Methemoglobin reductase activity (MHR), measured by the Hegesh assay, decreases as normal red cells age. This probably accounts for the slightly increased concentration of methemoglobin in older red cells. Exaggeration of normal MHR age lability was demonstrated in several individuals with various molecular abnormalities of NADH-diaphorase and was associated with more striking accumulation of methemoglobin in the older red cells of those patients. Diaphorase activity, measured by the Scott assay, does not exhibit age lability in normal red cells or the cells of affected patients. While diaphorase uses either NADH or NADPH as a cofactor, methemoglobin reductase requires NADH. Methemoglobin reductase is found in hemolysates but not in membrane preparations. While approximately 1% of total red cell NADH-diaphorase activity is membrane-associated. The specific activity of NADH-diaphorase is approximately 60 times greater than that of methemoglobin reductase. Whether the two activities represent distinct enzyme proteins or differing substrate affinities of the same enzyme awaits adaptation of the Hegesh assay to enzyme electrophoresis.

CONGENITAL METHEMOGLOBINEMIA usually results from deficiency of a red cell enzyme that reduces methemoglobin to hemoglobin.\(^1\) Scott demonstrated that patients with this condition were deficient in red cell NADH-diaphorase activity.\(^2\) The assay for this enzyme measures the reduction of a dye, 2,6 dichlorophenol-indophenol (DCIP). The genetic heterogeneity of this clinical entity became apparent after several patients were reported with congenital methemoglobinemia whose red cells contained electrophoretic variants of diaphorase activity.\(^3\)\(^6\)

Keitt, Smith, and Jandl observed one patient with congenital methemoglobinemia, in whose erythrocytes, the concentration of methemoglobin was greater in older cells than in younger cells.\(^7\) They also reported that the concentration of methemoglobin increased, though less dramatically, in older normal red cells. Rigas and Koler noted a 25% decrease in NADH diaphorase activity in the older cells of two individuals (a patient with hemoglobin H and...
a young red cell population and a normal control) in contrast to Keitt and his co-workers who found no such decrease in a single patient.

A red cell NADPH-diaphorase has been described by Huennekens, Caffrey, Basford, and Gabrio. An asymptomatic individual deficient in this enzyme has been described. Keitt and co-workers suggested that the accumulation of methemoglobin as red cells age might be secondary to the gradual depletion of cellular NADPH. Glutathione and ascorbic acid reduction systems have also been invoked as mechanisms which prevent heme oxidation, but their contribution is small and nonenzymatic. While the hexose monophosphate shunt (HMPS) activity is known to decrease in older red cells, the physiologic significance of this secondary mechanism has yet to be established.

Recently, Hegesh, Calmanovici, and Avron reported a more specific assay for methemoglobin reductase (MHR). This assay measures the reduction of a methemoglobin-ferrocyanide complex. We have utilized this new assay to study normal subjects and several patients with congenital methemoglobinemia. The correlation between MHR and NADH-diaphorase has been consistent in normal individuals and in patients with moderately deficient activity and methemoglobinemia.\(^6\)

Comparison of these enzyme activities reveals several significant differences. Age lability of MHR was demonstrated in normal red cells and presumably accounts for the accumulation of methemoglobin in older cells. This tendency was markedly exaggerated in several methemoglobinemic patients, obviating the necessity to invoke secondary mechanisms to explain greater increases in the concentration of methemoglobin in the older red cells of these individuals. In addition, MHR was found to require NADH and not NADPH as a cofactor. Finally, no MHR activity was membrane-associated while a very small portion of red cell diaphorase was consistently found in membrane preparations. Thus, it would appear that the behavior of MHR more closely parallels the pathophysiology of methemoglobinemia due to enzyme deficiency.

