THE RÔLE OF PYRIDINE NUCLEOTIDES IN THE REDUCTION OF METHEMOGLOBIN

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In the course of investigations concerning the use of *p*-aminopropiophenone-induced methemoglobinemia in the prevention of cyanide poisoning (1) and of the physiological impairments incident to methemoglobinemia (2, 3), it became of interest to study the mechanism of action between methylene blue and hemoglobin or methemoglobin. It has been shown, on the one hand, that methemoglobin is produced when methylene blue is injected into the normal organism (4, 5). On the basis of this action, methylene blue has been classified as a methemoglobin former and recommended as an antidote in the treatment of cyanide poisoning (5). On the other hand, it has also been demonstrated that the reduction of methemoglobin is accelerated when the dye is injected into the methemoglobinemic animal (6, 7). The present study is concerned with a further analysis *in vitro* of the reduction of methemoglobin in intact methemoglobinemic erythrocytes and in hemolysates, and with the possible rôle of pyridine nucleotides in this reduction.

EXPERIMENTAL

Methemoglobinemic cells were prepared *in vivo* by the injection of *p*-aminopropiophenone (2.5 to 3.0 mg. per kilo in 2 to 3 cc. of redistilled propylene glycol) into dogs. After approximately 1 hour most of these dogs exhibited symptoms of methemoglobin poisoning, and their erythrocytes showed a conversion of 60 to 80 per cent of their pigment to methemoglobin. Approximately 20 to 30 cc. of blood from normal or methemoglobinemic dogs were withdrawn by heart puncture and heparinized. The centrifuged cells were washed twice with 4 or 5 volumes of Ringer-phosphate solution of the following composition: 0.13 M NaCl, 0.0095 M $CaCl_2$, 0.0031 M KCl, 0.0015 M $MgSO_4$, and 0.01 M phosphate buffer, pH 7.40. After two such washings, glucose could no longer be detected. The cells were then

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diluted to the original blood volume with Ringer-phosphate solution and filtered through gauze to prevent the accidental introduction of small clots.

Hemolysates were prepared by centrifuging the normal or methemoglobinemic washed cell suspensions and removing the supernatant solution. The cells were frozen in an ice-salt mixture with continuous stirring, and appropriate amounts of precooled water were added. In certain experiments, hemolysis was performed in the presence of nicotinamide so as to yield a final concentration of 0.16 M nicotinamide in the hemolysate. The cells were immersed in a water bath at about 30° and thawed with constant stirring. After standing at room temperature for 30 minutes, the solutions were again centrifuged for 15 minutes and the clear supernatant liquid was withdrawn for use in the experiments.

Purified methemoglobin was prepared from guinea pig blood by the method of Warburg and Reid (8) and dialyzed at 0–4° against distilled water until nitrites could no longer be detected in the methemoglobin solution. The solution was diluted to a suitable volume with 0.05 M phosphate buffer (pH 7.40) and kept at 0–4° until use.

Sodium lactate solution was prepared by neutralizing lactic acid, C.P., with NaOH to phenol red. This solution showed no optical activity and was considered to consist of racemized sodium lactate. Sodium hexose diphosphate was obtained from the calcium salt (Schwarz) by treatment with the calculated amount of sodium oxalate and addition of a few drops of dilute $CaCl_2$ solution to the filtrate to insure the absence of oxalate ions. Methylene blue (U. S. P.), nicotinamide (U. S. P.), and dextrose (anhydrous, C.P.) were used.

Diphosphopyridine nucleotide (DPN) was prepared from pressed bakers' yeast according to the directions of Williamson and Green (9). The purity of the preparation was determined spectrophotometrically by measuring the change in absorption upon treatment with $Na_2S_2O_4$ at 340 mμ, and estimated to be about 10 to 20 per cent. Since no standard samples of DPN were available, and only a first approximation of the purity was required, this method was chosen because of its convenience. Reduced diphosphopyridine nucleotide (DPN-H_2) was prepared according to Green and Dewan (10) except that $Na_2S_2O_4$ in the solid state rather than in solution was added to the DPN solution. The excess $Na_2S_2O_4$ was destroyed by passing oxygen in a vigorous stream through the solution for 30 minutes. If the solution was cloudy after the treatment with $Na_2S_2O_4$, it was centrifuged and the supernatant filtered.

