ELECTROPHORESIS OF RED CELL NADH- AND NADPH-DIAPHORASES IN NORMAL SUBJECTS AND PATIENTS WITH CONGENITAL METHEMOGLOBINEMIA*

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Erythrocyte NADH-and NADPH-diaphorases (so-called methemoglobin reductases) have been shown to be different enzymes. A deficiency of NADH-diaphorase activity results in congenital methemoglobinemia (Gibson, 1948; Scott and Griffith, 1959; Jaffé, 1966b). Further understanding of this abnormality has been hampered by the fact that no satisfactory method for staining diaphorases after gel electrophoresis has been devised previously.

We now present a method for staining both diaphorases after starch gel electrophoresis and describe the results obtained with normal and NADH-diaphorase-deficient red cells. In one subject with congenital methemoglobinemia an electrophoretic variant (California) was found; in two other cases, this abnormality was not found and it is therefore believed that deficiency of NADH-diaphorase is genetically and biochemically heterogeneous.

MATERIAL AND METHODS

NADH-diaphorase activity in red cells was assayed according to Scott and McGraw (1962). For electrophoresis the washed cells were hemolyzed in 1 volume of cold distilled water, frozen and

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thawed three times and centrifuged at 31,000 g for 30 minutes. In some experiments the hemolysates were freed from hemoglobin by treatment with DEAE cellulose, using a modification of the method described by Hennessey et al. (1962). The hemoglobin-free extracts were concentrated by dialysis against 0.1 M Tris-HCl buffer, 1 mM EDTA, pH 7.4, under reduced pressure. A buffer system composed of 0.1 M Tris, 4.5 mM EDTA adjusted to pH 9.3 at 25° C with HCl was used in the electrode compartments. The starch gel was prepared with the same buffer diluted 1/10. Electrophoresis was performed horizontally at 4°C for four hours with an average voltage gradient of 20-25V/cm. The sliced gel was stained with a freshly prepared mixture composed of 1.3 mM NADH or NADPH, 1.2 mM 3 (4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide (MTT) and 0.06 mM 2,6-dichlorophenol indophenol (DCIP) in a 0.25 M Tris-HCl buffer, pH 8.4. The gels were developed at room temperature in the dark for at least three hours after application of the staining mixture. The diaphorase activity appeared as a dark-blue band.

The technique of staining described is based upon the capacity of DCIP to serve as an effective electron acceptor from NADH and NADPH in the presence of diaphorase (Scott and McGraw, 1962). We have found that at pH levels above 8.0, reduced (leuco-) DCIP readily reduces MTT, causing the formation of the purple-blue insoluble formazan derivative. When the defluorescence of NADH or NADPH in the presence of DCIP was followed, defluorescing bands were seen in the same position in which the formazan bands were obtained with the system including MTT.

RESULTS AND DISCUSSION

With NADH, a narrow diaphorase band appears in a position

behind hemoglobin A (Fig. 1). If NADPH is used as substrate, a relatively weaker band appears, migrating far ahead of Hb A. This band is somewhat broad and probably duplicated, but distinct resolution into two bands could not be achieved. When sufficiently large amounts of hemolysates are placed on the gel some cross-activity is observed: the NADH-diaphorase band is slightly stained in the presence of NADPH and conversely the NADPH-diaphorase stains weakly with NADH. In control experiments, in which no reduced coenzyme is included in the developing mixture, no band can be seen. The relationship of these electrophoretically distinct enzymes to the purified enzyme fractions described by Scott et al. (1965) is presently unknown.

No electrophoretic polymorphism could be found among 200 adult subjects investigated. Although it has been shown that cord blood has a low NADH-diaphorase activity (Ross, 1963), we found no electrophoretic peculiarity among 10 cord blood samples studied.

The red cell diaphorases from three non-related subjects with congenital methemoglobinemia were studied by gel electrophoresis. The red cells of the first patient had 10% of normal NADH diaphorase activity. This patient belongs to the family previously reported by Fialkow et al. (1965) (Case IV:9). After starch gel electrophoresis no NADH-diaphorase band could be seen. The mother and four other siblings known to be heterozygotes on the basis of enzyme assay had weaker NADH-diaphorase band with normal electrophoretic mobility. Electrophoresis of hemoglobin-free concentrated extracts gave the same results. The second patient, whose red cells contained approximately 10% of normal NADH-diaphorase activity, displayed a very weak band with normal mobility on gel electrophoresis. However, in hemoglobin-free

concentrated extracts the activity was lost and no band was seen. The red cells of the third patient with congenital methemoglobinemia and NADH-diaphorase deficiency had a residual diaphorase activity of approximately 25% of normal. The electrophoresis of crude hemolysates showed complete absence of a normal NADH-diaphorase band, while a weak faster band was seen (Fig. 1). This band was fairly strong in a hemoglobin-free extract, concentrated 6 times with respect to NADH-diaphorase activity, in which no other band could be seen. In normal control, hemoglobin-free, concentrated enzyme the same pattern as in normal crude hemolysates was seen and there was no band comparable to the fast-moving band observed in this patient. The patient's daughter had approximately 55% of normal red cell NADH-diaphorase activity. Electrophoretic examination of crude hemolysate and hemoglobin-free preparation displayed two bands: one with a normal mobility, the other identical to the fast band found in the mother.

As expected the electrophoretic pattern of the NADPH-diaphorase was normal in all the cases.

The unique band observed in the third homozygote (Fig. 1), representing a NADH-diaphorase with modified electrophoretic properties and relatively high residual activity has been tentatively named the California variant. The first two cases belong, most probably, to a different type of deficiency since their residual NADH-diaphorase activity is much lower and they do not display an electrophoretically abnormal band.

The relationship of the different types of NADH-diaphorase deficiency which we have described to NADH-diaphorase deficiency described among many different population groups (Jaffé, 1966b) requires clarification. It will also be of interest to determine whether the association of mental retardation with methemo-

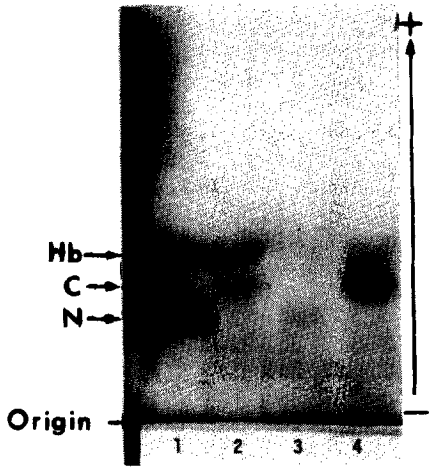

Figure 1

Gel electrophoresis of red cell NADH-diaphorase. Method in text. Hb: position of hemoglobin A; N: position of normal NADH-diaphorase; C: position of California variant of NADH-diaphorase. Channel 1: normal hemolysate; Channel 2: "California variant" hemolysate; Channel 3: normal hemoglobin-free extract; Channel 4: California variant, hemoglobin-free extract. The enzyme activity in Channel 1 and 4 is the same. In channel 2 and 3 the enzyme activity is 1/3 of that in channels 1 and 4.

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globinemia (Lamy et al., 1963; Fialkow et al., 1965; Jaffé et al., 1966) is due to a single variant. It is hoped that the method of enzyme electrophoresis described in this communication will prove helpful in answering some of these questions.

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