JCI The Journal of Clinical Investigation

THE REDUCTION OF METHEMOGLOBIN IN HUMAN ERYTHROCYTES INCUBATED WITH PURINE NUCLEOSIDES

Ernst R. Jaffé

J Clin Invest. 1959;38(9):1555-1563. https://doi.org/10.1172/JCI103934.

Research Article

THE REDUCTION OF METHEMOGLOBIN IN HUMAN ERYTHROCYTES INCUBATED WITH PURINE NUCLEOSIDES * †

By ERNST R. JAFFÉ

(From the Department of Medicine, Albert Einstein College of Medicine and the Bronx Municipal Hospital Center, New York, N. Y.)

(Submitted for publication March 30, 1959; accepted April 23, 1959)

This study is concerned with the effect of purine nucleosides and related compounds on the reduction of methemoglobin to hemoglobin in human erythrocytes *in vitro*. Other investigators have reported that mammalian erythrocytes can reduce methemoglobin to hemoglobin when the cells are incubated with glucose (2), lactate (3), glyceraldehyde, fructose (4), fumaric acid, malic acid (5), mannose, galactose (6), formaldehyde (7) and other aliphatic and aromatic aldehydes (8). Previous studies in this and other laboratories have demonstrated that normal human erythrocytes can metabolize the pentose moiety of certain purine nucleosides to lactic acid *in vitro* (9, 10). This metabolic activity is associated with enhanced resistance to osmotic stress (11, 12), increased organic phosphate esters (11), retention of potassium (11) and extrusion of sodium (13), maintenance of reduced glutathione (14, 15) and some prolongation of viability of erythrocytes stored *in vitro* (11, 16, 17).

In the present investigation it was observed that normal human erythrocytes in which 60 to 80 per cent of the hemoglobin was chemically oxidized *in vitro* could reduce a considerable portion of the methemoglobin to hemoglobin when incubated with certain purine nucleosides and sugars. This effect was associated with active metabolism of the compounds by the cells. The metabolism of these compounds and their effects on the reduction of methemoglobin in erythrocytes obtained from a patient with congenital methemoglobinemia were also studied.

MATERIALS AND METHODS

Whole blood was obtained from normal adults and was anticoagulated with about 1 mg. dry heparin per 10 ml. of blood. The volume of packed erythrocytes was determined by the method of Wintrobe (18). A portion of the blood was incubated at 37° C. with occasional mixing, after the addition of 0.6 to 0.9 mg. of sodium nitrite per ml. of packed erythrocytes. The sodium nitrite was dissolved in 1 ml. of isotonic sodium chloride solution. After 40 minutes' incubation, the treated blood was centrifuged at 1,500 G for seven minutes and the erythrocytes were washed three times with about three volumes of 0.9 per cent sodium chloride solution; the final centrifugation was carried out in a Servall Refrigerated Angle Centrifuge for 10 minutes at 1,900 G and at 4° C. (SS-1 rotor). A 25 per cent suspension of the treated and washed erythrocytes in isotonic sodium chloride solution was prepared and the actual volume of packed erythrocytes was determined. This procedure resulted in the conversion of 60 to 80 per cent of the hemoglobin to methemoglobin. Four ml. portions of the erythrocyte suspension were added to 25 ml. Erlenmeyer flasks containing 2 ml. of isotonic sodium chloride-sodium phosphate buffer solution (equal volumes of 0.9 per cent sodium chloride solution and isotonic sodium phosphate buffer, pH 7.3) in which were dissolved the compounds to be studied. The pH of these final suspensions was 7.3 to 7.4. The flasks were stoppered loosely with cotton plugs and incubated at 37° C. in a Dubnoff metabolic shaker-incubator oscillating 92 cycles per minute. In experiments where hemolysates were used, 50 per cent suspensions of treated and washed erythrocytes were prepared and the erythrocytes were hemolyzed by twice freezing and thawing rapidly. Two ml. of the hemolysate was added to flasks containing 3 ml. of distilled water, 1 ml. of isotonic sodium phosphate buffer, pH 7.3, and the dissolved compounds.

At intervals during the incubation, 0.4 ml. portions of each suspension were transferred to 10 ml. of 0.0167 M phosphate buffer, pH 6.6, and the methemoglobin concentration was determined by the method of Evelyn and Malloy (19), adapted for the Coleman Junior Spectrophotometer. The amount of methemoglobin was expressed as the per cent of the total hemoglobin pigment. The methemoglobin concentrations of the whole blood prior to treatment with sodium nitrite and of the erythrocyte suspensions or hemolysates prior to incubation were also determined. Duplicate samples assayed by this

* This work was presented in part at the Eastern Section Meeting of the American Federation for Clinical Research at Boston, Mass., December 12, 1958 (1).

