251
University of California
Berkeley, Calif. 94720