The erythrocyte suspensions, hemolysates, or methemoglobin solutions, prepared as described above, were mixed in equal proportions with solutions of the various substrates in 25 or 50 cc. Erlenmeyer flasks. The flasks were immersed in a water bath at 38° and shaken at the rate of 110 oscillations

per minute. Duplicate samples for the analytical determinations were withdrawn from the reaction mixtures at the times stated in Tables I to III. Methemoglobin was determined spectrophotometrically in duplicate aliquots, immediately upon withdrawal of the sample, by a slight modification of the method of Evelyn and Malloy (11). The presence of methylene blue in the concentrations used in the experiments was found not to interfere with the determination of methemoglobin. Lactic acid was determined by the method of Barker and Summerson (12), and the amount of total blood pigment iron according to the method of Drabkin (13).

Results

Oxidation of Hemoglobin and Reduction of Methemoglobin by Methylene Blue—Warburg, Kubowitz, and Christian (14), using the evolution of oxygen as a measure of methemoglobin formation, found that methylene blue oxidized hemoglobin to methemoglobin in intact, washed erythrocytes or in hemolysates prepared from them, but that in the presence of added glucose methylene blue reduced methemoglobin in intact erythrocytes. These findings were confirmed and extended in the present study with the spectrophotometric technique. It was found that, if the reaction was permitted to proceed for a sufficient length of time, complete oxidation of hemoglobin by methylene blue occurred both in hemolysates and in washed intact erythrocytes. Thus, at a concentration of 0.27 mM methylene blue, 72 per cent of the blood pigment from washed and laked erythrocytes was oxidized to methemoglobin in 4 hours, 94 per cent in 24 hours, and 100 per cent in 44 hours at 38°. In the presence of glucose, oxidation by methylene blue was retarded in intact erythrocytes but not in hemolysates.

The addition of glucose or lactate to methemoglobinemic cells caused reduction, provided that methylene blue was present and the cells were intact. It may be mentioned that glucose was more effective than twice its molar concentration of *dl*-lactate. Thus, in a typical experiment, the concentration of methemoglobin decreased in 2 hours from an initial value of 84 to 28 per cent in the presence of 56 mM glucose and 0.13 mM methylene blue; in the presence of 110 mM *dl*-lactate and the same concentration of methylene blue, the methemoglobin concentration decreased to only 48 per cent. No reduction was observed in hemolysates from methemoglobinemic cells, prepared as described above, in the presence of methylene blue and either glucose, lactate, or hexose diphosphate.

The results of Warburg *et al.* (14) as well as those obtained in the present study suggest that during hemolysis a factor, essential in the reduction of methemoglobin in the presence of substrate and methylene blue, is lost. That the process of glycolysis is linked with the reduction of methemoglobin is implied by the work of Kiese (15) and of Drabkin (16) who showed that

iodoacetate and fluoride inhibit this reduction. In confirmation of their results, it was found that a concentration of 100 mM fluoride or 1 mM iodoacetate completely suppressed the reduction in the presence of glucose and methylene blue.

Although glycolysis appeared to be necessary for the reduction of methemoglobin, the extent of the reduction did not parallel the extent of lactic acid formation when glucose was used as substrate. Thus, in a typical experiment no methemoglobin was reduced and 9.3 micromoles of lactic acid were formed in 10 cc. of reaction mixture during 100 minutes in the absence of methylene blue; in the presence of 0.0013 mM methylene blue, 4.2 micromoles of methemoglobin were reduced but the amount of lactic acid formed was essentially the same, 8.7 micromoles. These results indicate that the connection between glycolysis and methemoglobin reduction in the presence of methylene blue does not consist of a simple interaction between glucose or lactate and methylene blue.

