Reduction of Methemoglobin in Human Adult and Cord Blood Erythrocytes Incubated with Glucose or Inosine.* (31704)

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(Introduced by Mero Nocenti)
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The usual concentration of about 1% methemoglobin in normal human erythrocytes is maintained as the result of an equilibrium between the rate at which hemoglobin is oxidized and the rate at which methemoglobin is reduced(1). Increased concentrations of methemoglobin can result from exposure to drugs or chemicals which increase the rate of oxidation or can result from impairment of the capacity of erythrocytes to reduce methemoglobin. The tendency of newborns and infants to develop toxic methemoglobinemia has been attributed to a transient deficiency in the activity of the reduced diphosphopyridine nucleotide $NADH_2$-methemoglobin reductase system in their erythrocytes(2,3). Evidence to support this conclusion has been obtained with intact erythrocytes and with hemolysates(2-5). Other possible explanations for the increased susceptibility of newborns, such as altered metabolic activity of their erythrocytes, have not been evaluated completely.

The rate of reduction of methemoglobin by the erythrocytes of adults upon incubation with inosine is greater than the rate observed with glucose(6). Inosine is metabolized by purine nucleoside phosphorylase to yield ribose phosphate, thereby bypassing the activities of hexokinase and phosphofructokinase in the initial part of the Embden-Meyerhof pathway of glycolysis, but permitting the generation of $NADH_2$ through the activity of glyceraldehyde-3-PO_4 dehydrogenase(7,8). A rate-limiting step in the reduction of methemoglobin with glucose as substrate, therefore, may be the production of $NADH_2$. If there exists a deficient enzymatic reaction early in anaerobic glycolysis in the erythrocytes of newborns, this step could be bypassed by using inosine as substrate. Since there is no known deficiency in the utilization of inosine, erythrocytes from cord blood should reduce methemoglobin as rapidly as do the erythrocytes of adults when incubated with inosine, provided the activity of the $NADH_2$-methemoglobin reductase system is not deficient. The rates of reduction of methemoglobin by suspensions of erythrocytes from cord blood and from adults, therefore, have been determined during incubation with glucose or inosine.

Materials and methods. Thirteen blood samples, obtained from umbilical cords prior to the delivery of the placenta, were paired with samples collected from healthy adults within 1 hour. All samples, anticoagulated with acid-citrate-dextrose solution (NIH Formula A, 1 volume to 4 volumes of blood), were used within 3 hours. For each sample, a volume of blood containing 2 ml of erythrocytes was added to 2 ml of freshly prepared 0.145 M $NaNO_2$ solution to oxidize hemoglobin to methemoglobin. This mixture was incubated for 20 minutes at room temperature with frequent mixing. The supernatant solution was removed after centrifugation for 5 minutes at 4°C and 1,500 × *g* and the erythrocytes were washed with centrifugation 5 times with 10 volumes of a cold 9:1 mixture of 0.154 M NaCl solution and 0.067 M Sorensen's phosphate buffer, pH 7.4. The washed erythrocytes were resuspended in 5.5 volumes of a 1:1 mixture of the NaCl solution and phosphate buffer which contained sodium penicillin (267 units/ml) and streptomycin sulfate (167 μg/ml) to inhibit bacterial growth. Four ml of this suspension (packed cell volume about 15%) were added to each of 3 25-ml Erlenmeyer flasks and were in-

* These studies were supported, in part, by grants HE-2803 and HE-10041 from USPHS.

† Career Scientist of Health Research Council of City of New York (I-169).

TABLE I. Mean Rates of Reduction of Methemoglobin After Incubation of Erythrocyte Suspensions for 6 Hours.

	% per hour*		
	Control	Glucose	Inosine
Adult blood	1.6 ± .5	4.6 ± .7	8.4 ± .8
Cord "	1.5 ± .7	3.7 ± .6	5.9 ±1.2
P value	>.50	<.01	<.01

* Rates ± standard deviation.

cubated for 2 hours at 37°C in a metabolic shaker to deplete endogenous substrates. Two ml of either the 1:1 mixture of NaCl solution and phosphate buffer (Control) or of this mixture containing glucose or inosine (20 μm/ml) were added to the 3 flasks. Incubation was continued and the proportion of methemoglobin present was determined by the method of Evelyn and Malloy(9) at this time and after 2, 4 and 6 hours. At time 0, the concentration of methemoglobin was 85 to 95% in all suspensions. The pH of the suspensions was measured before and after incubation.

Erythrocyte suspensions prepared from 8 pairs of cord and adult blood samples not treated with $NaNO_2$ but otherwise handled in an identical fashion were incubated with the Control buffer solution. Methemoglobin determinations were performed after 0, 12, 24 and 36 hours of incubation to estimate the rate of spontaneous methemoglobin formation.