**MATERIALS AND METHODS**

Blood specimens, obtained from patients and normal subjects, were anticoagulated with preservative-free heparin (0.1 mg/ml). Routine blood counts were performed. Red cell methemoglobin content was measured by the method of Evelyn and Malloy. Lysates were prepared and the activities of NADH- and NADPH-diaphorase were measured as described by Scott. MHR activity was measured by the method of Hegesh, Calmanovici, and Avron. The activities of these enzymes were also measured in red cell ghosts prepared by the method of Nakao, Kurashina, and Nakao. Stromal protein content was measured by the method of Lowry and co-workers. Agarose electrophoresis, performed by the method of Bloom and Zarkowski, was used to detect the enzyme variants of NADH-diaphorase. Starch block electrophoresis of hemoglobin was performed by the method of Gerald and co-workers. Red cells were separated according to age by centrifugation. The mean red cell age of each population was assessed by the activity of glutamic oxaloacetic transaminase (GOT).

**CASE REPORTS**

The propositus, M. P., presented with neonatal methemoglobinemia due to transient deficiency of MHR. Methemoglobin was 2.5 g/100 ml (15%) at 3 days of age. The activity of red cell NADH-diaphorase was 6 U, a value consistent with homozygous deficiency, as
Fig. 1. Agarose electrophoresis of red cell enzymes stained for NADH-diaphorase.\(^5\) The anode is at top. The pattern on the left is that of a normal control with a single broad band. The pattern on the right is that of patient R. P. The gel was run for 2 hr, instead of 45 min, to resolve the two rapidly migrating isoenzymes. Note cathodal migration of hemoglobin at the bottom. The unequal staining of the R.P. enzyme bands was not consistent.

defined by Scott.\(^8\) On electrophoresis, two faint bands of NADH-diaphorase activity were observed: a normal band and one with more rapid mobility. This patient is now 2 yr old. She has grown and developed normally. The activities of MHR and NADH-diaphorase in her cells have been normal since the age of 10 mo and she no longer has detectable levels of methemoglobin. The two bands of diaphorase activity are now easily demonstrated by electrophoresis.

R. P., the mother of the propositus, has always been asymptomatic. She had almost normal MHR activity, no methemoglobinemia and has been previously reported as having only the “Boston fast” variant of NADH-diaphorase.\(^5,6\) The genetic features were not clarified until a prolonged electrophoretic analysis demonstrated that this isoenzyme was, in fact, made up of two bands of enzyme activity (Fig. 1).

Da. P. is the father of the propositus and is normal. C. P. and Di. P. are siblings of the propositus. L. V. and G. V. are the maternal grandparents of the propositus. The pedigree is consistent with the hypothesis that genes for these enzyme variants are allelic (Fig. 2). Hemoglobin electrophoresis was normal in all members of this family.

**Patient K. G.**

This individual has been the subject of several previous reports.\(^5,7\) Keitt demonstrated that the methemoglobin content of her older cells was 21% compared to 7% in her younger cells. She is currently under treatment with methylene blue. NADH-diaphorase activity was 29 \(\text{U}\) and MHR activity was 0.45 \(\text{U}\). Her red cells contain a slow variant of diaphorase activity, previously designated as “Boston slow”\(^6\) and a broad faint blur of activity extending from the slow band, through the area that would contain the normal band.\(^5\) Hemoglobin electrophoresis was previously reported to be normal.\(^7\)

**RESULTS**

**The Effects of Aging**

Red cells from normal individuals were separated according to age. Because of variability among subjects, the mean enzyme activities of young and old populations could not be compared. Therefore, the young and old cells from the same individual were compared using the ratio: top specific activity/
Fig. 3. The ratio of top/bottom enzyme specific activity. The cross-hatched areas include 1 S.D. on each side of the means of the normal subjects (solid lines). Circles, ratio in normal subjects, triangles, ratio in members of P. Family (initials next to symbol); squares, ratio in K.G. diaphorase (ratio for MHR not shown because it is so far off scale). Analysis by two-tailed t test demonstrated that the means were significantly different (p < 0.02). The efficacy of separation was demonstrated by analysis for hemolysate GOT. The mean top/bottom specific activity was 1.94 ± 0.63 with a range of 1.2 to 3.1.

bottom specific activity. The mean ratio of MHR was 1.15, indicating that younger cells had 15% greater activity of that enzyme than did older cells from the same subject (Fig. 3 and Table 1). In no instances was activity greater in older, denser cells (i.e., the ratio was never less than 1.00). In contrast, the activity of NADH-diaphorase did not decrease with age, confirming previous studies. The mean transaminase activity of younger cells was nearly twice that of older cells (range 1.2–3.1), confirming the efficacy of separation.

