The enzymic defect of hereditary methemoglobinemia: diaphorase

We wish to report the absence in red cells of persons with hereditary methemoglobinemia[1] of an enzyme which catalyzes the reduction of methemoglobin by reduced diphosphopyridine nucleotide. This enzyme also acts as a cytochrome *c* reductase and as a diaphorase, and its presence or absence in hemolyzates is most readily demonstrated by its ability to catalyze the reduction of 2,6-dichlorobenzenoneindophenol[2].

A typical experiment is shown in Fig. 1. In the absence of hemolyzate, an excess of DPNH reduces the dye at a rate that is first order with respect to dye concentration. The addition of oxidized normal hemolyzate increases the rate of reduction of the dye, while the addition of oxidized methemoglobinemic hemolyzate decreases the observed rate of reduction. This decrease is due to a rapid and reversible reaction between reduced 2,6-dichlorobenzenoneindophenol and methemoglobin, whereby the total reducible substrate is increased by the addition of methemoglobin in the hemolyzate. When correction is made for the additional substrate (equivalent in this case to 0.096 μmole 2,6-dichlorobenzenoneindophenol) the rate with the methemoglobinemic hemolyzate is indistinguishable from the non-enzymic rate.

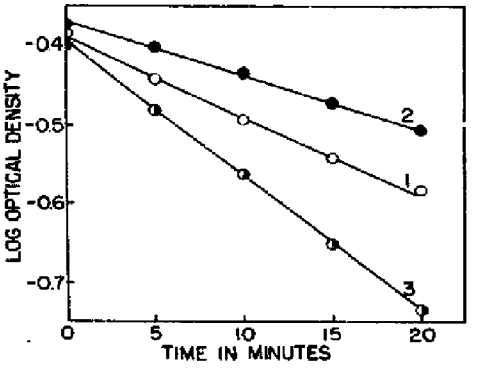

Fig. 1. Diaphorase activity of oxidized hemolyzates. Hemolyzate of nitrite-treated cells equivalent to 3.25 mg hemoglobin; tris(hydroxymethyl)aminomethane hydrochloride, pH 7.55, 20 μmoles; sodium ethylenediaminetetraacetate, 1 μmole; sodium 2,6-dichlorobenzenone-indophenol, 0.17 μmole; DPNH, 0.88 μmole; vol., 3 ml; temperature, 23°. Absorbancy determined at 600 mμ. 1, no hemolyzate; 2, methemoglobinemic hemolyzate; 3, normal hemolyzate.

Results with hemolyzates from a series of persons with methemoglobinemia and of control subjects are shown in Table I. The net rate of reduction of dye in all methemoglobinemic hemolyzates was essentially zero while an easily demonstrable reduction has been found in all normal hemolyzates. That there was no enzymic activity in methemoglobinemic hemolyzates was further demonstrated by the effect of DPNH concentration. The non-enzymic rate and the rate in the presence of methemoglobinemic hemolyzates were directly proportional to initial DPNH concentration while the rate with normal hemolyzates was little affected by moderate changes (0.44 to 1.32 μmoles) in this concentration. When TPNH was used as a substrate, the rate of dye reduction was less than 10% of that found with DPNH.

Recently it has become apparent that there are two types of hereditary methemoglobinemia. One type which is due to the presence of an abnormal hemoglobin is inherited as a dominant and is readily distinguished by spectrophotometric or electrophoretic methods[3]. The second type, of which the cases in Alaska are representative[1], is recessive and is associated with an abnormally slow rate of methemoglobin reduction

Abbreviations: DPNH, TPNH, reduced di- and triphosphopyridine nucleotide.

TABLE I

DIAPHORASE ACTIVITY OF HEMOLYZATES

Rates are first-order reaction constants measured as in Fig. 1 and corrected for non-enzymic reaction. Methemoglobinemic subjects have the code numbers previously used[1].

Methemoglobinemic subjects		*Control subjects*	
Subject	*Diaphorase activity* min^{-1}	*Subject*	*Diaphorase activity* min^{-1}
III-4	0.000	A	0.015
V-2	0.000	B	0.009
IV-1	0.000	C	0.022
VI-1	0.001	B	0.013
VI-2	0.001		
VII-2	0.001	B	0.014
VII-2	0.001	B	0.013
VII-2	0.002	C	0.032
VII-2	0.001	Nine various	0.019–0.062

in red cells[4,5]. GIBSON[6] from indirect evidence postulated that a DPNH diaphorase was lacking in red cells of persons with the enzymic type of methemoglobinemia. The present paper gives the first direct evidence that such is indeed the case. Lack of this enzyme would appear to explain the salient characteristic of the disease—the presence of from 5–60 % of the hemoglobin as methemoglobin. That only part of the hemoglobin is present as methemoglobin in these people may be due to non-enzymic reduction of methemoglobin. A very slow rate of methemoglobin reduction is observed in methemoglobinemic red cells[1] and a slightly more rapid rate is observed with DPNH or TPNH in methemoglobinemic hemolyzates. The factors responsible for this residual reduction are not known, although ascorbic acid appears to be one of them[1].

