NADH Diaphorase: An Inherited Variant Associated with Normal Methemoglobin Reduction

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Four different electrophoretic phenotypes of NADH diaphorase (methemoglobin reductase) have been described, all in association with methemoglobinemia due to deficiency of the enzyme (Kaplan and Beutler 1967; West et al. 1967; Bloom and Zarkowsky 1969). In this report we describe two additional electrophoretic phenotypes, one of which was shown to be inherited and to be unaccompanied by methemoglobinemia, abnormal catalytic function, or unusual physical properties.

MATERIALS AND METHODS

Blood from 378 normal blood donors was collected in ACD solution, the plasma removed, and the remaining packed red cells washed three times with 0.9% NaCl solution. Hemolysates for electrophoresis were prepared by dilution and mixing of the packed cells with one volume of water and one-half volume of toluene. Stroma-free lysates, extracted after high-speed centrifugation, were subjected to vertical starch-gel electrophoresis (Smithies 1959) for 18 hours at 4° C and 6 v/centimeter in sodium phosphate buffer, *p*H 7, 0.1 M in the buffer trays and 0.005 M in the gel. Enzymatic activity was localized as described by Kaplan and Beutler (1967). In some instances, the electrophoretic patterns were also examined after dialysis overnight against two changes of gel buffer or after one of the following substances was added to the lysate: 20 mM mercaptoethanol, 1.0 mM or 2.5 mM disodium adenosine triphosphate added immediately prior to application of hemolysate to starch gel; 1 mM NADH added with and without preincubation at 37° C for one hour; and 25 mM neutralized, oxidized glutathione added with preincubation for 30 minutes at 37° C.

Diaphorase assays (Scott 1960), carried out in duplicate with a correction for dye reduction in the absence of coenzyme, were performed on untreated samples and after heat and urea inactivation (Parr and Fitch 1964; Carter et al. 1966).

Methemoglobin levels were determined by the method of Evelyn and Malloy as described by Hawk (1954). In vitro methemoglobin reduction of intact cells was studied in the presence of glucose or lactate (Jaffé 1959).

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RESULTS

In all but two samples, the NADH diaphorase electrophoretic pattern in fresh hemolysates consisted of a single band of enzyme activity similar to that previously reported (Kaplan and Beutler 1967) (table 1). The remaining two samples, from unrelated Caucasians, contained a second band with faster mobility. Figure 1 shows the double-banded pattern of these two samples (Mr. K. and Mrs. G.) compared with the single band of the normal enzyme.

TABLE 1

FREQUENCY OF ENZYME PHENOTYPES IN THREE RACIAL GROUPS

Ethnic Group	No. of Variants	No. of Subjects
Caucasian	2	175
Negro	0	125
Oriental*	0	78
Total	2	378

* Mixed, mostly Japanese.

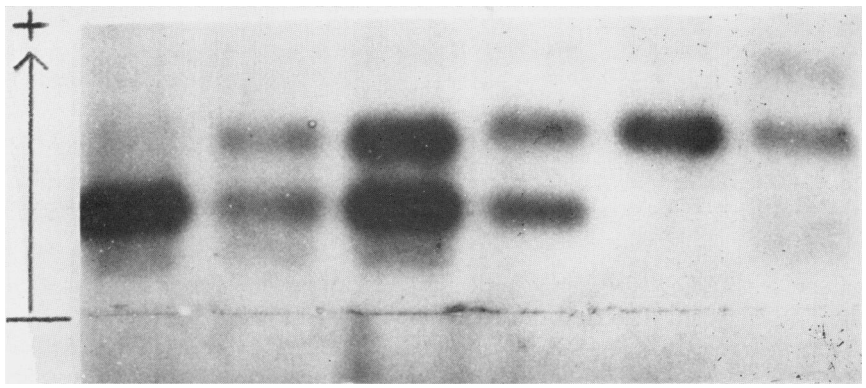

FIG. 1.—Starch-gel electrophoresis of red-cell NADH diaphorase. From left: slot 1, normal homozygous subject; slot 2, Mr. K.; slot 3, Mrs. G.; slot 4, partially "aged" sample from normal subject; slot 5, completely "aged" sample from normal subject; slot 6, completely "aged" sample from Mrs. G.

