PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDE DIAPHORASE FROM METHEMOGLOBINEMIC ERYTHROCYTES

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Diphosphopyridine nucleotide diaphorase activity has been reported as lacking in red cells of persons with hereditary methemoglobinemia (Scott, 1960), but whether the enzyme was missing or was inhibited was uncertain. To settle this point, a method of purification of this enzyme from normal red cells (Scott and McGraw, 1962) was applied to methemoglobinemic red cells.

A small amount of DPNH diaphorase activity can be measured in hemolyzates from methemoglobinemic red cells. The average amount of this activity in hemolyzates from 20 persons with hereditary methemoglobinemia was about 10 per cent of that found in normal hemolyzates. Since this amount of activity was scarcely greater than the error of the method, there was doubt at first that it was real. However, the fact that the activity can be purified as shown below leaves no question of its reality.

Red cells from one pint of normal blood and one pint of methemoglobinemic blood were subjected in parallel to the purification procedure previously described (Scott and McGraw, 1962),

except that the final chromatographic step was omitted to conserve material. The same methods of assay and definitions of units and specific activity were used.

TABLE 1

Comparison of Purified Diaphorase from Normal and Methemoglobinemic Cells

	Methemoglobinemic Enzyme	Normal Enzyme
Total Activity of 1 Pint Blood, Units	36	141
Yield of Purified Enzyme, Units	1.2	6.8
Specific Activity of Purified Enzyme, Units/mg Protein	0.8	9.4
Purification Factor, Specific Activity of Sample/Specific Activity of Original Hemolyzate	1700	5400
Turnover, Moles DPNH Oxidized per Mole Flavin	2900	2600

As shown in Table 1, the small amount of activity in methemoglobinemic cells was concentrated by the same purification procedure as was the normal enzyme. Since the per cent yield was about equal in the two preparations, there was no evidence of inhibition of a normal enzyme in methemoglobinemic cells. The amount of flavin in the two preparations was closely proportional to enzyme activity, and the turnover per mole flavin was comparable to that found for a more highly purified enzyme (Scott and McGraw, 1962), (2200 moles DPNH oxidized per mole flavin in a preparation with a specific activity of 80). It can be concluded that the normal enzyme is in fact lacking in methemoglobinemic blood since if it

were inhibited, and if this inhibition had persisted throughout purification, the amount of flavin in the purified methemoglobinemic enzyme was insufficient to allow the presence of an inhibited normal diaphorase.

The presence of even a small amount of normal diaphorase in methemoglobinemic cells is difficult to explain in terms of genetic theory. Therefore, differences in properties of the normal and methemoglobinemic enzymes were sought, and three differences were in fact found. The heat stability of the two enzymes differed as shown in Figure 1. The methemoglobinemic enzyme was inactivated at pH 8.5 and 51° at a rate such that K, the first order reaction constant, was 0.152 $min.^{-1}$. The normal enzyme was more stable and the resulting curve could be that of a mixture of enzymes of varying stability.

The methemoglobinemic enzyme was also less stable at low pH. At pH 4.7 and 23°, 90 per cent of the methemoglobinemic enzyme was inactivated in 10 minutes, while 55 per cent of the activity of the normal enzyme was retained after 30 minutes at this pH. The methemoglobinemic enzyme was also more active at low pH values; the ratio of activity at pH 6.9 (phosphate buffer) to activity at pH 7.6 (tris buffer) was 1.4 for the normal enzyme and 2.1 for the methemoglobinemic enzyme. Further studies of the methemoglobinemic enzyme were prevented by lack of material.

The most probable explanation is that the DPNH diaphorase of normal cells is a mixture of two enzymes, the major one of which is lacking in methemoglobinemia. Thus the heat denaturation of the normal enzyme in Figure 1 follows a curve that would

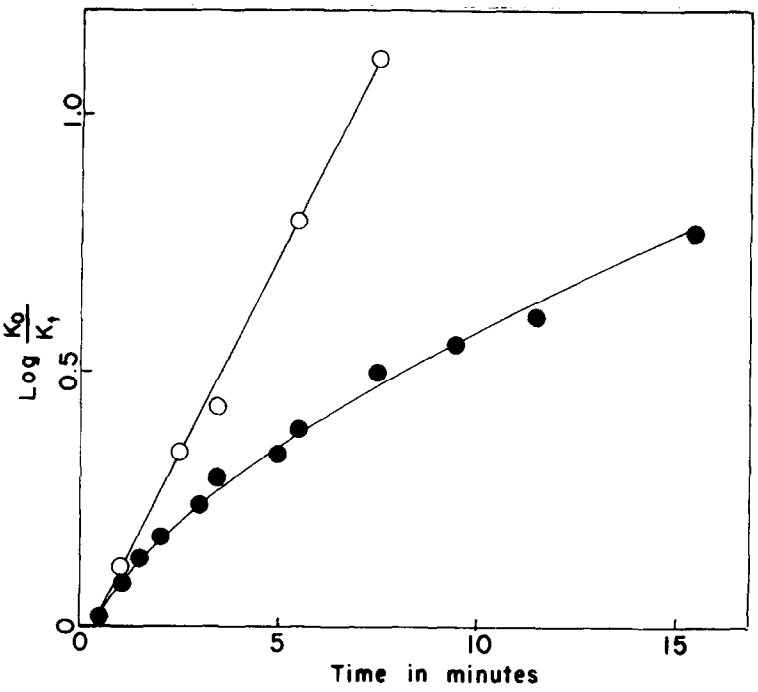

Figure 1. Heat stability of normal and methemoglobinemic diaphorase. K_0 is the first order reaction constant for reduction of 2,6-dichlorobenzenoneindophenol before heating of the enzyme; K_t the same constant after heating for t minutes. Temperature 51°, pH 8.6 (tris buffer).
● - Normal enzyme; © - methemoglobinemic enzyme.

be anticipated if the normal enzyme were a mixture of 40 per cent of a heat-labile enzyme (K = .152 min.$^{-1}$) and 60 per cent of a more stable enzyme (K = .035 min.$^{-1}$). The acid stability of the normal enzyme suggests a mixture of similar proportions of two enzymes, one destroyed at pH 4.7 and the other stable at this pH. Direct proof of the presence of two enzymes in normal cells must, however, await their separate purification from this source.

References

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