The effects of cell aging upon the activities of these enzymes was also studied in the patients (Fig. 3, Table 1). In R.P. and C.P., MHR was more age-labile than normal. This corresponded to a small but consistent increase in the methemoglobin concentration in their older cells. In contrast, the diaphorase activity of R.P.'s younger cells was only 12% greater than that of her older cells. In Da. P. and Di. P., whose MHR stability was normal, no significant in-

<table>
<thead>
<tr>
<th>SUBJECT</th>
<th>PHENOTYPE</th>
<th>% MHR TOP</th>
<th>% MHR BOTTOM</th>
<th>MHR TOP MHR BOTTOM = RATIO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Da.P.</td>
<td></td>
<td>0</td>
<td>0</td>
<td>1.50 = 1.07</td>
</tr>
<tr>
<td>R.P.</td>
<td></td>
<td>0.5</td>
<td>1.5</td>
<td>1.35 = 0.80</td>
</tr>
<tr>
<td>C.P.</td>
<td></td>
<td>1.1</td>
<td>1.7</td>
<td>1.35 = 0.85</td>
</tr>
<tr>
<td>Di. P.</td>
<td></td>
<td>0</td>
<td>0.2</td>
<td>1.75 = 1.20</td>
</tr>
<tr>
<td>K.G</td>
<td>BOSTON SLOW</td>
<td>7</td>
<td>21</td>
<td>1.16 = 9.7</td>
</tr>
</tbody>
</table>

The key for symbols is the same as that for pedigree (Fig. 3). MHR activity is expressed as mumoles per minute per milligram methemoglobin.
creases in methemoglobin concentration were observed in their older cells. The propositus was not tested in this manner because of her age and the large volume of blood required.

The age lability of MHR in K.G. was even more striking. The ratios of top/bottom specific activity were 9.7 for MHR (old cells contained only 10% of the enzyme capacity of younger cells) and 0.75 for NADH-diaphorase. The diaphorase stability data are consistent with those of Keitt. The dramatic age lability of MHR observed in this patient probably accounts for the marked increase in concentration of methemoglobin that Keitt noted in her older red cells.

**Cellular Distribution of NADH-diaphorase and Methemoglobin Reductase**

The NADH-diaphorase and MHR activities of normal hemolysate and membranes were analyzed by the Scott and Hegesh assays, respectively (Table 2). Both hemolysates and ghosts possessed diaphorase activity, using either NADH or NADPH as substrate. The activity of hemolysate diaphorase was greater when NADH was the cofactor. Since ghosts resuspended to original volume contain approximately 1 mg protein per ml, membrane-associated NADH-diaphorase activity represents approximately 1% of the total red cell activity.* No MHR activity was observed in ghosts with either pyridine nucleotide. Hemolysate MHR activity was demonstrated only with NADH.

**DISCUSSION**

Ample precedent exists for the production of functional enzyme deficiency by alteration of primary enzyme structure. The data presented indicate that normal MHR is probably age-labile. Our data with regard to diaphorase conflict with the data of Rigas and Koler but agree with the data of Keitt, Smith, and Jandl. In both previous studies, however, only one normal individual was studied, while we have examined five normal subjects and several individuals with variant isoenzymes. It seems likely that enzyme lability accounts for the slight accumulation of methemoglobin noted by Keitt in normal red cells as they age. Therefore, it seems unnecessary to hypothesize a decline in activity of any age-dependent NADPH-, GSH- or ascorbate-linked methemoglobin reductase system to explain this observation. Although these systems may deteriorate slowly with aging of red cells, their clinical significance with respect to the reduction of methemoglobin has never been demonstrated and the age-lability of MHR shown in the present study provides a reasonable explanation for all of the findings. The absence of methemoglobinemia in Sass' patient with deficient activity of NADPH-diaphorase strongly suggests that system is of no physiologic importance. This is consistent with the inability of NADPH to act as a cofactor, in vitro, for MHR.

