A STARCH-GEL ELECTROPHORETIC METHOD FOR THE STUDY OF DIAPHORASE ISOZYMES AND PRELIMINARY RESULTS WITH SHEEP AND HUMAN ERYTHROCYTES

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Received September 17, 1967

In the human erythrocyte, an enzyme called diaphorase catalyzes the reduction of 2, 6-dichlorophenol indophenol and is probably identical to an enzyme catalyzing DPNH-dependent methemoglobin reduction. This identity is made probable by the observation that, in the enzyme deficiency type of congenital methemoglobinemia, in which DPNH-catalyzed methemoglobin reduction is deficient, there is also a deficiency of diaphorase activity (Scott and Griffith, 1959). The identity of diaphorase and DPNH-dependent methemoglobin reductase has not yet been established in other than human erythrocytes.

Diaphorase activity of erythrocytes can be assayed conveniently by measuring the rate of reduction of dichlorophenol indophenol spectrophotometrically (Scott and Griffith, 1959). The decrease in optical density, as the oxidized blue dye is reduced to its colorless state, is measured at

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a wave length of 600 mμ. A method for identifying diaphorase isozymes after starch-gel electrophoresis has been developed in our laboratory by incubating the gel in the presence of dichlorophenol indophenol and DPNH, with the site of the isozymes being indicated by decolorization of the blue dye. This method has been used for the study of diaphorase isozymes from hemolysates of sheep and human erythrocytes.

METHODS

Venous blood from either sheep or human subjects was drawn in heparin or acid-citrate-dextrose as anticoagulants. The sheep utilized in this study were breeds or crosses of breeds maintained at the Michigan State University College of Veterinary Medicine. Human subjects were either laboratory and professional personnel or healthy adult inmates of the Southern Michigan State Prison at Jackson, Michigan.

Our technique of preparing hemolysates for electrophoresis has been previously described (Shows, et al., 1964). Electrophoresis was carried out essentially as performed by Fildes and Harris (1966) for adenylate kinase except that a pH of 8.0 rather than 7.0 was used in both bridge and gel buffers, and electrophoresis was carried out vertically rather than horizontally. The gel was stained for identification of diaphorase activity by preparing an agar overlay containing 0.75 percent Ionagar, 1.76×10^{-4} M DPNH, 1.4×10^{-4} M 2,6-dichlorophenol indophenol, in a 0.05 M tris buffer, at pH 8.0. The agar was dissolved in approximately three-fourths of the tris buffer by heating to boiling. This was allowed to cool, and after it reached a temperature of 40°C., the additional reagents, dissolved in the remaining one-fourth of the tris buffer, were added to the agar mixture. It is important that the DPNH and dichlorophenol indophenol not be mixed until the overlay is ready to pour, because of nonenzymatic

reduction of the dye by DPNH. After thorough mixing, the agar mixture was poured over the starch gel, and the gel incubated for approximately one hour at 37°C.

RESULTS

A. Studies of Sheep Erythrocytes

Slots 3, 4, and 5 of Figure 1 demonstrate the three diaphorase isozyme patterns observed in sheep erythrocytes. The isozyme bands

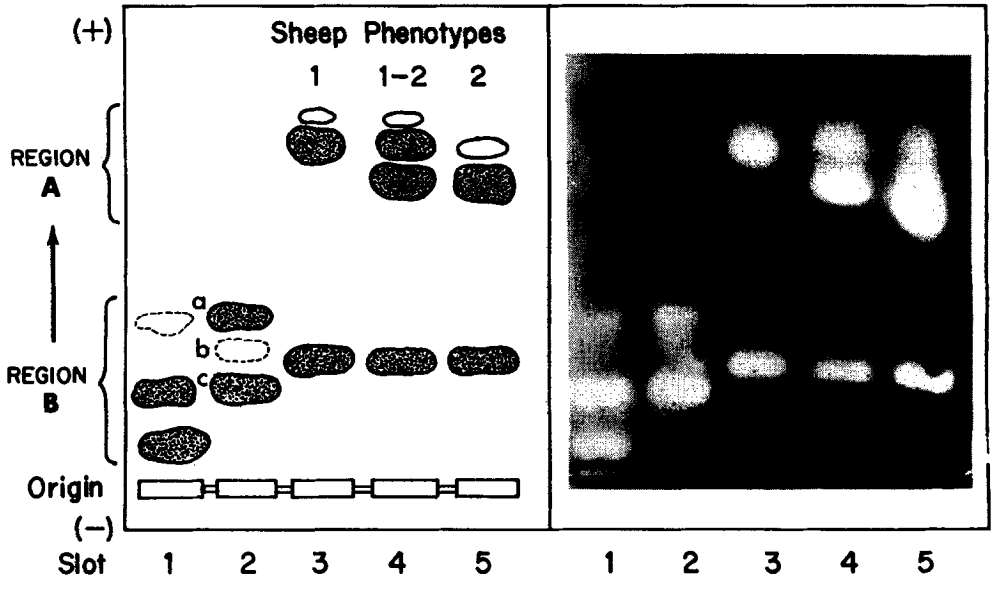

Figure 1. Electrophoretic patterns of diaphorase isozymes of sheep and human erythrocytes. On the right is a photograph of a starch gel, on the left a drawing interpreting the photograph. Slot 1 contains an unusual human variant, slot 2 contains a common human pattern (see text), and slots 3-5 contain sheep hemolysates of the three observed phenotypes. The letters a, b, and c denote enzyme bands in slot 2. In slot 5, in the photograph, there is some trailing and curvature of the slow band in region A, because this slot is at the edge of the gel. In the drawing, the pattern in slot 5 is drawn according to the pattern usually seen in the 2 phenotype of sheep.

denoted region A show genetic variation. Analysis of our preliminary breeding data in the only three mating types observed (Table 1) is compatible with the hypothesis that the three phenotypic patterns are produced by a simple, single locus, two allele system.

