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A Stability Study of the Esterase D Isoenzymes

Evidence of variations in human red cell esterase was first presented by Tashian [*1-3*]. With starch gel, borate buffer electrophoresis followed by azo-dye-coupled staining procedures, zones of esterase activity were classified as A_1, A_2, A_3, B, and C. Population variants of these esterase enzymes have proven to be either exceedingly rare or nonexistent and consequently have no forensic application.

In 1973, Hopkinson et al [*4*] defined another esterase activity, esterase D (EsD), with "fluorogenic" substrate overlays following starch gel electrophoresis. They characterized the isoenzymes of EsD by electrophoretic mobilities, staining properties, pH profiles, and tissue distribution. Family and population studies were also made.

Recognizing the forensic potential of gene frequencies of EsD polymorphism, Parkin and Adams [*5*] in 1975 applied a modified thin-layer electrophoresis technique using a tris-(hydroxymethyl)aminomethane-citrate-borate-lithium hydroxide buffer to bloodstain phenotyping. Among their observations was a recognition of difficulty in accurately typing increasingly aged dried stains. In view of the reported alterations in band patterns with older stains, a comprehensive stability evaluation of these isoenzymes has been conducted in this laboratory. Since fluid blood samples submitted for analysis may contain a variety of anticoagulants and preservatives and may be subjected to different temperature environments in transit, a systematic study of the effects of these factors has also been done.

Method

Samples

Age of Dried Bloodstains—Dried bloodstains from 18 donors (eleven EsD 1, five EsD 2-1, and two EsD 2) were made by finger-prick onto cotton cloth. These were stored at room temperature and analyzed on alternate days (thrice weekly) until the electrophoretic patterns became uninterpretable. Dried bloodstains from freshly collected control samples of the three phenotypes were included on each plate.

Effect of Anticoagulants and Temperatures—Blood from one donor of each phenotype was drawn by venipuncture into a set of Vacutainer® tubes (Becton-Dickinson) containing the following anticoagulants:

heparin—143 units of sodium heparin per 7 ml of blood;

oxalate—4 mg of potassium oxalate and 6 mg of ammonium oxalate per 5 ml of blood;

EDTA—0.07 ml of 15% potassium ethylenediaminetetraacetic acid containing 0.014 mg of potassium sorbate per 7 ml of blood;

blank—no anticoagulant or preservative; and

citrate-fluoride[2]—50 mg sodium citrate and 100 mg of sodium fluoride per 10 ml of blood.

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[2] Non-vacuum tubes supplied by the Centre of Forensic Sciences to law enforcement agencies in Ontario for collection of blood samples for alcohol determinations.

Immediately after collection, blood from each tube was divided into three lots and stored at 3°C, 37°C, and room temperature (approximately 22°C). Every other day, an aliquot from each specimen was air-dried on a piece of clean cotton cloth and analyzed within 48 h of stain preparation. Staining and analysis of the samples continued as the fluid blood aged until the electrophoretic patterns became uninterpretable. Freshly collected control samples of the three phenotypes were included in each plate.

Materials

The electrophoretic technique used was essentially that described by Parkin and Adams [5], with slight modification. The support medium was 1-mm-thick 10% starch gel. The gel buffer consisted of 6.75 m*M* Tris, 1.8 m*M* citric acid, 2.2 m*M* boric acid, and 0.2 m*M* lithium hydroxide, pH 7.4, and the tank buffer was composed of 440 m*M* boric acid and 40 m*M* lithium hydroxide, pH 7.2. For the substrate buffer 0.05*M* sodium acetate was adjusted to pH 6.9 with acetic acid. The substrate mixture was prepared by dissolving 5 mg 4-methyl umbelliferyl acetate in two drops of acetone and immediately mixing it with 10 ml of substrate buffer.

Procedure

A portion of approximately 3 to 4 mm^2 was cut from each sample stain and soaked in one drop of 0.05*M* Cleland's reagent (dithiothreitol) in gel buffer for approximately 15 min. A slit was cut in the gel approximately 60 mm from the cathode and a piece of Whatman No. 3 filter paper (approximately 6 by 1 mm) saturated with the stain extract was inserted into the slit.

Electrophoresis was carried out at 15 V/cm toward the anode at 4°C for 3 h. After electrophoresis, a sheet of Whatman No. 3 filter paper was placed on top of the gel on the anodal side of the origin, saturated with the substrate mixture, and allowed to react at room temperature for 10 min.

At the end of reaction with the substrate overlay, each plate was photographed under ultraviolet light with a Polaroid Land camera[3] (Fig. 1). The results were read from the photograph "blind" and independently by three people. Densitometer[4] tracings were made from the Polaroid negatives when aged samples approached the end point of readability.