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*This was calculated as follows: Total soluble enzyme activity/ml blood = enzyme activity/mg hgb X hgb concentration (mg/ml). The normal hgb in our laboratory is 15 g/100 ml (150 mg/ml). Total membrane-associated enzyme activity/ml ghosts = enzyme activity (u/mg ghost protein) X protein concentration (mg/ml). The normal ghosts suspension used contained approximately 1 mg protein/ml.
The data in the P. family (Table 1) suggest that the presence of the slower of the two fast diaphorase variants is associated with excessive age lability. Di. P., who carried the faster and the normal isoenzymes, showed normal age lability of MHR and did not accumulate methemoglobin in her older red cells. C.P. and R.P., who carried the slower variant (with the normal and faster ones, respectively), were abnormal in these respects. Unfortunately, the grandparents were not available for retesting after the introduction of the Hegesh assay to verify this conclusion.

Even more exaggerated age lability of MHR was observed in the unrelated patient, K.G., who carried a different isoenzyme of NADH-diaphorase and this probably explains her more severe symptomatology and "pseudomosaicism."

The relationship of the diaphorase system to MHR remains unclear. The occurrence of isoenzyme variants of NADH-diaphorase in individuals who also carry an MHR with abnormal physical properties (i.e., exaggerated age-lability) seems more than coincidental. Hegesh and co-workers have recently demonstrated that these enzyme activities are both associated with a protein (or proteins) of 30,000 molecular weight, in contrast to the NADPH-diaphorase activity which is associated with a smaller protein that has no NADPH-MHR activity.

We have demonstrated an increased specific activity of NADH-diaphorase, compared to MHR which may reflect differing affinities of the same enzyme for various electron acceptors. This difference in specific activity may explain some of the contrasting features of the two activities. For example, the decrement of MHR activity due to aging might not be detected in the larger total NADH-diaphorase activity and the small proportion of NADH-diaphorase that is membrane associated might not be detectable as a similar proportion of the smaller specific MHR activity. Whether either of these activities truly measures "methemoglobin reductase" activity is unproven, as both assays

Table 2. Comparison of Diaphorase and Methemoglobin Reductase Activity in Normal Hemolysates and Ghosts Using NADH and NADPH as Substrate

<table>
<thead>
<tr>
<th></th>
<th>Hemolysates Diaphorase* µmoles/min/mg Methemoglobin</th>
<th>MHR µmoles/min/mg Methemoglobin</th>
<th>Ghosts Diaphorase* µmoles/min/mg Protein</th>
<th>MHR µmoles/min/mg Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH</td>
<td>138 ± 28</td>
<td>2.26 ± 0.49</td>
<td>230 ± 110</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(74 ± 15)</td>
<td></td>
<td>(38 ± 18)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = 8</td>
<td>N = 28</td>
<td>N = 5</td>
<td>N = 17</td>
</tr>
<tr>
<td></td>
<td>99 ± 9</td>
<td>0</td>
<td>320 ± 30</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>(52 ± 5)</td>
<td></td>
<td>(53 ± 5)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>N = 3</td>
<td>N = 3</td>
<td>N = 3</td>
<td></td>
</tr>
</tbody>
</table>

*Hemolysate diaphorase activity is expressed as µmole of dye reduced per minute per milligram methemoglobin. Ghost diaphorase activity is expressed as µmole of dye reduced per minute per milligram protein. This was calculated from the measured molar extinction coefficient of our lot of 2,6 dichlorophenol-indophenol (Eastman Organic Chemicals, Rochester, N.Y.) at pH 7.0 and 20°C, 1.64 × 10⁴. The activity is also expressed in the more familiar Scott units (in parentheses); i.e., ΔOD/min/3.25 mg methemoglobin × 10⁴ (for hemolysates or ΔOD/min/mg protein × 10⁴ (for ghosts).
require an additive (the dye or ferricyanide) to demonstrate reducing power. Whether they represent distinct enzyme proteins must await the development of a technique for the staining of MHR activity following electrophoresis.

ACKNOWLEDGMENT

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