TECHNICAL NOTE

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The Determination of Carbonic Anhydrase-2 Phenotypes in Dried Bloodstains by Cellulose Acetate Electrophoresis

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ABSTRACT: A new, simple, and rapid method for phenotyping carbonic anhydrase-2 (CA_2) on bloodstains 15 weeks old was performed. Separation of the CA_2 isozymes was accomplished by cellulose acetate electrophoresis. The staining substrate was fluorescein diacetate.

KEYWORDS: pathology and biology, genetic typing, carbonic anhydrase, fluorescein diacetate, cellulose acetate, hemoglobin, electrophoresis

Carbonic anhydrase (carbonate dehydratase, 4.2.1.1) isozymes CA_1 and CA_2 are second to hemoglobin as the major protein component of erythrocytes. The enzyme has a molecular weight of approximately 29 700 and catalyzes the reversible conversion of carbon dioxide and water to carbonic acid. Both CA_1 and CA_2 are monomeric and are determined by autosomal loci [*1*].

Variants of CA_1 have been identified but their frequency is extremely low. In 1971, Moore et al [*2*] used a specific antiserum to CA_2 and discovered by immunoelectrophoresis a genetically determined polymorphism in this enzyme among blacks. Availability and expense of this antiserum were negative factors in the adoption of their procedure. Hopkinson et al [*3*] developed a simple and inexpensive method for the detection of CA_2. Their staining procedure was based on CA_2 catalyzing the hydrolysis of fluorescein diacetate, thereby liberating fluorescein, which fluoresces bright yellow under long-wave ultraviolet light.

Hughes [*4*] described a starch gel electrophoresis procedure for the determination of CA_2 isozymes in dried bloodstains. The procedure required a 4-h electrophoresis run and was able to phenotype bloodstains up to four weeks old.

This report describes a cellulose acetate electrophoresis procedure for the phenotyping

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of CA_2. This procedure has the advantage of a shorter electrophoresis run time, excellent sensitivity, and, for lysates and relatively fresh stains, the simultaneous identification of the common hemoglobin variants. Bloodstains up to 15 weeks old were phenotyped.

Materials and Methods

A Beckman Microzone electrophoresis system with accessories, 57- by 145-mm Type 11200BB Sartorius cellulose acetate membranes, and 250-μL polyethylene tubes were used.

The tank buffer consisted of 4.5 g tris(hydroxymethyl)aminomethane (Tris) and 21.8 g glycine made up to 1 L with deionized water, pH 8.4. The membrane buffer was the same as the tank buffer.

For the reaction buffer, 3.6 g dibasic sodium phosphate and 0.05 g magnesium chloride were dissolved in 250 mL deionized water. The pH was adjusted to 7.5 with 1*N* hydrochloric acid.

To prepare the reaction substrate mixture, 100 mg of agarose (Sigma Type I) was dissolved by boiling it in 10 mL of the reaction buffer; it was then cooled to 60°C. Approximately 4 mg of fluorescein diacetate was dissolved in a few drops of acetone. The agarose was combined with the dissolved fluorescein diacetate and immediately poured into two or three square plastic petri dishes.

Preparation of Samples

Blood samples were obtained from autopsies as well as from a local hospital. Hemolysates were prepared by adding two volumes of deionized water to one volume of packed erythrocytes. Dried bloodstains were prepared by placing a small amount of whole blood onto a clean piece of cotton gauze. The stains were then stored at room temperature. For the extraction of bloodstains, dry blood chips or several fibers of bloodstained cloth were placed into a 250-μL tube and extracted with a minimal volume of 0.05*M* Cleland's reagent (dithiothreitol). The bloodstain was allowed to extract for at least 15 min. Longer extraction times were required for older stains.

Electrophoresis

A cellulose acetate membrane was floated on a portion of the Tris-glycine tank buffer. The membrane was blotted between filter paper and placed on the tank bridge. The bridge was then placed into the electrophoresis tank, which had previously been filled with cold tank buffer. One application of approximately 0.25 μL of hemolysate was applied to the middle of the membrane. Bloodstain extractions were applied two to four times depending on the age and the concentration of the stain. Electrophoresis was carried out at room temperature at a constant voltage of 500 V for 45 min.