Results and Discussion

Age of Dried Bloodstains

Figure 2 illustrates the electrophoretic patterns of the three EsD phenotypes, 1, 2-1, and 2, obtainable from both freshly prepared hemolysates and dried bloodstains. The two homozygous (EsD 1 and 2) patterns consist of three isoenzyme bands, each displaying decreasing intensities toward the anode, with the third and weakest band of EsD 1 corresponding in electrophoretic mobility to the first and strongest band of EsD 2 [5]. The anodal order of the band positions may thus be designated as EsD 1 Positions 1, 2, and 3 and EsD 2 Positions 3, 4, and 5. In terms of electrophoretic mobility, the heterozygous form of the enzyme (EsD 2-1) appears to be a simple sum of the two homozygous forms showing five isoenzyme bands corresponding to the positions of Bands 1 to 5 of the homozygous forms. However, the activity of the 2-1 pattern is such that, in terms of relative band intensities,

[3]Polaroid camera, Model 545 film holder, with accessory mount. Film: Polaroid 4 by 5 Land film, Type 55/positive-negative. Illumination: No. 4 General Electric black-light bulb (ultraviolet), Type F-15 T8-BLB.

[4]Vitatron TLD 100 universal densitometer.

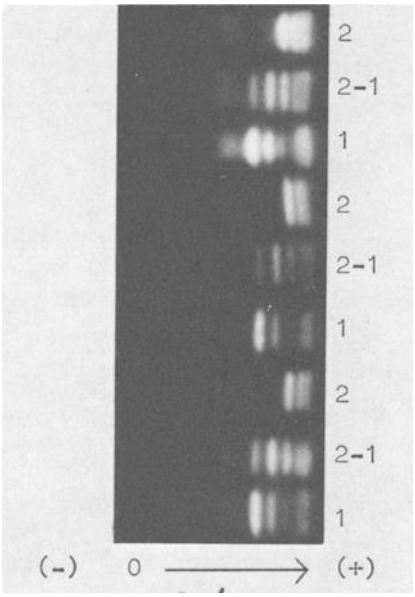

FIG. 1—*An EsD plate* (O = *origin*).

2 > 3 >1. Bands 4 and 5 occurring in the EsD 2 and 2-1 patterns are known to have mobilities approximating esterase A_2 banding and are often poorly resolved; they are consequently not considered significant in EsD phenotyping and are not illustrated in the figures.

One of the first observations made with aging bloodstains was a change in band intensities of phenotypes 1 and 2-1 with time. Specifically, Band 1 in EsD 1 loses its intensity more rapidly than Bands 2 and 3, and with EsD 2-1, Band 2 loses its intensity more rapidly than Bands 1 and 3. However, when EsD 1 bloodstains were allowed to age further, the same pat-

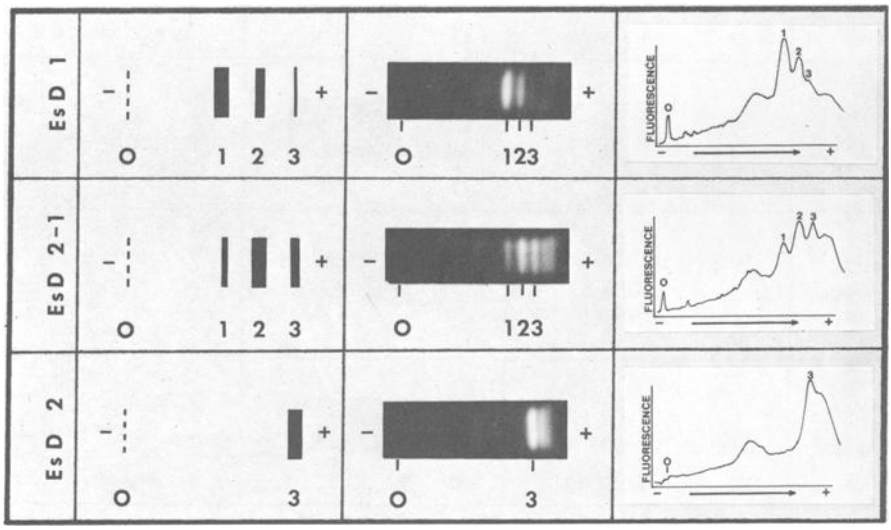

FIG. 2—*Electrophoretic patterns of the three EsD phenotypes, 1, 2-1, and 2* (O = *origin*).

tern emerged as with aged EsD 2-1 stains; all three bands in both phenotypes were of equal intensity (Figs. 3 and 4). No similar difficulty was encountered with the EsD 2 pattern. These observations led to the establishment of the following criteria:

1. To differentiate 1 and 2-1 patterns, the banding must display unequal intensities. At least one band must be more intense than the others.
2. If total activity is low or if the bands are diffused, the pattern is not considered readable.