† This investigation was supported by grants from the National Heart Institute of the National Institutes of Health, United States Public Health Service (Grant H-2803), the Atomic Energy Commission [Contract AT (30-1) 1855], and the Office of Naval Research [Contract Nonr-1765 (00)].

spectrophotometric method agreed within ± 1.5 per cent. In a few experiments, the methemoglobin concentrations were also estimated from the difference between the total hemoglobin content, determined by comparison with a cyanmethemoglobin standard (20), and the hemoglobin content determined by the oxygen binding capacity of the erythrocyte suspensions, determined in the laboratory of Dr. Charles W. Frank. The spectrophotometric and the gasometric methods agreed within ± 4.5 per cent. The spectrophotometric method described recently by Mills and Randall (21) was employed in some experiments to permit the calculation of both methemoglobin and choleglobin concentrations. The methemoglobin concentrations by this method and by the method of Evelyn and Malloy agreed within ± 2 per cent. Significant choleglobin concentrations were not detected in erythrocyte suspensions during 22 hours of incubation with the various nucleoside and carbohydrate substrates and only 1.8 per cent was present in a suspension incubated with ascorbic acid.

Penicillin G and streptomycin sulfate, 1 mg. of each, were added to the incubation flasks in most of the experiments detailed in this paper. Even after 22 hours of incubation there was no bacterial contamination as evidenced by the absence of growth when aliquots of the suspensions were cultured on blood agar and in infusion broth. Identical results in the reduction of methemoglobin to hemoglobin were observed, however, in experiments performed with and without added antibiotics.

Lactic acid determinations were performed by the method of Barker and Summerson (22) on clear filtrates of perchloric acid extracts prepared by adding one volume of each erythrocyte suspension to six volumes of cold 2 per cent perchloric acid after the suspensions had been incubated for six hours.

Determinations of the pH of the erythrocyte suspensions after 22 hours of incubation were made with a Beckman Model G glass electrode pH meter. The pH of the control suspensions ranged from 7.2 to 7.5, but the pH of the suspensions incubated with the various compounds did not differ from that of the control by more than ± 0.1 pH unit. These changes in the pH could not be correlated with the effect of the compounds on the reduction of methemoglobin to hemoglobin.

The degree of spontaneous hemolysis that occurred during incubation was determined by adding 0.2 ml. aliquots of each suspension to 2 ml. of isotonic sodium chloride solution and 2 ml. of distilled water. The amount of hemoglobin was determined, after centrifugation and dilution of 1 ml. of the supernatant solution with 4 ml. of distilled water, by measuring the optical density at 540 mμ in a Beckman DU spectrophotometer. The per cent spontaneous hemolysis was then calculated by relating the optical density of the isotonic sodium chloride solution sample to the distilled water sample whose hemolysis was taken as 100 per cent. Spontaneous hemolysis, even after 22 hours of incubation, did not exceed 4 per cent.

Most of the compounds used in these experiments were obtained commercially. The 2,6-diaminopurine riboside (2,6-diamino-9-β-D-ribofuranosyl purine) and the adenine glucoside (9-β-D-glucopyranosyl adenine) were synthesized and provided by Dr. Bertram A. Lowy. Samples of purine riboside (9-β-D-ribofuranosyl purine) and adenosine-1-N-oxide were generously provided by Dr. George B. Brown. Mr. David Schwarz of Schwarz Laboratories supplied the adenosine-5′-phosphoramide. Dr. Henry M. Kissman of the Lederle Laboratories Division of the American Cyanamid Company kindly supplied the Puromycin Hydrochloride® (6-dimethylamino-9 (3′-p-methoxy-L-phenylalanyl amino-β-D-ribofuranosyl) purine), dimethyl-adenosine (6-dimethyl-amino-9-β-D-ribofuranosyl purine), amino-adenosine (6-amino-9 (3′-amino-3′-deoxy-β-D-ribofuranosyl) purine), aminonucleoside (6-dimethyl-amino-9 (3′-amino-3′-deoxy-β-D-ribofuranosyl) purine) and aminoribose (3-amino-3-deoxyribose) hydrochloride. The concentrations of the compounds were expressed as micromoles per milliliter of packed erythrocytes and were contained in a final volume of 6 ml.

Case history. The patient (E. D., Hospital No. 30216) was a 30 year old Puerto Rican woman who was seen in the Prenatal Clinic of the Bronx Municipal Hospital Center during the eighth month of her fifth pregnancy in November, 1957. At that time cyanosis of the hands, lips and vaginal mucosa was noted. She reported frequent headaches, slight exertional dyspnea and easy fatigability. Except for these findings, physical examination was entirely normal with a normal intrauterine pregnancy. The hemoglobin concentration was 11.6 Gm. per cent, the hematocrit 40 per cent and the erythrocytes appeared slightly hypochromic on the stained blood smear. The reticulocyte count ranged from 0.8 to 1.5 per cent. The white blood cell count, differential count, urine analysis, serology, roentgenogram of the chest and liver chemistries were within normal limits. Spectrophotometric examination of hemolysates prepared from whole blood and from washed erythrocytes obtained from a sample of the patient's blood revealed the absorption spectrum of methemoglobin as characterized by a peak at 630 mμ that disappeared on the addition of sodium cyanide. On repeated examinations the concentration of methemoglobin ranged between 23 and 26 per cent, but on one occasion was 31 per cent. One hour after the intravenous administration of methylene blue, 1 mg. per Kg., the patient's mucous membranes were of a normal pink color and the concentration of methemoglobin was 1 per cent. The methemoglobin concentration was maintained at a level of 9 to 13 per cent by the daily oral administration of 500 mg. of ascorbic acid and was accompanied by relief of her symptoms. That the abnormal hemoglobin in the erythrocytes of this patient was actually normal methemoglobin and not hemoglobin M was further established by spectrophotometric analysis of a hemolysate treated with potassium ferricyanide (23) and by starch block electrophoresis of a hemolysate kindly performed by Miss Margaret Pease through the cooperation of Dr. Park S. Gerald.