Immediately after electrophoresis, the ends of the membrane were cut off and the membrane was placed facedown onto the reaction plate. Care was taken not to trap any air between the membrane and the plate. The reaction plate was incubated at 37°C for 5 to 10 min and then viewed under long-wave ultraviolet light. The CA_2 isozyme bands were seen as bright yellow fluorescent bands on a dark background. The phenotypes were recorded and the membrane was washed in tap water and placed between two blotters to dry. This procedure permits the membrane to be preserved for future study or review.

Results and Discussion

Figure 1 illustrates the three common genetic phenotypes of CA_2 and four hemoglobin types. CA_2 1-1 consists of a single fluorescent band with the most cathodic migration.

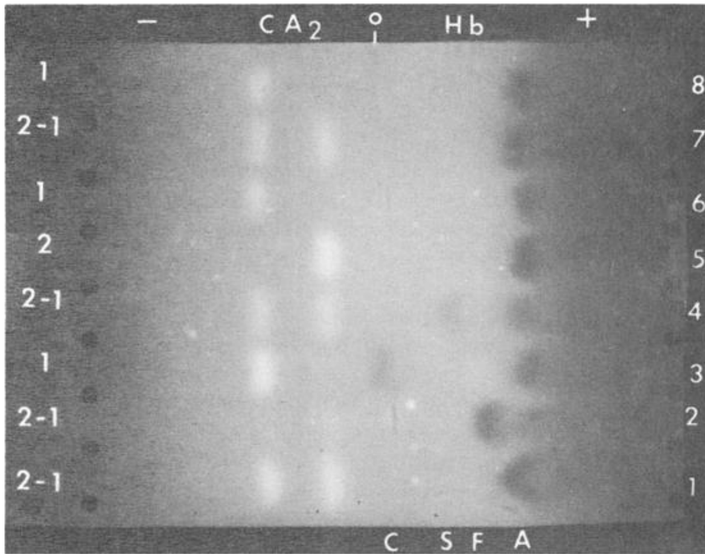

FIG. 1—*Three common phenotypes of carbonic anhydrase-2 and four hemoglobin types.*

CA_2 2-2 is represented by a single fluorescent band nearer the origin but also cathodic. The heterozygote CA_2 2-1 has both bands present in equal intensity. In practice the CA_2 2-1 bands each appear to have approximately half the intensity of a homozygous CA_2 1-1 or CA_2 2-2 band. Figure 1 also shows a fetal cord blood sample. The sample is a CA_2 phenotype 2-1 and shows approximately 10 to 20% of the CA_2 activity of adult erythrocytes, as described by Harris and Hopkinson [*1*]. All of the CA_2 bands are cleanly separated and their phenotypes unambiguous. Four common hemoglobin variants are illustrated in Fig. 1. They are, in order of increasing anodic migration, C, S, F, and A. The hemoglobin bands also show clean separation and the phenotyping of these common variants is unambiguous.

Bloodstains of various ages were tested. In Fig. 2, Samples 3, 4, 6, and 7 are 14-day-old bloodstains. The indicated CA_2 phenotypes are easily identified and do not differ in quality from the hemolysate standards.

Hughes [*4*] reported that bloodstains four weeks old could be successfully phenotyped. Figure 3 illustrates the CA_2 phenotyping of bloodstains 15 weeks old. The successful phenotyping of older bloodstains may be attributed to a more basic pH phosphate reaction buffer. The higher pH reaction buffer, on cellulose acetate, intensified the CA_2 bands without increasing background, thus increasing sensitivity. This is particularly important in CA_2 2-1 phenotypes. The CA_2 bands in the 15-week-old stains show a slightly more cathodic migration; however, the common phenotypes are still easily identified. The cathodal shift may be due to an increased ionic concentration at the origin as a result of multiple sample applications. No storage bands were observed in these bloodstains. Incubation of the reaction plate for an extended time did result in the development of another weakly fluorescent band. This band has been shown to be the CA_1^1 isoenzyme [*4*]. The band remained virtually on the origin and did not present any problems for interpretation.