With these criteria, no error was encountered in grouping stains up to four weeks of age. Stains older than four weeks could not be grouped. When case control bloodstains were tested, some stains between three and four weeks old yielded inconclusive results (Table 1).

Effect of Anticoagulants and Temperatures

Fluid blood incubated at 37°C for one day was readily and correctly grouped regardless of the anticoagulant; however, by the third day at 37°C all the fluid samples had deteriorated to the extent that none of the phenotypes could be identified.

After five weeks of storage at 3°C, phenotypes of all fluid samples were easily identified regardless of anticoagulant. The stability limit was not established for this storage temperature. Slight changes in the relative intensities of the isoenzyme bands were evident, but they were not severe enough to cause any difficulty in typing.

Figure 5 illustrates the effects of different anticoagulants on the survival times of EsD phenotypes in fluid blood samples stored at room temperature (approximately 22°C). In no instance was there evidence of transition between patterns. With time, pattern changes paralleled those observed in aging stains or faded uniformly to the point where, using the reading criteria, the results were inconclusive.

Clearly, fluid blood either without anticoagulant or with citrate-fluoride provided maximum stability of the isoenzymes for up to 28 days. Heparin-treated samples were readable

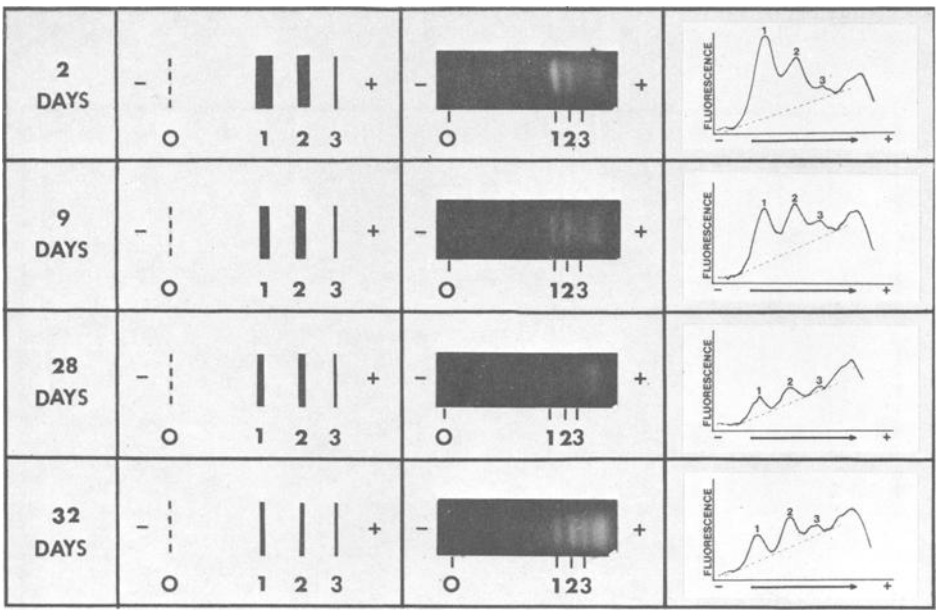

FIG. 3—*Changes in EsD 1 isoenzyme pattern as observed in progressively aging bloodstains* (O = *origin; dotted lines in the densitometer tracings represent adjusted baselines*).