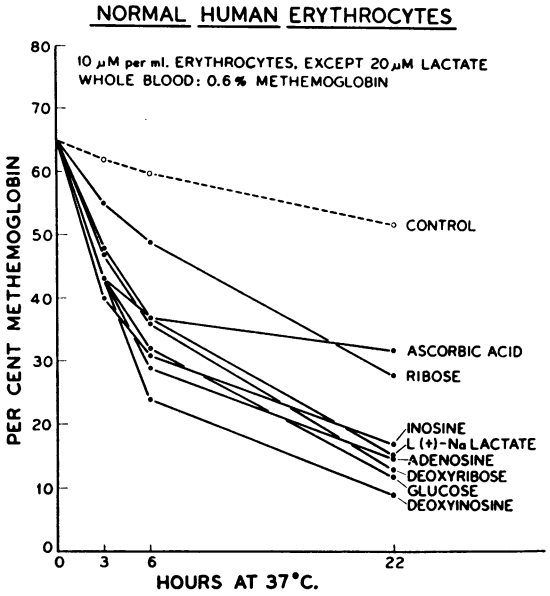

FIG. 1. EFFECT OF INCUBATION WITH VARIOUS COMPOUNDS ON THE REDUCTION OF METHEMOGLOBIN IN NORMAL HUMAN ERYTHROCYTES

RESULTS AND DISCUSSION

Normal human erythrocytes

Data from a typical experiment are presented in Figure 1 and Table I, Column A. Incubation with 10 μMoles per ml. of erythrocytes of inosine, adenosine, deoxyribose, glucose and deoxyinosine, as well as L (+)-sodium lactate, present in twice the molar concentration of the other compounds, resulted in the reduction of methemoglobin to hemoglobin at a much greater rate than occurred in the control erythrocyte suspension incubated without substrate. After six hours of incubation, almost identical amounts of lactic acid were present in the perchloric acid extracts of the erythrocyte suspensions incubated with adenosine, inosine and deoxyinosine, while perhaps slightly more was found in the suspension incubated with glucose. Although about 30 per cent less lactic acid had been formed after six hours of incubation with deoxyribose, the effect on methemoglobin conversion was only slightly less than that of the purine nucleosides and glucose. The extent of reduction of methemoglobin by deoxyribose was indistinguishable from that of the purine nucleosides and glucose after 22 hours. Incubation with ribose resulted in slower reduction of methemoglobin to hemoglobin than did incubation with the other compounds, and after six hours only about one quarter as much lactic acid was present as in the suspensions incubated with the purine nucleosides and glucose. L (+)-sodium lactate, 20 μMoles per ml. of erythrocytes, affected the reduction of methemoglobin at about the same rate as deoxyribose, 10 μMoles per ml. After 22 hours, however, the effect of lactate was essentially the same as that of the purine nucleosides and sugars. About 30 per cent of the lactate added had disappeared from the total erythrocyte suspension after six hours of incubation. Although initially ascorbic acid had almost the same effect as the various purine nucleosides, glucose, deoxyribose and lactate, this compound was the least effective after 22 hours of incubation. From the data presented in Table II it is apparent that incubation with guanosine, 2,6-diaminopurine riboside, xanthosine, deoxyadenosine and deoxyguanosine also had a marked effect on the reduction of methemoglobin to hemoglobin. In every instance, after 22 hours of incubation, the effect of the purine deoxyriboside was slightly greater than the effect of the corresponding purine riboside. The reason for this observed difference in effect is obscure. Although incubation with fructose and galactose promoted the reduction of methemoglobin to hemoglobin, these hexoses were less effective than an equimolar amount of glucose. A significant effect from incubation with fumaric acid could be demonstrated only

TABLE I

Lactic acid present after six hours of incubation of A) normal human erythrocytes and B) erythrocytes of congenital methemoglobinemia with purine nucleosides and sugars

Compound	A Normal human erythrocytes	B Erythrocytes of congenital methemoglobinemia
	*μMoles/ml.**	*μMoles/ml.**
Glucose	1.09	1.51
Inosine	1.02	1.18
Adenosine	0.99	1.18
Deoxyinosine	0.96	1.07
Deoxyribose	0.69	0.73
L (+)-sodium lactate	0.68	0.87
Ribose	0.25	0.31
Ascorbic acid	0	0

* Micromoles of lactic acid per micromole of substrate added per milliliter of erythrocytes, corrected for control.

TABLE II

The effect of purine nucleosides, sugars and related compounds on the reduction of methemoglobin in normal human erythrocytes

Compound	Concentration*	Initial methemoglobin	Methemoglobin after incubation for: 3 hrs.	6 hrs.	22 hrs.
		%	%	%	%
Control		70	69	67	62
Adenosine	8		47	38	24
Deoxyadenosine	8		50	31	18
Control		65	65	62	50
Inosine	10		42	28	13
Guanosine	10		44	30	12
Deoxyguanosine	10		45	24	5
Control		62	59	60	47
Adenosine	8		43	32	12
2, 6-DAPR†	8		44	31	10
Xanthosine	8		50	40	13
Control		77	73	73	63
Glucose	9		57	43	20
Fructose	9		68	56	32
Galactose	9		69	63	37
Fumaric acid	19		68	64	48
Control		65	65	63	56
Adenosine	10		42	29	15
Aminoadenosine	10		63	60	39

* Concentration in micromoles per milliliter of erythrocytes.

