J Forensic Sci, Jan. 1978, Vol. 23, No. 1

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Phenotyping of Erythrocyte Acid Phosphatase in Fresh Blood and in Bloodstains on Cellulose Acetate

The genetically controlled polymorphic enzyme system of human erythrocyte acid phosphatase (EAP) has considerable usefulness as a discriminating factor in blood because five common phenotypes can be separated electrophoretically as distinct patterns.

Hopkinson et al [*1*] described the conventional use of starch gel as the supporting medium for the electrophoretic separation of the isoenzymes of EAP. This method requires between 5 to 16 h, and additional time is needed for the preparation of plates and for sample insertion. Furthermore, this method requires an elaborate cooling system.

Sonneborn [*2*], using cellulose acetate as the supporting medium, reduced the electrophoresis time to 105 min and the development time to 70 min. Neither of these methods produced electrophoretograms in which the band pattern were always distinct and easily discernible.

In this laboratory a new electrophoretic method, based on techniques previously described by Grunbaum [*3*], is in routine use in the phenotyping of EAP and several other variants in fresh human blood. Eight blood samples may be simultaneously picked up and applied to a cellulose acetate membrane and then subjected to electrophoresis. The resultant EAP bands can be clearly discerned and identified within 45 to 50 min. Phenotype patterns on the electrophoretograms may be kept indefinitely to provide permanent records. This, of course, is not possible when gel is used as a supporting medium. This method is also used successfully with aged bloodstains.

Materials and Methods

Equipment and Reagents

1. Beckman Microzone® Electrophoresis System and accessories (Beckman Instruments, Inc., Fullerton, Calif.).
2. An automatic multiple sample applicator and accessories described by Grunbaum [*4*].
3. Cellulose acetate membranes, 5.5 by 14.5 cm (Sartorius Filters, Inc., South San Francisco, Calif.).

The preparation of these materials was financially assisted through a federal grant from the Law Enforcement Assistance Administration (LEAA) and the California Office of Criminal Justice Planning (OCJP) and under Title I of the Crime Control Act of 1973. The opinions, findings, and conclusions in this publication are those of the authors and not necessarily those of OCJP or LEAA. Received for publication 23 May 1977; accepted for publication 15 June 1977.

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4. Polyethylene tubes, 1.5 ml, cone-shaped (Cole Scientific, Calabasas, Calif.).

5. Buffers—For the cell buffer, the following chemicals were dissolved in 800 ml distilled water: 4.41 g sodium citrate (0.015*M*); 3.38 g monobasic sodium phosphate (0.0245*M*); 0.372 g ethylenediaminetetraacetic acetate (EDTA) (disodium salt). The pH was then adjusted with sodium hydroxide (1 to 5*N*) to 5.9 and finally brought to 1 litre volume. For the membrane buffer, one part cell buffer was diluted with 3.75 parts distilled water (26.5 ml diluted to 100 ml). For the developing mixture buffer, 10.5 g citric acid (0.05*M*) and 2.0 g sodium hydroxide (0.05*M*) were dissolved in 800 ml distilled water. The pH was adjusted to 5.0 with sodium hydroxide (1*N*) and the volume was adjusted to 1 litre.

6. Developing substrate mixture—Noble agar, 250 mg (Difco Laboratories, Detroit, Mich.) was dissolved by boiling in 10 ml developing mixture buffer and cooled to 55°C. Approximately 2 mg of 4-methylumbelliferyl phosphate was dissolved in 10 ml developing mixture buffer. These mixtures were combined and 15% by volume glycerol was added. This was stirred and poured at once into three or four square plastic petri dishes, which were then covered with their complementary plastic tops. The procedure for plate preparation was similar to the one described by Grunbaum [*4*] for phenotyping of phosphoglucomutase. In actuality, many plates were prepared at once and stored in a refrigerator for several days of use. The workload was not substantially different in preparing one or multiple gel substrate plates.

Method

To prepare the hemolysates, whole blood was centrifuged and the plasma separated. An aliquot of packed cells (approximately 200 μl) was added to a disposable, micro polyethylene tube that contained 0.5 ml distilled water. The unwashed cells were twice frozen and thawed by placing the tube first in liquid nitrogen and then in lukewarm water. This process produced sufficient hemolysis. About 25 μl of each hemolysate was placed in a numbered well in the sample holder for the eventual transfer to the cellulose acetate membrane by the multiple sample applicator.

For the bloodstain extraction, several fibers of bloodstained cloth or dry blood chips were placed into the well of the sample holder. A minimal volume of 0.05*M* Cleland's reagent (dithiothreitol, Sigma No. D-8255) was added to the well contents and allowed to soak in for 5 to 15 min. For more efficient extraction, the liquid in the well was sucked into a capillary and expelled very carefully to avoid any air bubbling. This process was repeated two to five times.