The enzyme from normal cells has been purified over 100-fold by fractional salting-out with $(NH_4)_2SO_4$ and adsorption on $Ca_3(PO_4)_2$ gel. Methemoglobinemic red cells when subjected to the same purification procedure gave no perceptible enzymic activity. The purified enzyme resembled that found in hemolyzates in that it had one-tenth the activity with TPNH that it had with DPNH. The relative rates of reduction of the dye, cytochrome *c*, and methemoglobin by purified enzyme at pH 7.2 and 23° were 15,000:130:1. This is in agreement with the results in whole cells and hemolyzates, where the rate of methemoglobin reduction in cells at 37° is 1/8,000 the rate of dye reduction in hemolyzates at 23°. Since the rate of reduction with methemoglobin is so low compared to the other activities it is possible that methemoglobin reduction is a reaction secondary to some other function of the enzyme. This appears unlikely however because the only known clinical findings in this condition are the presence of methemoglobin in red cells and a compensatory polycythemia.

Although HUENNEKENS *et al.*[7] have reported the isolation of a "methemoglobin reductase" from red cells, there is some doubt that their enzyme is in fact the agent responsible for methemoglobin reduction *in vivo* since methylene blue is an obligatory intermediate in their system. The enzyme reported here is not the system found by HUENNEKENS *et al.* since our system does not require methylene blue for methemo-

globin reduction and has a different pyridine nucleotide specificity. Furthermore, extracts prepared by their method of treatment of hemolyzates with chloroform and ethanol, or by boiling, were inactive in our system.

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1 E. M. SCOTT AND D. D. HOSKINS, *Blood*, 13 (1958) 795.
2 H. EDELHOCH, O. HAYAISHI AND L. J. TEPLY, *J. Biol. Chem.*, 197 (1952) 97.
3 P. S. GERALD AND P. GEORGE, *Science*, 129 (1959) 393.
4 H. BARCROFT, Q. H. GIBSON, D. C. HARRISON AND J. MCMURRAY, *Clin. Sci.*, 5 (1945) 145.
5 R. F. SIEVERS AND J. B. RYON, *Arch. Int. Med.*, 76 (1945) 299.
6 Q. H. GIBSON, *Biochem. J.*, 42 (1948) 13.
7 F. M. HUENNEKENS, R. W. CAFFREY, R. E. BASFORD AND B. W. GABRIO, *J. Biol. Chem.*, 227 (1957) 261.

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Pyrophosphate formation in cell-free extracts of *Escherichia coli*

In respiring cells of *Acetobacter suboxidans*, yeast[1], and *Merulius lacrymans*[2], the acid-labile phosphate in PP has a more rapid turnover than that in ATP and the other nucleoside di- and tri-phosphates. This suggests that the formation of PP is to some extent independent of ATP, and consequently arises by some separate phosphorylating pathway.

Intact cells of *Escherichia coli* behave similarly to other micro-organisms studied. PP is rapidly labelled when *E. coli* cells respire in the presence of $^{32}P_i$. ATP is more slowly labelled. In the presence of excess carrier P_i, the specific activity of PP approaches about half the specific activity of P_i, while the specific activity of ATP approaches that of PP.

Cell-free extracts of *E. coli* contain a very active PPase, which rapidly splits added PP. The extracts contain, however, a small amount of PP, which seems to be protected from the action of the hydrolyzing enzyme. This PP fraction is rapidly labelled by added $^{32}P_i$.

An extract of *E. coli* was made by treating the cell paste in aqueous suspension with an ultrasonic vibrator. The pH in the liquid after ultrasonic treatment was close to 5.5. The suspension was first freed from remaining cells, then centrifuged at 10,000 $\times$ *g* for 10 h, and then the speed was increased to 35,000 $\times$ *g* for 45 min. The precipitate was resuspended in Tris buffer, pH 8.0, to give a highly viscous liquid of pH 7.5. After centrifugation at 35,000 $\times$ *g* for 45 min, a slightly turbid, reddish supernatant, and a ropy precipitate were obtained. The supernatant was used in these experiments to study the incorporation of $^{32}P_i$ into PP and ATP. Endogenous respiration and phosphorylation was high. In some experiments alcohol dehydrogenase, DPN, and alcohol were added to test the effect of DPNH.

Abbreviations: PP, inorganic pyrophosphate; P_i, inorganic orthophosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Tris, tris(hydroxymethyl)aminomethane; DPN, DPNH, oxidized and reduced diphosphopyridine nucleotide.