Table 1

Number of lambs of various diaphorase isozyme phenotypes arising from the three mating types observed.

Parental Mating Types		Number of Lambs of Various Phenotypes		
Male	Female	1	1-2	2
1	1	8	0	0
1	1-2	5	13	0
1	2	0	4	0

The observed frequencies of the three diaphorase phenotypes in the various breeds of sheep studied are given in Table 2. Assuming that our genetic hypothesis for the inheritance of phenotypes is correct, it is apparent that both alleles are present in all breeds sampled. Gene frequencies have not been calculated because a very small number of rams sired most of the animals in the flocks.

Table 2

Number of sheep of the three diaphorase phenotypes in the various breeds and crosses studied

Breed	Number of Sheep of Various Phenotypes		
	1	1-2	2
Tunis	14	13	5
Tunis, Suffolk, Shrop, and Cheviot crosses	9	8	1
Dorset and Suffolk crosses	27	7	0
Western Whiteface	7	8	2
Total	57	36	8

Sheep erythrocytes also exhibit a second region of diaphorase activity, termed region B in Figure 1. No genetic variation was detected in this second region.

B. Studies of Human Erythrocytes

The diaphorase isozyme pattern of human hemolysates usually showed two bands, labelled "a" and "c" in Figure 1 (slot 2). Often a minor component appeared between these bands (labelled "b" in slot 2 of Figure 1). All of these bands appear in a region analogous to Region B of the sheep patterns. The pattern shown in slot 1 of Figure 1 is an unusual variant, the only one of which we have seen in several hundred samples from Negroes and Caucasians. Family studies were not possible in this individual because of the absence of any known living relatives. The isozyme pattern in this adult Negro male was always reproducible.

The leading band (band "a" of slot 2) frequently showed variation in intensity between individuals. The pattern in slot 2 of Figure 1 shows a fairly intense leading band. Other samples, had, at the most, only a trace of activity in this location. The tendency to have strong leading bands seemed to be familial; when such a pattern occurred in an individual, a similar pattern could usually be demonstrated in one of the parents. However, these variations in strength of band "a" were not always reproducible. An individual whose blood had shown a heavy leading band on a number of occasions did not always show it on a subsequent occasion. It appears that there are physiological or technical variables influencing the strength of band "a" in human hemolysates, over which we have not been able to gain control, although we have worked for over one year, modifying our technique in many ways, in an effort to obtain reproducibility. Manipulations which have been tried include the following:

Drawing the blood in various types of anticoagulant (heparin, EDTA, ACD); preparation of the hemolysate by freezing and thawing, or sonication; omitting the toluene extraction in hemolysate preparation; the addition of urea, 2-mercaptoethanol, EDTA, or DPN to the red cells before or after hemolysis; variation in the gel pH from 6.5 to 9.0; the use of tris-borate-EDTA, tris maleate-EDTA, tris-succinic acid, and L-histidine as gel buffers; addition of DPN to the gel; protecting the enzyme against temperature inactivation by running the electrophoresis at temperatures below 0°C.; and the use of starch, acrylamide, and cellulose acetate as supporting media. None of these modifications have resulted in improved reproducibility in the strength of band "a" of the human diaphorase isozyme patterns.

DISCUSSION

The purposes of this paper have been 1) to present a simple method for the electrophoretic demonstration of the isozymes of the enzyme, diaphorase, in whole hemolysates, 2) to demonstrate polymorphic variation in this isozymic system in sheep erythrocytes, and 3) to illustrate the presence of diaphorase isozymes, with possible genetically determined variation, in human erythrocytes.

With respect to the sheep system there appear to be two loci determining enzymes that catalyze the diaphorase reaction. One locus, specifying the faster migrating bands, (region A), shows genetic variation. This variation does not effect the rate of migration of the slower migrating band (region B), suggesting that the latter is controlled by a second locus.

With respect to the human diaphorase isozyme system, one probable genetic variant (slot 1 of Figure 1) has been identified. This variant pattern has been reproducible in multiple samples over the period of one

year. The interpretation of the variation in strength of band "a", commonly present in both Negroes and Caucasians, but not always reproducible, awaits further study.

The enzymes under study appear to be specific, or nearly so, for DPNH. Substitution of TPNH for DPNH in the staining overlay resulted in only a very faint band in region B of sheep hemolysates. No other bands of activity were noted in sheep or human hemolysates. It should be of interest to use this starch gel technique on hemolysates of patients with congenital methemoglobinemia arising from a deficiency of DPNH-catalyzed methemoglobin reduction (Gibson, 1948).

While this work was underway we became aware that a somewhat similar technique had been developed in the laboratory of E. R. Huehns of the University of London, and is being applied to the study of partially purified diaphorase enzymes from human erythrocytes.

ACKNOWLEDGMENTS

We wish to acknowledge the cooperation and assistance of the Parke, Davis Company, including the use of facilities at the Southern Michigan State Prison at Jackson, Michigan, and the cooperation of inmate volunteers of the prison.

This work was supported in part by USPHS research grant AM-09381, USPHS Career Development Award 1-K3-AM-7959 (GJB), USPHS training grant T01-GM-00071, and in part by the Research and Development Command, Office of the Surgeon General, Department of the Army, under contract DA-49-193-MD-2855 with the Department of Medicine, University of Michigan. This is Contribution No. 267 to the Army Research Program on Malaria.

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