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Electrophoresis of Esterase D in Fresh Blood and in Bloodstains on Cellulose Acetate

Esterase D (EsD) was first phenotyped by Hopkinson et al [*1*] using starch gel electrophoresis. Three phenotypes were described: 1-1, 1-2, and 2-2. Bender and Frank [*2*] detected a new EsD phenotype, which they named 3-1. Typing of EsD in bloodstains with starch gel has been reported by Parkin and Adams [*3*]. Esterase D isoenzymes have also been separated by electrophoresis with polyacrylamide gel, agarose gel, and cellulose acetate by Köster et al [*4*]. They observed poor results with cellulose acetate and discontinued its use. Since the results with phenotyping EsD on cellulose acetate in our laboratory are unambiguous, fast, reproducible, and economical, it is obvious that good results depend on the technology employed.

At this laboratory, 10 000 fresh blood specimens from human blood donors have been typed for EsD on cellulose acetate membranes within the past year. We have found cellulose acetate to be the best electrophoretic supporting medium for EsD phenotype determination in both fresh blood and 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 [*5*].
3. Cellulose acetate membranes, 5.5 by 14.5 cm (Sartorius Filters, Inc., South San Francisco, Calif.).
4. Polyethylene tubes, 1.5 ml, cone-shaped (Cole Scientific, Calabasas, Calif.).
5. Buffers—For fresh blood, the cell buffer was made by dissolving the following chemicals in 800 ml distilled water: 1.88 g Trizma Base (Sigma Chemical Co. T-1503), 0.87 g citric acid, 0.28 g boric acid, and 0.042 g lithium hydroxide. The pH was then adjusted to 7.2 with hydrochloric acid and finally brought to 1 litre volume. Undiluted cell

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buffer was use as the membrane buffer. The developing mixture buffer was 0.05*M* sodium acetate buffer, pH 5.7.

For bloodstains, the cell buffer was made by dissolving the following chemicals in 800 ml distilled water: 12.11 g Trizma Base (Sigma Chemical Co. T-1503), 11.62 g maleic acid (Sigma M-0375), 2.92 g ethylenediaminetetraacetic acid (EDTA) (Sigma ED-2SS), and 2.03 g magnesium chloride (Sigma M-0250). The pH was adjusted to 7.4 with 40% sodium hydroxide, and the mixture was brought to one litre volume. A 1:10 dilution of the cell buffer was used as the membrane buffer.

6. Developing substrate mixture—Noble agar, 1.0 g (Difco Laboratories, Detroit, Mich.), was dissolved by boiling in 80 ml of developing mixture buffer and cooled to 55°C. Approximately 15 mg of 4-methylumbelliferyl acetate was dissolved in a minimal amount of acetone and this solution added to 20 ml of developing mixture buffer. The two mixtures were combined, stirred, and poured at once into 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 [*6*] for phenotyping phosphoglucomutase. Actually about 20 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

Fresh Blood

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, 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 electrophoresis, a cellulose acetate membrane was saturated in the membrane buffer, blotted between filter papers, and placed onto the bridge of the cell. The bridge with the membrane was placed into the electrophoresis cell, which was previously charged with cold (about 4°C) cell buffer. Four applications of about 0.25 μl each of hemolysate were applied to the membrane. Electrophoresis was carried out for 30 min at a constant 300 V, and the amperage started at 2.0 mA and rose to 2.5 mA.

Immediately after electrophoresis the cellulose acetate membrane was cut at each end with a razor blade approximately 10 mm outside the rows of index holes. The membrane was then carefully placed facedown on the reaction gel surface in such a way as to prevent trapped air bubbles. After 5 min at room temperature, the pattern may be observed with transmitted long-wave ultraviolet light and photographed. The membrane can be removed from the reaction gel and dried between blotters at room temperature. The pattern remains visible and is enhanced by wetting the membrane with water.