† 2,6-Diaminopurine riboside.

when this compound was present in twice the molar concentration of the other sugars. These experiments demonstrated that the compounds effective in promoting the reduction of methemoglobin to hemoglobin included purine ribosides (adenosine, guanosine, inosine, 2,6-diaminopurine riboside and xanthosine), purine deoxyribosides (deoxyadenosine, deoxyguanosine and deoxyinosine), hexoses (glucose, galactose and fructose), pentoses (ribose and deoxyribose), fumaric acid, L (+)-sodium lactate and ascorbic acid.

Although the precise mechanisms by which methemoglobin in intact erythrocytes is reduced to hemoglobin are still unknown, there is considerable evidence that one or more enzyme systems are involved. The normal process of methemoglobin reduction is dependent upon the integrity of the erythrocyte (2, 24), is associated with carbohydrate metabolism (4, 25) and requires the regeneration of reduced pyridine nucleotides (4, 26). Recently, partial purification, identification and characterization of an enzyme from human erythrocytes whose existence was implied by the investigations of Warburg and Christian (24) and which was named methemoglobin reductase (Hämiglobinreduktase) by Kiese (4) have been reported (27–30). It was postulated (28, 30) that this enzyme has two prosthetic groups: *1*) an unknown carrier, perhaps ionic iron, which is detached upon hemolysis and purification and that can be substituted for by methylene blue or other autoxidizable dyes, and *2*) a tightly bound iron porphyrin moiety. This enzyme requires reduced triphosphopyridine nucleotide (TPNH), but it can also utilize reduced diphosphopyridine nucleotide (DPNH) at a slower rate. There may exist, however, in normal human erythrocytes two separate reductases, one dependent upon TPNH and the other dependent upon DPNH (4, 31, 32). It has been suggested that the system that utilizes DPNH is the one that reduces methemoglobin to hemoglobin in normal human erythrocytes in the absence of methylene blue (32).

Metabolic pathways for the regeneration of either of the two reduced pyridine nucleotides are present within normal human erythrocytes. For example, reduction of triphosphopyridine nucleotide (TPN) to TPNH can occur via the hexose-monophosphate shunt upon metabolism of glucose-6-phosphate to 6-phosphogluconate and 6-phosphogluconic acid to ribulose-5-phosphate. Reduction of diphosphopyridine nucleotide (DPN) to DPNH can occur by way of the Embden-Meyerhof pathway upon the oxidation of glyceraldehyde-3-phosphate to 1,3-diphosphoglycerate and by the oxidation of lactate to pyruvate.

The results of the experiments described here may be explained by the metabolism of the ribose or deoxyribose portion of the purine nucleosides by way of the hexose-monophosphate shunt and the Embden-Meyerhof pathways with the regeneration of reduced pyridine nucleotides. Phosphorolytic cleavage of inosine or guanosine by erythrocyte purine nucleoside phosphorylase (33–35) results in the formation of ribose-1-phosphate that can be isomerized to ribose-5-phosphate. This latter compound can enter the hexose-monophosphate shunt with the formation of hexose phosphate and triose phosphate (36) which may be metabolized further by way of the Embden-Meyerhof pathway. Adenosine is deaminated to inosine by an active erythrocyte adenosine deaminase (37), but the reactions involved in the

utilization of 2,6-diaminopurine riboside and xanthosine are not yet known. Since erythrocyte purine nucleoside phosphorylase can apparently also cleave deoxyinosine (33), deoxyribose from the deoxyribosides could also enter a metabolic pathway the details of which have not been elucidated in the erythrocyte. The observation that ribose and deoxyribose were effective in promoting the reduction of methemoglobin to hemoglobin and that lactic acid accumulated when erythrocytes were incubated with these pentoses is of some interest. Other investigators have stated that ribose is not metabolized by erythrocytes *in vitro* (38), although increased oxygen uptake by rabbit erythrocytes incubated with ribose, arabinose and xylose plus methylene blue has been reported (39). The pathways by which ribose and deoxyribose are metabolized are unknown, but they might involve phosphorylation and entry into the hexose-monophosphate shunt and Embden-Meyerhof pathway. Glucose, fructose and galactose are metabolized by these pathways with the regeneration of reduced pyridine nucleotides. Fumaric acid may be metabolized to malic acid by fumarase and the latter can be oxidized to oxaloacetate by malic acid dehydrogenase with the reduction of DPN to DPNH. These two enzymes of the tricarboxylic acid cycle are known to be present in mature mammalian erythrocytes (38). Since the metabolism of these various substrates can lead to the regeneration of DPNH, the reported observations are consistent with the concept that the DPNH-dependent mechanism is the one that normally reduces methemoglobin to hemoglobin in normal human erythrocytes. Differences in the effectiveness of these various compounds may reflect differences in the permeability of the erythrocyte or differences in the rates at which the metabolic reactions proceed within the cell.