This method has been used in actual case work to aid the investigators in determining if an unknown bloodstain could have originated from an individual of Negroid ancestry. The CA_2 and Hb phenotypes are also used in frequency calculations for a given bloodstain.

To date, 250 blood samples from American blacks have been examined by this method.

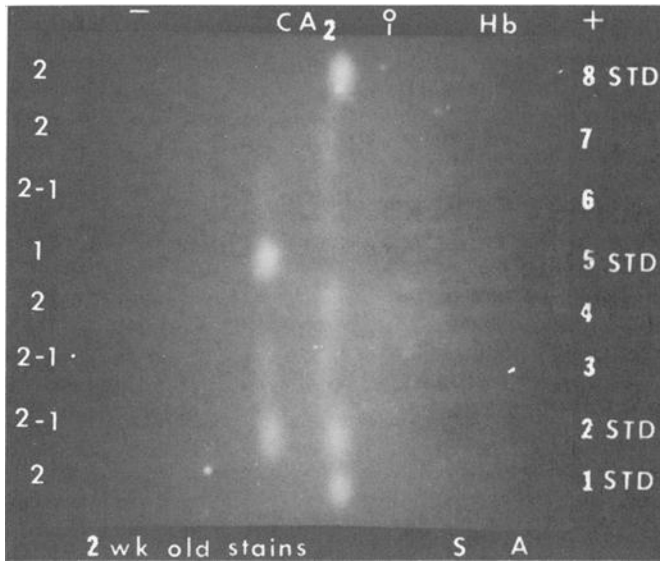

FIG. 2—*Carbonic anhydrase-2 phenotyping of two-week-old bloodstains.*

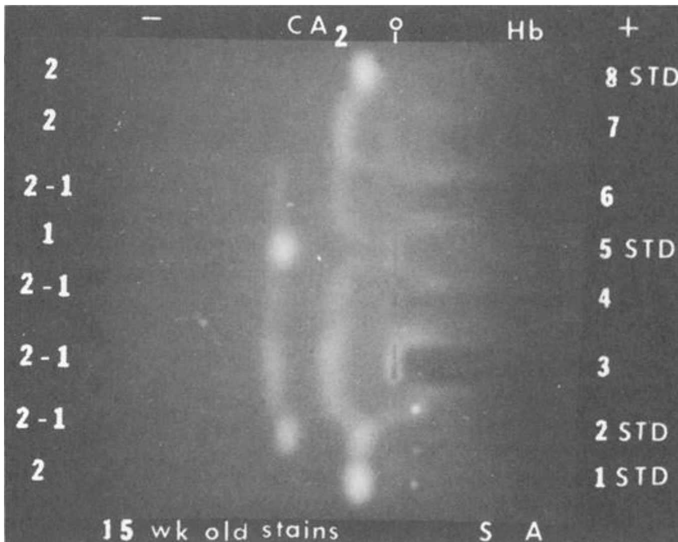

FIG. 3—*Carbonic anhydrase-2 phenotyping of 15-week-old bloodstains.*

The observed phenotypic frequencies are these: CA_2 1-1, 83.6%; CA_2 2-1, 15.9%; and CA_2 2-2, 0.5%, with an observed gene frequency of $CA_2^1 = 0.916$ and $CA_2^2 = 0.084$. The phenotype and gene frequencies compare favorably with previously published data [*2,4*] and are in good agreement with the Hardy-Weinberg equilibrium.

Summary

Determination of CA_2 genetically controlled variants was achieved in 45 min by cellulose acetate electrophoresis. Fluorescent patterns characteristic of specific phenotypes were observed after the isozymes reacted with a fluorescein diacetate substrate. Fluorescent band patterns were observed under long-wave ultraviolet light within 5 min after incubation at 37°C. Dried bloodstains 15 weeks old were successfully phenotyped by this method.

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