Bloodstains

Whenever possible a few dry blood chips were scraped from the surface of stained material for analysis. When there were no chips, a few fibers of cloth containing the bloodstain were placed in the sample holder. A minimal volume of 0.05*M* Cleland's reagent (dithiothreitol, Sigma D-8255) was added to the stain and the mixture was allowed to soak for 5 to 10 min.

The cellulose acetate membrane was saturated with 1:10 dilution of the bloodstain buf-

fer and treated as for electrophoresis of fresh blood. Two applications of the blood extract were applied to the cellulose acetate membrane close to the cathode. Electrophoresis was carried out for 60 min at 200 V. The amperage started at 0.4 mA and rose after 60 min to 4.0 mA.

The method for detection is the same as for fresh blood. However, developing of visible patterns at 37°C may take 15 min or longer, depending on the persistence of the EsD enzymes and the quality of the stain.

Results and Discussion

Figure 1 shows a cellulose acetate electrophoretogram of 16 specimens phenotyped simultaneously. In the routine screening of thousands of samples for a population fre-

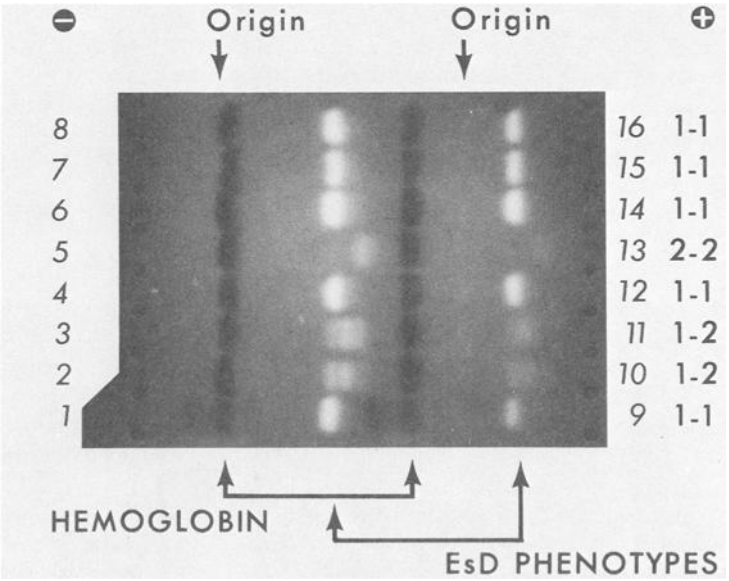

FIG. 1—*This cellulose acetate electrophoretogram shows the three common phenotypes of EsD from human blood, namely, 1-1, 1-2, and 2-2. The eight random samples in each row are duplicates to show that a given phenotype pattern is identical, regardless of whether the sample is placed on the cathodic or anodic side of the membrane. Thus 16 specimens can be phenotyped simultaneously.*

quency study, this laboratory has made a considerable savings in time of analyses and cost of materials by phenotyping 16 samples on a single membrane. The two rows of eight each are duplicates and were derived from eight random blood donors. Placement on the cathodic or anodic side appears to make no difference in the patterns of identical samples. The hemoglobin pattern seen in Positions 1 and 9 is from a person with sickle-cell trait.

In Fig. 2 the three common types of EsD, 1-1, 1-2, and 2-2, are shown in Positions 1, 2, and 3 and then again in Positions 5, 6, and 7. The very rare EsD variant 1-3 is seen in Positions 4 and 8. The duplicate pattern of each phenotype is identical with respect to resolution, intensity, and space on the membrane. This is a result of uniform sample size and placement on the cellulose acetate membrane. The resolution of the individual EsD isoenzymes in Figs. 1 and 2 is so clear that a diagram is not necessary.