The electrophoretic pattern of samples from some donors, which had been refrigerated briefly (one to two days) at 4° C, developed an additional anodal zone which was initially thought to represent a fast-moving variant. However, this confusion was subsequently resolved when it became apparent that the "aging" zone of the real variants had a faster migration than the aging zone of the "normal" phenotype. Moreover, further aging of both normal and variant enzymes resulted in complete disappearance of the original zones of activity (fig. 1).

The basis of the aging phenomenon has not been elucidated. It was shown to be independent of the anticoagulant used in sample collection. No interconversion could

be demonstrated with heat or dialysis, or by addition of sulfhydryl reagents, NADH, ATP, leukocyte, or platelet extracts. This ability to change during aging was not always consistent when blood specimens were obtained from the same person on more than one occasion. The diaphorase from some donors never developed aging zones even after six months of storage. However, when these hemolysates were mixed with equal volumes of hemolysates containing aged enzyme and stored at 4° C for two or three days, only the aged pattern was detectable on electrophoresis.

Mr. K. was not available for further study. However, the migration rate of his unusual isozyme was demonstrated on several occasions to be slightly slower than the variant enzyme from Mrs. G.

Seven additional members of Mrs. G.'s family (of Scotch-Irish and English origin) were found to have her unusual phenotype. The mean diaphorase activity of affected family members was 0.0038 with a range of 0.0032–0.0043, which is within the normal range established in our laboratory (ΔA_{600} per minute = 0.0039 ± 0.0009). Red cells from Mrs. G. contained 0.3% methemoglobin, and their rate of methemoglobin reduction was normal.

As shown in figure 2, the inheritance pattern was consistent with that of autosomal

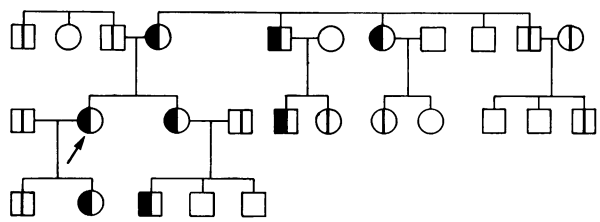

FIG. 2.—G family pedigree. Divided symbols: subjects homozygous for the normal diaphorase; half-shaded symbols: subjects heterozygous for normal and variant enzyme; open symbols: subjects not tested.

codominance. Studies of other genetic markers revealed absence of close genetic linkage of the structural locus for NADH diaphorase to the loci for the ABO, Rhesus and Duffy blood-group systems, Gc and haptoglobin serum groups, and the PGM_1 locus of phosphoglucomutase. These findings were in accord with previous studies on families with NADH diaphorase deficiency (Scott et al. 1963; Scott and Wright 1969).

Electrophoretic studies of the crude hemolysate of Mrs. G. showed that activity of both the normal and the variant isozyme was almost completely destroyed by incubating the hemolysate at 50° C for five minutes. Incubation at 45° C for 40 minutes reduced the activity about 50%, as did preincubation in 1.5 M urea. No change in the migration of either normal or variant enzyme was observed after partial heat inactivation or preincubation with oxidized glutathione, mercaptoethanol, NADH, and ATP.

DISCUSSION

In contrast with previously reported electrophoretic variants of NADH diaphorase (Kaplan and Beutler 1967; West et al. 1967; Bloom and Zarkowsky 1969), the un-

usual enzyme in Mrs. G.'s family had apparently normal activity and was distinguishable from the usual type only by its difference in charge.

A pattern of autosomal inheritance observed in Mrs. G.'s family was also reported in families with deficiency of this enzyme (Scott 1960). An autosomal structural gene locus is also strongly supported by the coexistence of two, presumably allelic, gene products in the red cells of both affected males and females.