For the electrophoresis, a cellulose acetate membrane was saturated in the 1:3.75 dilution of the cell buffer, blotted between filter papers, and placed onto the bridge of the cell. The holder with the membrane was placed into the electrophoresis cell, which had previously been filled with cold (about 4°C) cell buffer. One application of about 0.25 μl hemolysate was applied to the cathodic side of the center of the membrane. The sample holder, multiple applicator, and electrophoresis cell were extremely well indexed and integrated. Consequently, sample placement relative to the cathode and anode was precise and reproducible (see Fig. 1). Electrophoresis was carried out at a constant voltage of 250 V for 40 min. The starting amperage was about 2 mA and slowly rose to about 3.5 to 4.0 mA. This rise is amperage was not sufficient to cause any thermal convection.

Immediately after electrophoresis, the ends of the membrane were cut off with a razor blade about 10 mm outside of each row of index holes. The membrane was lifted and placed facedown onto the developing gel substrate mixture. Care was taken to avoid trapping any air and to make complete contact. The membrane was incubated at 37°C for 10 min or less, then viewed under long-wave ultraviolet light. The isoenzymes showed fluorescent bands where the 4-methylumbelliferyl phosphate had been hydrolyzed to 4-methylumbelliferone. The EAP isoenzyme patterns were recorded photographically when the individual

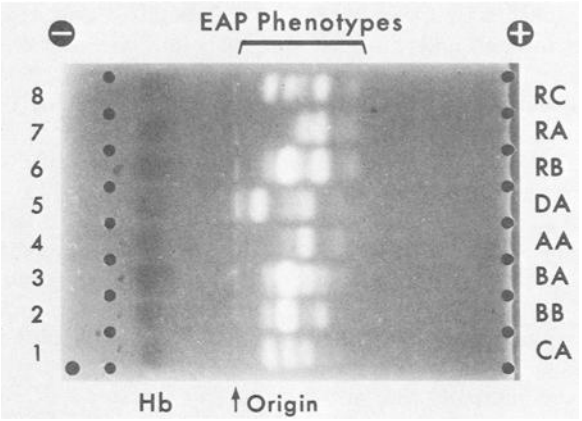

FIG. 1—*The cellulose acetate electrophoretogram shows four common* (AA, BA, BB, *and* CA) *and four rare* (DA, RB, RA, *and* RC) *phenotypes of EAP from human blood.*

isoenzymes showed optimal intensity. The membrane was removed from the gel, layered between two clean blotters, and placed in a drying frame. The frame was placed in an oven and subjected to 110 °C for 10 min. The proteins were thus denatured and the enzyme pattern was immobilized and prevented from further diffusion. In this fashion, the cellulose acetate electrophoretogram was permanently preserved so that it could be studied again.

Results and Discussion

The quality of an electrophoretic pattern depends on many variables. Voltage and time of electrophoresis are variables that can be controlled. Changes in pH, molarity, thermal convection, diffusion, consistency of the supporting medium, and electroendosmosis are not readily controlled. However, the effect of these variables may be significantly reduced by shortening the length of time of electrophoresis to the absolute minimum and keeping the sample size as small as possible. For instance, thermal convection and the consequent diffusion with blurred resolution may be insignificant if electrophoresis time is brief. Also, if a sample is large and broad relative to the electrodes, the resolution will be adversely affected. A narrow sample application will result in a higher resolution. These factors were carefully considered in working out the method for the phenotyping of EAP.

Figure 1 is an eight-sample cellulose acetate electrophoretogram showing four common phenotypes of EAP, namely AA, BB, BA, and CA, and four rare EAP phenotypes, DA, RB, RA, and RC. At this time we have analyzed some 10 000 samples of blood and we found many EAP types of CB and the homozygous EAP phenotype C.

In Fig. 2, Samples 1 and 8 are fresh standards of EAP types BA and AA, respectively. Samples 2 through 7 are from 30-day-old bloodstains on cloth. The indicated EAP types are unmistakably recognizable and they do not differ in quality from the fresh specimens. The addition of Cleland's reagent to aged bloodstains seems to restore the phenotype patterns to their original condition. Without this reagent, degradation products may appear and interfere with correct identification.

The intensity of EAP activity is dramatically heightened through use of glycerol in the developing substrate mixture. The effects of glycerol and other alcohols on the EAP reaction rate was reported by Luffman and Harris in 1967 [5]. Sensabaugh and Golden [6] added glycerol to the starch gel supporting medium and noticed an increase of fluorescence. They observed that the addition of glycerol to the substrate mixture had little