Figure 3 shows a cellulose acetate electrophoretogram of the three common phenotypes of EsD in a two-week-old bloodstain on cloth. The respective phenotypes are clearly dis-

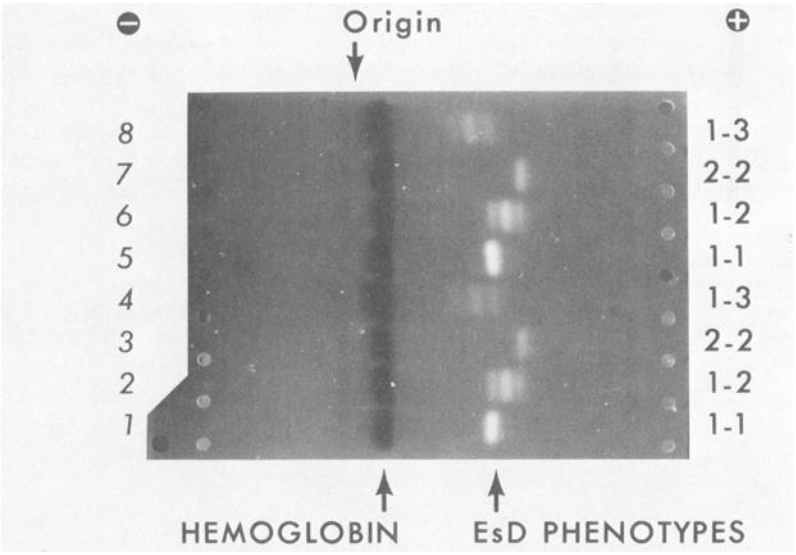

FIG. 2—*This electrophoretogram shows the rare phenotype pattern EsD, the 1-3 type, when run simultaneously with standards of the common EsD types.*

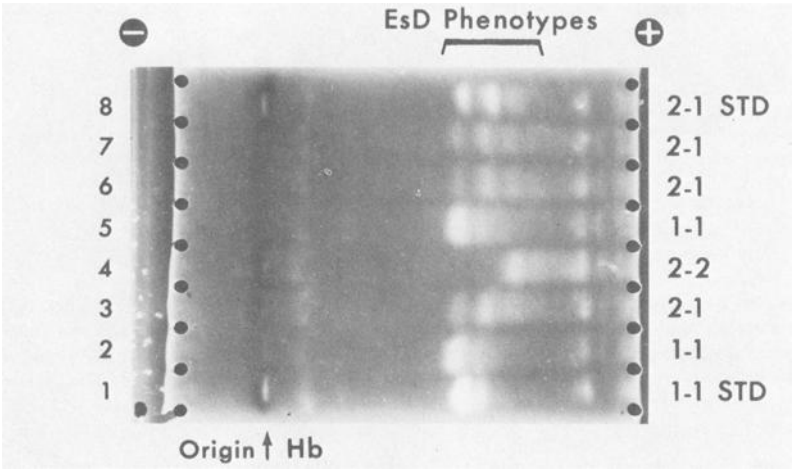

FIG. 3—*This cellulose acetate electrophoretogram shows EsD phenotypes from two-week-old bloodstains. Samples 1 and 8 are fresh blood, phenotypes 1-1 and 2-1, respectively, which are used as standards.*

tinguishable when compared to fresh standards in Positions 1 and 8. Four-week-old laboratory-prepared bloodstains on cloth were easily phenotyped. This method has been used in actual casework to phenotype for EsD in bloodstains up to two months old. Since no two random bloodstains received in a criminalistics laboratory will have an identical history, identification of the exact EsD phenotype may at times be difficult and inconclusive. However, the procedure described in this paper permits the investigating criminalist to exploit a bloodstain thoroughly for all the EsD that may persist.

Summary

Three common variants of EsD and one rare type have been phenotyped by electrophoresis of red blood cell hemolysates on cellulose acetate. When this method is used with fresh blood, 1 μl hemolysate consisting of one part cells to three parts water is subjected to electrophoresis for 30 min. The isoenzymes are then subjected to reaction with 4-methylumbelliferyl acetate. Fluorescent band patterns can be observed under ultraviolet light after 5 min at room temperature. As many as 16 samples can be analyzed simultaneously on the same membrane. Electrophoresis of dried bloodstains requires 60 min, and phenotype development takes 15 min at 37°C.

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