Rôle of Pyridine Nucleotides in Reduction of Methemoglobin—In view of the above results, it was considered possible that the lack of reduction of methemoglobin by methylene blue in hemolyzed cells was caused by the absence of pyridine nucleotides from hemolysates, since there is evidence that pyridine nucleotides disappear rapidly upon lysis of erythrocytes (17) and homogenization of several animal tissues. The disappearance may be counteracted by the addition of nicotinamide during the process of disintegration of the cell structure (18, 19). Consequently, the effect of nicotinamide, present at the time of hemolysis, upon the reduction of methemoglobin was tested.

Table I shows the extent of reduction of methemoglobin in hemolysates in the presence of nicotinamide and various substrates. No significant reduction occurred in the presence of nicotinamide and methylene blue when glucose was used as a substrate. With hexose diphosphate, 5 to 13 (average 9) per cent of the methemoglobin originally present was reduced in 1 hour when nicotinamide but no methylene blue was present. The addition of methylene blue to this system caused a much more rapid reduction; in nine experiments, an average of 33 per cent of the methemoglobin originally present was reduced in 1 hour under these conditions. This reduction was completely inhibited by the addition of 1 mM iodoacetate. The presence of nicotinamide also led to a substantial reduction of methemoglobin when lactate was chosen as the substrate.

The above data indicated that either DPN or triphosphopyridine nucleotide or both were necessary for the reduction of methemoglobin by lactate or hexose diphosphate. The reaction mechanism was assumed to consist of a reduction of pyridine nucleotides by these substrates, and a subsequent interaction between the reduced pyridine nucleotide and met-

TABLE I

Effect of Nicotinamide (NCA) and Methylene Blue (MB) on Reduction of Methemoglobin in Hemolysates with Glucose, Lactate, and Hexose Diphosphate (HDP) As Substrates

The initial concentration of methemoglobin was 60 to 80 per cent of the total blood pigment.

Fraction of methemoglobin reduced						
During 60 min. in presence of					During 120 min. in presence of	
2.8 mM glucose, 0.13 mM MB	160 mM NCA, 2.8 mM glucose, 0.13 mM MB	2.8 mM HDP, 0.13 mM MB	160 mM NCA, 2.8 mM HDP	160 mM NCA, 2.8 mM HDP, 0.13 mM MB	20 mM lactate, 0.13 mM MB	160 mM NCA, 20 mM lactate, 0.13 mM MB
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
0	0	0	5	27	2	7
3	2	0	7	31	0	18
3	4	0	9	31		20
		1	11	32		
			13	33		
				33		
				36		
				36		
				39		

TABLE II

Reduction of Purified Methemoglobin (MetHb) by Reduced Diphosphopyridine Nucleotide ($DPN\text{-}H_2$) in Presence of Methylene Blue (MB)

The composition of the reaction mixture is expressed in terms of the absolute amounts of reactants, in micromoles, present in a volume of 6 cc. The concentration of methemoglobin is expressed as per cent of total blood pigment.

Composition of reaction mixture	Concentration of methemoglobin after incubation at 38° for		
	0 min.	15 min.	20 min.
	per cent	*per cent*	*per cent*
0.044 MetHb*	90	90	
0.044 " + 0.29 $DPN\text{-}H_2$	90	83	
0.044 " + 0.29 " + 0.027 MB	90	12	
0.044 " + 0.29 DPN + 0.027 "	90	90	
0.068 "	98		98
0.068 " + 0.44 $DPN\text{-}H_2$	98		85
0.068 " + 0.44 " + 0.027 MB	98		8

* The controls are treated with a solution which contained the same amount of $Na_2S_2O_4$ as was used in the reduction of DPN and which had been exposed to oxygen for 30 minutes. The amounts of DPN and $DPN\text{-}H_2$ given in the table are estimated on the basis of spectrophotometric assay of the preparation as described in the text.

hemoglobin, mediated by methylene blue. Table II shows the reaction of DPN-H_2 with purified methemoglobin, in the presence of methylene blue. It may be seen that methemoglobin was reduced to some extent directly by DPN-H_2 and that this reaction was greatly accelerated in the presence of methylene blue. No reduction occurred when DPN instead of DPN-H_2 was used. The rate of reduction of methemoglobin in this system was rapid; no further change in the concentration of methemoglobin was found after incubation for approximately 15 minutes at 38°. Table III shows that the extent of reduction of methemoglobin under these conditions was a function of the amount of reduced pyridine nucleotide added to the system.