When a hemolysate of normal human erythrocytes rather than intact erythrocytes was incubated with some of the effective compounds, only ascorbic acid had an effect on the concentration of methemoglobin after three hours of incubation (Table III). It should be noted that no attempt was made to inhibit the destruction of pyridine nucleotides that is known to occur when animal tissues are disrupted (40). The observation that incubation with ascorbic acid leads to the reduction of methemoglobin in a hemolysate, as well as in intact erythrocytes, is compatible with the belief that ascorbic acid acts directly on methemoglobin (41). This experiment with a hemolysate confirms the importance of an intact erythrocyte for methemoglobin reduction in the absence of methylene blue or other autoxidizable dyes. In addition, it demonstrates that the compounds effective with intact erythrocytes did not exert their effect by reducing methemoglobin to hemoglobin directly.

TABLE III

The effect of various compounds on the reduction of methemoglobin in a hemolysate of normal human erythrocytes *

Compound	Methemoglobin after incubation for 3 hrs.
	%
Control	72
Glucose	74
Glucose-6-phosphate	71
Adenosine	72
Deoxyadenosine	73
Ribose	75
Deoxyribose	70
Ascorbic acid	34

* Initial concentration of methemoglobin was 72 per cent. Concentration of compounds was 19 μMoles per ml. of erythrocytes.

The effect of methylene blue on the reduction of methemoglobin may be due to the ability of this dye to serve in an electron transport system (2). It may, however, also be due, in part, to the activation or stimulation of the hexose-monophosphate shunt pathway (4, 42, 43). The incubation of normal human erythrocytes containing methemoglobin with glucose, inosine or deoxyinosine plus methylene blue resulted in marked acceleration in the reduction of methemoglobin to hemoglobin (Table IV, Part A). The failure of methylene blue to enhance greatly the effect of ribose and deoxyribose is not readily explained. It is conceivable that the permeability of the erythrocyte to ribose is so limited that no acceleration from addition of methylene blue could be demonstrated. If deoxyribose is metabolized by way of the Embden-Meyerhof pathway, only a limited increase in the rate of methemoglobin reduction would be expected since the methemoglobin reduction system that utilizes DPNH is not enhanced markedly by this dye (4, 32, 42).

TABLE IV

The effect of methylene blue on the ability of some compounds to enhance the reduction of methemoglobin in human erythrocytes

Compound	Concentration*	Initial methemoglobin	Methemoglobin after incubation for 3 hrs.	
			Without meth. blue	With meth. blue†
		%	%	%
A. Normal human erythrocytes				
Control		68	64	59
Inosine	10		44	11
Glucose	10		49	3
L (+)-sodium lactate	19		48	34
Control		67	65	62
Ribose	10		58	51
Deoxyribose	10		52	47
Control		65	62	57
Inosine	10		40	9
Deoxyinosine	10		44	14
B. Erythrocytes of congenital methemoglobinemia				
Control		68	77	72
Glucose	10		74	4
Inosine	10		74	10
L (+)-sodium lactate	20		75	69

* Concentration in micromoles per milliliter of erythrocytes.

† Concentration of methylene blue was 0.12 μMoles per ml. of erythrocytes.

This latter effect was also shown by the minimal increase in the effect of L (+)-sodium lactate when methylene blue was added to the erythrocyte suspension.

The specificity of the effect on the reduction of methemoglobin to hemoglobin is demonstrated by those compounds that did not increase the rate of reduction of methemoglobin above that observed in the control suspensions (Table V). Compounds closely related in structure to the effective purine nucleosides were ineffective. Incubation with purine riboside, adenosine-1-N-oxide, adenine glucoside, dimethyl-adenosine, aminonucleoside and Puromycin Hydrochloride®, as well as with the purine, adenine, did not result in either enhanced reduction of methemoglobin to hemoglobin or in the production of lactic acid. A slight effect on methemoglobin reduction was observed with aminoadenosine (Table II) and about 13 per cent as much lactic acid was present after six hours of incubation as with an equimolar amount of adenosine. It remains to be determined if this finding represented true metabolism of the amino-adenosine or if there occurred a small amount of non-enzymatic hydrolysis of the compound with the formation of adenosine. When the purine portion

TABLE V

Compounds ineffective in enhancing the reduction of methemoglobin to hemoglobin when incubated with normal human erythrocytes

Compound	μMoles*	Compound	μMoles*
Pyrimidine ribosides		Intermediates of carbohydrate metabolism and related compounds	
Cytidine	8		
Uridine	8		
Pyrimidine deoxyribosides		Sucrose	9
Deoxycytidine	8		
Deoxyuridine	8	Glucose-6-phosphate	10
Thymidine	8	Fructose-1,6-diphosphate	11
		Ribose-5-phosphate	11
Nucleotides		2,3-diphosphoglycerate	11
Adenosine-5′-phosphate	11		
Adenosine diphosphate	11	Aminoribose	10
Adenosine triphosphate	11		
Adenosine-5′-phosphoramide	10	D (-)-sodium lactate	19
		Sodium pyruvate	17
Related compounds			
Adenine	7	Miscellaneous compounds	
Adenosine-1-N-oxide	8, 10	Aspartic acid	10
Dimethyl-adenosine	10	Cysteine	10
Aminonucleoside	10	Glucuronic acid	10
Adenine glucoside	8, 10	Glucuronolactone	10
Purine riboside	8	Glutathione, reduced	10
Puromycin Hydrochloride®	10	Sodium thiosulfate	10