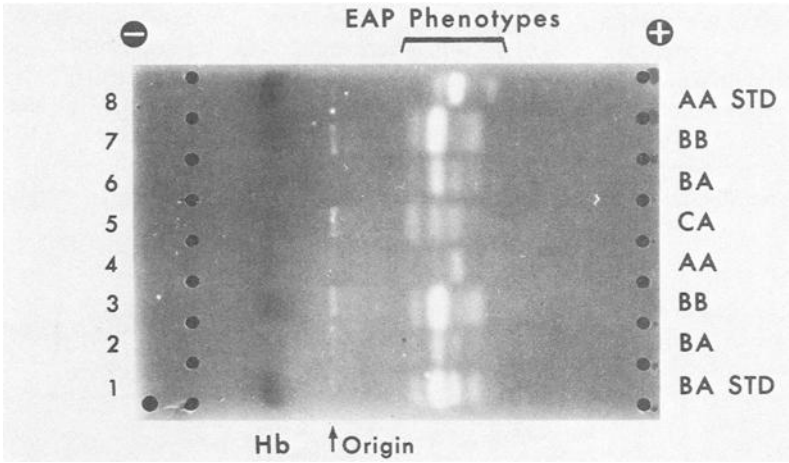

FIG. 2—*Phenotyping of bloodstains for EAP on cellulose acetate membranes. Samples 1 and 8, which are types* BA *and* AA, *respectively, from fresh blood, serve as standards for comparison. Samples 2 through 7 are from six different bloodstains aged on cloth for 30 days. They are types* BA, BB, AA, CA, BA, *and* BB, *respectively.*

effect when used with starch gel. In this laboratory we have found that when glycerol is applied to the cellulose acetate membrane there is an undesirable slowing of the mobility of the EAP isoenzymes. However, when glycerol is added to the substrate mixture only and cellulose acetate is used as the supporting medium there is a dramatic increase in fluorescence.

Glycerol, when added to the substrate mixture, is effective for use with cellulose acetate and not with starch gel because of differences in the physical characteristics of the two supporting media. A band that appears after electrophoresis on cellulose acetate remains on the surface of the very thin membrane and is still highly concentrated at time of contact with the substrate containing glycerol; consequently, it reacts immediately. Conversely, the sample is embedded in the relatively thick layer of starch gel. After electrophoresis each band diffuses throughout the depth of the starch gel layer. This diffusion takes time, so the enzyme reaction with the substrate containing glycerol is delayed. Only that small diffuse part of the band that appears at the interface of the starch gel and substrate can interact with the glycerol since the glycerol does not readily diffuse into the starch gel.

A high intensity ultraviolet source is used to bring out the extremely sharp visible pattern of the highly fluorescent bands characteristic of any EAP phenotype. As with many enzymatic reactions following electrophoresis, the bands increase in sharpness and continue to widen over a period of time. However, the broadening of the bands eventually results in poor resolution. A black and white Polaroid® photograph should be taken as a record when the phenotype pattern is complete and resolution has not yet been adversely affected. In the procedure described, this time interval is at least 30 min.

This method for EAP phenotyping is extremely useful and economical in our study of the frequency distribution of genetic variants in a selected population. Since the method is also applicable to dried blood, it should be useful in the crime laboratory in increasing individualization of bloodstains submitted for identification.

Summary

This electrophoretic procedure phenotypes the genetically controlled variants of EAP. With a modified Beckman Microzone electrophoresis cell and cellulose acetate membranes,

eight samples of hemolysates are separated simultaneously into their respective polymorphic isoenzymes. After the isoenzymes react with 4-methylumbelliferyl phosphate for 5 to 10 min, fluorescent patterns characteristic of specific phenotypes are observed. The whole procedure, from sample placement with a semiautomatic applicator to phenotype identification, requires 50 min and 0.25 to 0.5 μl of hemolysate consisting of one part erythrocytes and three parts water. Bloodstains prepared on cotton cloth and aged at ambient laboratory conditions for 30 days are also readily phenotyped.

Acknowledgments

The authors wish to thank the administration of the Alameda Country Sheriff's Department for their encouragement and support for the work conducted in the bloodstain analysis program.

References

[1] Hopkinson, D. A., Spencer, N., and Harris, H., "Red Cell Acid Phosphatase Variants: A New Human Polymorphism," *Nature* (London), Vol. 199, 1963, pp. 969-971.

[2] Sonneborn, H. H., "Determination of Acid Phosphatase Isoenzymes by Cellulose Acetate Electrophoresis," *Humangenetik*, Vol. 18, 1973, pp. 355-356.

[3] Grunbaum, B. W., "A Micro Procedure for Fast Typing of the Genetic Variants of Phosphoglucomutase," *Journal of the Forensic Science Society*, Vol. 14, 1974, pp. 151-157.

[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] Luffman, J. and Harris H., "A Comparison of Some Properties of Human Red Cell Acid Phosphatase in Different Phenotypes," *Annals of Human Genetics*, Vol. 30, 1967, p. 387.

[6] Sensabaugh, G. F. and Golden, V. L., "Erythrocyte Acid Phosphatase Typing on Starch Gels Containing Glycerol," *Forensic Serology News*, Vol. 2, No. 6, 1977.

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