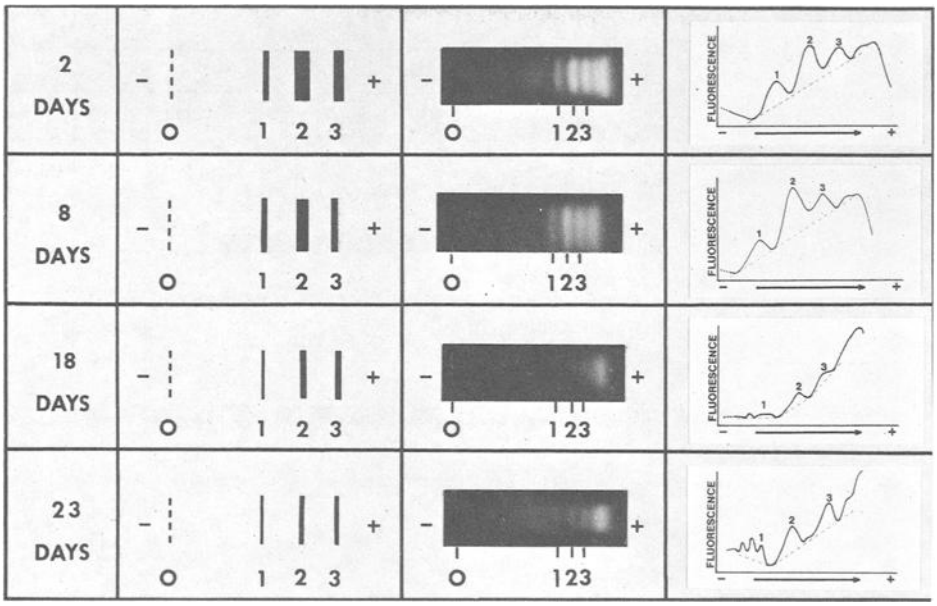

FIG. 4—*Changes in EsD 2-1 isoenzyme pattern as observed in progressively aging bloodstains* (O = *origin; dotted lines in the densitometer tracings represent adjusted baselines*).

TABLE 1—*Summary of EsD grouping of control blood samples.*

Source	Total Attempted	Conclusive Results	Inconclusive Results
Staff	48	48	0
Case control (Ontario)	366	334	32[a]

[a] Each of these samples displayed some evidence of deterioration. The resulting patterns did not satisfy our established criteria for reading.

until the 21st day, while oxalate and EDTA severely reduced the survival time to 14 and 3 days, respectively.

Since interpretation of the patterns becomes increasingly difficult as the isoenzymes age, densitometer tracings are of assistance. Screening the Polaroid negatives for light absorbance rather than making direct fluorescent readings on the plate effectively circumvents the problems of rapid band diffusion and gel drying following the substrate reaction phase. In Figs. 3 and 4, the dotted lines represent baseline adjustment for background fluorescence.

Preliminary studies of EsD in body fluids showed that when seminal stains and swabs of vaginal fluid were treated in the same manner as bloodstains, very weak EsD patterns corresponding to the blood patterns of the donors could be observed. However, even with stains within 24 h of age, the patterns were usually too weak for interpretation.

No detrimental effect on the EsD pattern was observed when blood was mixed with perspiration or saliva; however, slight distortion of the bands was observed with saliva because of slight digestion of the starch around the origin by the amylase.

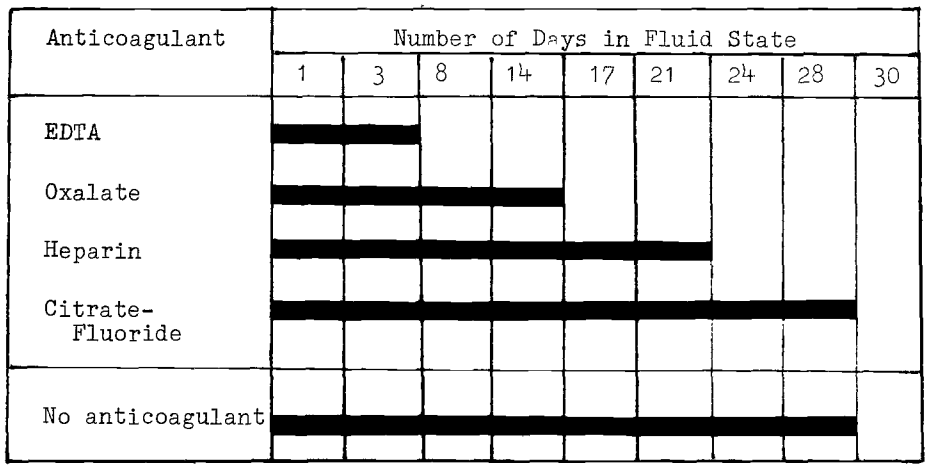

FIG. 5—*Relative survival times of EsD phenotypes in fluid blood containing various anticoagulants, stored at room temperature (approximately 22°C).*

On the basis of the 382 control samples collected to date from staff members and from cases in Ontario, the population distribution is as follows: EsD 1, 82.7% (316); EsD 2-1, 15.2% (58); and EsD 2, 2.1% (8).

Summary

A study of the changes in electrophoretic patterns of EsD 1 and 2-1 phenotypes in progressively aging bloodstains was carried out and resulted in the establishment of criteria for the proper interpretation of such patterns. With these criteria, the EsD isoenzymes in dried bloodstains were found to have a maximum stability of four weeks.

While degradation of the isoenzyme in fluid blood at 37°C was complete by the third day, instability was not detected until the sixth week in fluid samples stored at 3°C; the presence or type of anticoagulant had no observable effect on the 3°C samples. When fluid blood was stored at room temperature, the presence of anticoagulant and preservative did not enhance the stability of EsD. Citrate-fluoride did not produce any observable effect; heparin, oxalate, and EDTA increased the rate of degradation, with EDTA being the most detrimental, reducing the stable period to three days.

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