* Concentration in micromoles per milliliter of erythrocytes.

of the effective compounds was replaced by another group, there was no effect on the reduction of methemoglobin and no lactic acid production was observed (10). The pyrimidine ribosides (cytidine and uridine) and the pyrimidine deoxyribosides (deoxycytidine, deoxyuridine and thymidine) were ineffective. The nucleotides of adenosine (adenosine-5′-phosphate, adenosine diphosphate, adenosine triphosphate and adenosine-5′-phosphoramide) were also without effect on methemoglobin reduction. Incubation with a disaccharide (sucrose), sodium pyruvate, aminoribose and the optical isomer of the natural form of sodium lactate, *i.e.*, D (−)-sodium lactate, did not result in increased reduction of methemoglobin to hemoglobin. Several phosphorylated intermediates of carbohydrate metabolism (glucose-6-phosphate, fructose-1,6-diphosphate, ribose-5-phosphate and 2,3-diphosphoglycerate) were ineffective, probably as a result of the impermeability of the erythrocyte to phosphorylated sugars. Compounds unrelated in structure to the purine nucleosides were studied and were ineffective. These compounds included aspartic acid, sodium thiosulfate, glucuronic acid and glucuronolactone. Although it has been reported that reduced glutathione and cysteine will reduce methemoglobin in hemolysates (44), these compounds were ineffective in the intact erythrocyte system used in these studies.

Erythrocytes of congenital methemoglobinemia

Data from an experiment performed with the erythrocytes from the patient with congenital methemoglobinemia are presented in Figure 2 and Table I, Column B. It is apparent that incubating these erythrocytes with the purine nucleosides and sugars that promoted methemoglobin reduction in normal human erythrocytes did not result in significant reduction of methemoglobin to hemoglobin. Incubation with ascorbic acid, which is thought to act directly on methemoglobin (41), did lead to a decrease in the methemoglobin concentration. The quantity of lactic acid present after six hours of incubation was comparable to that observed with normal erythrocytes, except that perhaps slightly more lactate was produced from glucose. The significance of this apparent increased glucose utilization remains to be investigated. The data on lactic acid production con-

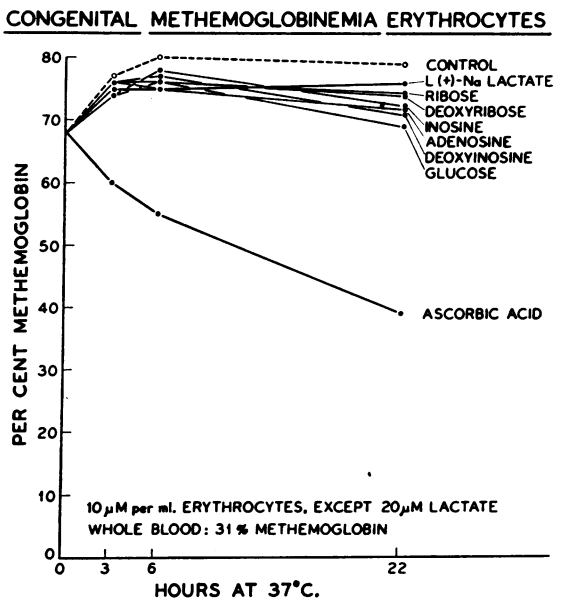

FIG. 2. EFFECT OF INCUBATION WITH VARIOUS COMPOUNDS ON THE REDUCTION OF METHEMOGLOBIN IN THE ERYTHROCYTES OF CONGENITAL METHEMOGLOBINEMIA

firm the observation that the erythrocytes of congenital methemoglobinemia can metabolize glucose (45) and they also demonstrate that these cells can metabolize ribose, deoxyribose and the pentose moiety of certain purine nucleosides.

The nature of the defect in the erythrocytes of congenital methemoglobinemia is unknown, but may involve the absence of an unknown electron carrier system between reduced pyridine nucleotides and methemoglobin (30, 42, 46). That the deficiency can be corrected by methylene blue has been reported by a number of investigators (32, 45, 47) and is further supported by the data in Table IV, Part B. The concentration of methemoglobin in the erythrocytes from the patient with congenital methemoglobinemia was increased by incubating the cells with sodium nitrite. Incubation of these treated erythrocytes with glucose or inosine plus methylene blue resulted in the reduction of methemoglobin to an extent equal to that observed in normal erythrocytes. Only a slight effect from L (+)-sodium lactate and methylene blue was noted. Since the pathways by which nucleosides and sugars are metabolized in the congenital methemoglobinemia erythrocytes would appear to be normal, it may be that methylene blue serves in a substitute electron transport sys-

tem between reduced pyridine nucleotides and methemoglobin in these cells (4, 42).

SUMMARY AND CONCLUSIONS

The incubation of normal human erythrocytes that contained 60 to 80 per cent methemoglobin with the purine nucleosides that were actively metabolized to lactic acid resulted in the reduction of a considerable portion of the methemoglobin to hemoglobin. The effective nucleosides included adenosine, guanosine, inosine, 2,6-diaminopurine riboside, xanthosine, deoxyadenosine, deoxyguanosine and deoxyinosine, and a slight effect was observed with aminoadenosine. The pentoses, ribose and deoxyribose, were also able to enhance the reduction of methemoglobin. In addition, the ability of glucose, galactose, fructose, fumaric acid and L (+)-sodium lactate to promote methemoglobin reduction was confirmed.