TABLE III

Effect of Concentration of DPN-H_2 on Extent of Methemoglobin Reduction

Each reaction mixture contained 0.045 mM methylene blue. A relative concentration of DPN-H_2 of 1 represented a concentration of 0.017 mg. of a reduced Williamson-Green preparation in 6.0 cc. of reaction mixture.

Relative concentration of DPN-H_2	Fraction of MetHb reduced in 30 min.
units	*per cent*
1	28
2	40
4	45
11	68
16	75
20	79

DISCUSSION

The present work has shown (*a*) that when nicotinamide, a known suppressor of pyridine nucleotide hydrolysis, is present hexose diphosphate or lactate can serve as substrate in the reduction of methemoglobin in hemolysates as well as in intact erythrocytes, while glucose is no longer utilized after hemolysis, and (*b*) that methemoglobin interacts with DPN-H_2 to yield hemoglobin. Both of these reactions are greatly accelerated by methylene blue. The findings indicate that, incident to hemolysis, processes other than destruction of pyridine nucleotides occur which abolish glycolysis in hemolysates. In view of the non-utilization of glucose in the reduction of methemoglobin in such a system it appears likely that one or more steps in the phosphorylating mechanism, leading to the production of hexose diphosphate from glucose, are abolished by hemolysis.

It is implied by the experiments here presented that the component in the Williamson-Green yeast preparation, active in the reduction of methemoglobin, is DPN-H_2. It is recognized that this preparation contains

substances other than DPN. However, several lines of evidence, derived from this study and the work of other investigators, support the conclusion previously expressed. Runnstroem, Lennerstrand, and Borei (20) observed that the addition of DPN to a system consisting of hemolyzed blood, hexose monophosphate or diphosphate, and methylene blue increased oxygen consumption and phosphorylations. Lennerstrand (17) reported that pyridine nucleotides of erythrocytes disappeared rapidly following lysis. Mann and Quastel (18) and Handler and Klein (19) demonstrated that nicotinamide in high concentrations was a specific competitive inhibitor of a nucleosidase which liberates nicotinamide from DPN during the disintegration of animal tissues. In the present study, reduction of methemoglobin in hemolysates proceeded only on addition of nicotinamide to the system during lysis. This reduction was abolished by the addition of low concentrations of iodoacetate, a known inhibitor of dehydrogenases which bring about reduction of DPN. The above findings indicate very strongly that the component in the Williamson-Green preparation, which, after treatment with $Na_2S_2O_4$, is active in methemoglobin reduction, is indeed DPN.

It appears that the essential feature of the reduction of methemoglobin within the intact erythrocyte or in solution is the interaction between DPN-H_2 and methemoglobin. Methylene blue mediates this reaction; it may be presumed to be reduced by DPN-H_2 to leucomethylene blue, which in turn reduces methemoglobin to hemoglobin. For the reducing action of methylene blue a continuous source of DPN-H_2 must thus be available. The removal of substrate, or the inhibition of glycolysis by fluoride or iodoacetate, interferes with the production of DPN-H_2; hemolysis results in the enzymatic destruction of DPN. If the formation of DPN-H_2 is prevented by any one of these means, leucomethylene blue is no longer regenerated in this system, and methylene blue acts purely as an oxidant.

SUMMARY

Reduction of methemoglobin by hexose diphosphate or lactate and methylene blue in hemolysates occurs only in the presence of nicotinamide during lysis. Glucose cannot function as substrate in this system, but is utilized in intact methemoglobinemic cells.

Addition of a partially purified preparation of reduced diphosphopyridine nucleotide from yeast to methemoglobin and methylene blue results in the rapid reduction of methemoglobin. Diphosphopyridine nucleotide in its oxidized form is ineffective in this system.

Conditions are defined for the action of methylene blue as an oxidant of hemoglobin or a reductant of methemoglobin.

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