The decreased activity of glutathione reductase reported to accompany methemoglobinemia due to NADH diaphorase deficiency has raised the possibility of a common subunit for these two enzymes (Jaffé 1966). If there were a shared subunit, a charge variation in that subunit would be reflected in altered mobility of both enzymes. However, Kaplan and Beutler (1968) found no change in the electrophoretic migration of NADH diaphorase in subjects with inherited charge variation of glutathione reductase. Similarly, in the present study, both Mr. K. and Mrs. G. had normal glutathione reductase patterns.

Although two examples of electrophoretic NADH diaphorase variation were found in unrelated donors among nearly 400 samples tested, the frequency of variation may be lower than 0.5%; other investigators found no similar variant isozyme patterns among several hundred samples (Kaplan and Beutler 1967; Brewer et al. 1967).

SUMMARY

Electrophoretic variation of NADH diaphorase was found in the red cells of two unrelated blood donors. Studies on the family of one of these donors were consistent with autosomal codominant inheritance of a variant allele. Metabolic and biochemical characteristics of this enzyme were indistinguishable from those of the usual type.

REFERENCES

Bloom, G. E., and Zarkowsky, H. S. 1969. Molecular heterogeneity of methemoglobinemia. *Proc. Soc. Pediat. Res.* **39**:59.

Brewer, G. J.; Eaton, J. W.; Knutsen, C. S.; and Beck, C. C. 1967. A starch gel electrophoretic method for the study of diaphorase isozymes and preliminary results with sheep and human erythrocytes. *Biochem. Biophys. Res. Commun.* **29**:198–204.

Carter, N. D.; Gould, S. R.; Parr, C. W.; and Walter, P. H. 1966. Differential inhibition of human red cell phosphogluconate dehydrogenase variants. *Biochem. J.* **99**:17P–18P.

Hawk, P. B. 1954. *Practical physiological chemistry.* 13th ed. Blakiston, New York.

Jaffé, E. R. 1959. The reduction of methemoglobin in human erythrocytes incubated with purine nucleosides. *J. Clin. Invest.* **38**:1555–1563.

Jaffé, E. R. 1966. Hereditary methemoglobinemias associated with abnormalities in the metabolism of erythrocytes. *Amer. J. Med.* **41**:786–798.

Kaplan, J.-C., and Beutler, E. 1967. Electrophoresis of red cell NADH- and NADPH-diaphorase in normal subjects and patients with congenital methemoglobinemia. *Biochem. Biophys. Res. Commun.* **29**:605–610.

Kaplan, J.-C., and Beutler, E. 1968. Electrophoretic study of glutathione reductase in human erythrocytes and leucocytes. *Nature* (London) **217**:256–258.

Parr, C. W., and Fitch, L. I. 1964. Hereditary partial deficiency of human-erythrocyte phosphogluconate dehydrogenase. *Biochem. J.* **93**:28C–30C.

Scott, E. M. 1960. The relation of diaphorase of human erythrocytes to inheritance of methemoglobinemia. *J. Clin. Invest.* **39**:1176–1179.

SCOTT, E. M.; LEWIS, M.; KAITA, H.; CHOWN, B.; and GIBLETT, E. R. 1963. The absence of close linkage of methemoglobinemia and blood group loci. *Amer. J. Hum. Genet.* **15**:493–494.

SCOTT, E. M., and WRIGHT, R. C. 1969. The absence of close linkage of methemoglobinemia and other loci. *Amer. J. Hum. Genet.* **21**:194–195.

SMITHIES, O. 1959. An improved procedure for starch-gel electrophoresis: further variations in the serum proteins of normal individuals. *Biochem. J.* **71**:585–587.

WEST, C. A.; GOMPERTS, B. D.; HEUHNS, E. R.; KESSEL, I.; and ASHBY, J. R. 1967. Demonstration of an enzyme variant in a case of congenital methaemoglobinaemia. *Brit. Med. J.* **4**:212–214.