The erythrocytes from a patient with congenital methemoglobinemia were unable to reduce methemoglobin to hemoglobin when incubated with the nucleosides and sugars that promoted the reduction of methemoglobin in normal erythrocytes, despite the ability of the congenital methemoglobinemia erythrocytes to produce comparable amounts of lactic acid. These observations are consistent with the concept that the defect in congenital methemoglobinemia lies in a failure in electron transport to methemoglobin.

Incubation with ascorbic acid resulted in the reduction of methemoglobin in normal erythrocytes and in hemolysates, as well as in the erythrocytes of congenital methemoglobinemia.

The addition of methylene blue to normal erythrocyte suspensions containing added glucose or purine nucleosides accelerated the rate of reduction of methemoglobin and permitted the erythrocytes of congenital methemoglobinemia to reduce methemoglobin in a normal manner.

The studies presented here suggest that compounds that can be metabolized by human erythrocytes by pathways that can lead to the reduction of pyridine nucleotides will promote the reduction of methemoglobin to hemoglobin, provided the necessary electron transport mechanism is intact.

ACKNOWLEDGMENT

The author is indebted to Doctors Sheldon Spielman, Sherman Karpen and Selig Neubardt of the Department of Obstetrics and Gynecology of the Bronx Municipal Hospital Center who made the patient with congenital methemoglobinemia available for study.

REFERENCES

1. Jaffé, E. R. The effect of purine nucleosides on the reduction of methemoglobin in human erythrocytes (abstract). Clin. Res. 1959, 7, 12.
2. Warburg, O., Kubowitz, F., and Christian, W. Über die katalytische Wirkung von Methylenblau in lebenden Zellen. Biochem. Z. 1930, **227**, 245.
3. Wendel, W. B. Oxidation of lactate by methemoglobin in erythrocytes with regeneration of hemoglobin. Proc. Soc. exp. Biol. (N. Y.) 1931, **28**, 401.
4. Kiese, M. Die Reduktion des Hämiglobins. Biochem. Z. 1944, **316**, 264.
5. Kiese, M., and Schwartzkopff-Jung, W. Die Reduktion des Hämiglobins. III. Reduktion des Hämiglobins und Stoffwechsel in roten Zellen. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1947, **204**, 267.
6. Spicer, S. S., Hanna, C. H., and Clark, A. M. Studies *in vitro* on methemoglobin reduction in dog erythrocytes. J. biol. Chem. 1949, **177**, 217.
7. Matthies, H. Methämoglobinrückbildung in Reticulocyten. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1956, **229**, 331.
8. Matthies, H. Die Wirkung von Aldehyden auf die Methämoglobinrückbildung in Erythrocyten. Biochem. Z. 1957, **329**, 341.
9. Rubinstein, D., Kashket, S., and Denstedt, O. F. Studies on the preservation of blood. IV. The influence of adenosine on the glycolytic activity of the erythrocyte during storage at 4° C. Canad. J. Biochem. 1956, **34**, 61.
10. Lowy, B. A., Jaffé, E. R., Vanderhoff, G. A., Crook, L., and London, I. M. The metabolism of purine nucleosides by the human erythrocyte *in vitro*. J. biol. Chem. 1958, **230**, 409.
11. Gabrio, B. W., Donohue, D. M., and Finch, C. A. Erythrocyte preservation. V. Relationship between chemical changes and viability of stored blood treated with adenosine. J. clin. Invest. 1955, **34**, 1509.
12. Jaffé, E. R., Lowy, B. A., Vanderhoff, G. A., Aisen, P., and London, I. M. The effects of nucleosides on the resistance of normal human erythrocytes to osmotic lysis. J. clin. Invest. 1957, **36**, 1498.
13. Harris, E. J., and Prankerd, T. A. J. The effect of adenosine on the movement of sodium between erythrocytes and the suspension medium (abstract). Biochem. J. 1955, **61**, xix.
14. Beutler, E., Robson, M., and Buttenwieser, E. The mechanism of glutathione destruction and protection in drug-sensitive and non-sensitive erythrocytes. *In vitro* studies. J. clin. Invest. 1957, **36**, 617.
15. Klebanoff, S. J. Glutathione metabolism. 2. The oxidation and reduction of glutathione in intact erythrocytes. Biochem. J. 1957, **65**, 423.

16. Prankerd, T. A. J. Revival of stored blood with guanosine and its successful transfusion. Lancet 1956, **1**, 469.
17. Lange, R. D., Crosby, W. H., Donohue, D. M., Finch, C. A., Gibson, J. G., II, McManus, T. J., and Strumia, M. M. Effect of inosine on red cell preservation. J. clin. Invest. 1958, **37**, 1485.
18. Wintrobe, M. M. Clinical Hematology, 4th ed. Philadelphia, Lea & Febiger, 1956, p. 367.
19. Evelyn, K. A., and Malloy, H. T. Microdetermination of oxyhemoglobin, methemoglobin, and sulfhemoglobin in a single sample of blood. J. biol. Chem. 1938, **126**, 655.
20. Crosby, W. H., and Houchin, D. N. Preparing standard solutions of cyanmethemoglobin. Blood 1957, **12**, 1132.
21. Mills, G. C., and Randall, H. P. Hemoglobin catabolism. II. The protection of hemoglobin from oxidative breakdown in the intact erythrocyte. J. biol. Chem. 1958, **232**, 589.
22. Barker, S. B., and Summerson, W. H. The colorimetric determination of lactic acid in biological material. J. biol. Chem. 1941, **138**, 535.
23. Gerald, P. S. The electrophoretic and spectroscopic characterization of hgb M. Blood 1958, **13**, 936.
24. Warburg, O., and Christian, W. Über Aktivierung der Robisonschen Hexose-Mono-Phosphorsäure in roten Blutzellen und die Gewinnung aktivierender Fermentlösungen. Biochem. Z. 1931, **242**, 206.
25. Drabkin, D. L. Maintenance of active hemoglobin—a function of erythrocytes (abstract). Fed. Proc. 1946, **5**, 132.
26. Gutmann, H. R., Jandorf, B. J., and Bodansky, O. The rôle of pyridine nucleotides in the reduction of methemoglobin. J. biol. Chem. 1947, **169**, 145.
27. Kiese, M., Schneider, C., and Waller, H. D. Hämiglobinreduktase. Naunyn-Schmiedeberg's Arch. exp. Path. Pharmak. 1957, **231**, 158.
28. Huennekens, F. M., Caffrey, R. W., Basford, R. E., Gabrio, B. W. Erythrocyte metabolism. IV. Isolation and properties of methemoglobin reductase. J. biol. Chem. 1957, **227**, 261.
29. Lonn, L., and Motulsky, A. G. Electrophoretic demonstration of a non-hemoglobin protein (methemoglobin reductase) in hemolysates (abstract). Clin. Res. Proc. 1957, **5**, 157.
30. Huennekens, F. M., Caffrey, R. W., and Gabrio, B. W. The electron transport sequence of methemoglobin reductase. Ann. N. Y. Acad. Sci. 1958, **75**, 167.
31. Wendel, W. B. Oxidations by erythrocytes and the catalytic influence of methylene blue. I. The oxidation of lactate to pyruvate. J. biol. Chem. 1933, **102**, 373.
32. Gibson, Q. H. The reduction of methaemoglobin in red blood cells and studies on the cause of idiopathic methaemoglobinaemia. Biochem. J. 1948, **42**, 13.
33. Sandberg, A. A., Lee, G. R., Cartwright, G. E., and Wintrobe, M. M. Purine nucleoside phosphorylase activity of blood. I. Erythrocytes. J. clin. Invest. 1955, **34**, 1823.
34. Huennekens, F. M., Nurk, E., and Gabrio, B. W. Erythrocyte metabolism. I. Purine nucleoside phosphorylase. J. biol. Chem. 1956, **221**, 971.
35. Tsuboi, K. K., and Hudson, P. B. Enzymes of the human erythrocyte. I. Purine nucleoside phosphorylase; isolation procedure. J. biol. Chem. 1957, **224**, 879.
36. Dische, Z. Synthesis of hexosemono- and diphosphate from adenosine and ribose-5-phosphate in human blood *in* Phosphorus Metabolism, W. D. McElroy and B. Glass, Eds. Baltimore, The Johns Hopkins Press, 1951, vol. I, p. 171.
37. Schaedel, M. L., and Schlenk, F. Adenosine and adenosine deaminase. Tex. Rep. Biol. Med. 1948, **6**, 176.
38. Denstedt, O. F. The enzymology of the erythrocyte *in* Blood Cells and Plasma Proteins, J. L. Tullis, Ed. New York, Academic Press Inc., 1953, p. 223.
39. Nossal, P. M. The metabolism of erythrocytes. I. Respiration in the absence and presence of methylene blue. Aust. J. exp. Biol. med. Sci. 1948, **26**, 123.
40. Handler, P., and Klein, J. R. The inactivation of pyridine nucleotides by animal tissues *in vitro*. J. biol. Chem. 1942, **143**, 49.
41. Barcroft, H., Gibson, Q. H., Harrison, D. C., and McMurray, J. Familial idiopathic methaemoglobinaemia and its treatment with ascorbic acid. Clin. Sci. 1945, **5**, 145.
42. Gibson, Q. H. Methaemoglobin and sulphaemoglobin *in* The Chemical Pathology of Animal Pigments, R. T. Williams, Ed. Biochemical Society Symposia No. 12, Cambridge, University Press, 1954, p. 55.
43. Brin, M., and Yonemoto, R. H. Stimulation of the glucose oxidative pathway in human erythrocytes by methylene blue. J. biol. Chem. 1958, **230**, 307.
44. Morrison, D. B., and Williams, E. F., Jr. Methemoglobin reduction by glutathione or cysteine. Science 1938, **87**, 15.
45. Eder, H. A., Finch, C., and McKee, R. W. Congenital methemoglobinemia. A clinical and biochemical study of a case. J. clin. Invest. 1949, **28**, 265.
46. Scott, E. M., and Hoskins, D. D. Hereditary methemoglobinemia in Alaskan Eskimos and Indians. Blood 1958, **13**, 795.
47. King, E. J., White, J. C., and Gilchrist, M. A case of idiopathic methaemoglobinaemia treated by ascorbic acid and methylene blue. J. Path. Bact. 1947, **59**, 181.