Results. In all 13 paired samples, a decreased rate of reduction of methemoglobin was observed when erythrocytes from cord blood were compared with erythrocytes from adults, whether incubated with glucose or with inosine (Table I). The difference between the mean rate of reduction for the 13 cord blood and 13 adult blood samples after incubation with either substrate also was statistically significant ($P<0.01$ by Student's t test). The rates of reduction were nearly linear throughout the 6-hour period of incubation (Fig. 1). The difference in the degree of redness between suspensions of cord blood and adult blood erythrocytes after incubation for 6 hours with the substrates was obvious. Only limited reduction occurred in the absence of added substrate, and there was no significant difference between cord blood and adult blood Controls (Table I).

The pH decreased during incubation by an average of 0.15 pH unit in the flasks with glucose and by 0.22 pH unit in the flasks with inosine. There was no difference, however, between the cord blood and adult blood erythrocyte suspensions in respect to the initial or post-incubation pH values. Spontaneous hemolysis after 6 hours was less than 1% in all erythrocyte suspensions.

The mean rates of spontaneous formation of methemoglobin in the 8 pairs of blood samples not treated with $NaNO_2$ were 0.50 ± 0.12% and 0.57 ± 0.05% methemoglobin per hour for adult blood and cord blood erythrocytes, respectively. These rates were not significantly different ($P = 0.49$).

Discussion. The decreased rate of reduction of methemoglobin by erythrocytes from cord blood upon incubation with either glucose or inosine is most consistent with the reported decrease in the activity of the $NADH_2$-methemoglobin reductase system in the erythrocytes of newborns(2). The only other defect likely to result in a slower rate of reduction of methemoglobin is a deficiency in the supply of $NADH_2$(8). A decrease in available $NADH_2$

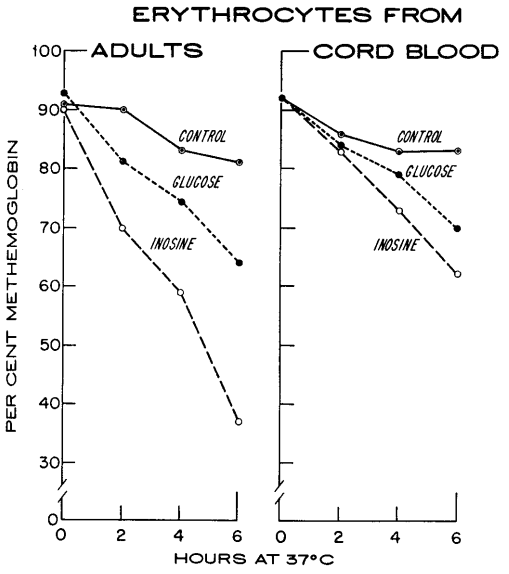

FIG. 1. The reduction of methemoglobin in a representative experiment with paired erythrocyte suspensions prepared from adult and cord blood and incubated without substrate (Control) and with glucose or inosine.

could result from a decrease in glycolysis with impaired activity of enzymes required for the generation of $NADH_2$, from a decrease in the content of NAD or from preferential utilization of $NADH_2$ in another metabolic reaction. Utilization of glucose and inosine and the activities of many enzymes in the Embden-Meyerhof and hexose monophosphate shunt pathways are known to be increased in erythrocytes from cord blood(10,11). The activity of phosphofructokinase, however, is decreased(12). The concentrations of oxidized and reduced pyridine nucleotides are essentially the same in erythrocytes of adult and cord blood(13). Increased utilization of $NADH_2$ through the activity of α-glycerophosphate dehydrogenase has been suggested as the explanation for the decreased ability to reduce methemoglobin observed with erythrocytes from cord blood(14). Increased accumulation of pyruvate would be expected if $NADH_2$ is utilized for α-glycerophosphate dehydrogenase activity. Little or no pyruvate accumulates when nitrite-treated erythrocytes from newborns are incubated with glucose, despite increased utilization of glucose and decreased reduction of methemoglobin(8). These observations support the conclusion that the potential supply of $NADH_2$ probably is not limited in the erythrocytes of cord blood. The reduction of methemoglobin by erythrocytes from cord blood is decreased even with inosine which can be metabolized by reactions that bypass the activity of phosphofructokinase.

An increased rate of oxidation of hemoglobin F, present in high concentration in erythrocytes from cord blood, upon exposure of intact cells to methemoglobin-forming agents or to inhibitors of metabolic reactions has been reported(1). Purified oxyhemoglobin F, however, was no more susceptible to spontaneous oxidation than was oxyhemoglobin A(1). In the present study, the rates of spontaneous methemoglobin formation in in substrate-depleted, intact cord blood and adult blood erythrocytes were not different. Thus, the spontaneous formation of methemoglobin under the conditions of these experiments would not explain the differences in the rates of reduction which were observed.

Summary. Impaired reduction of methemoglobin to hemoglobin by erythrocytes from cord blood is observed with either glucose or inosine as substrate. This observation is consonant with the hypothesis that the decreased reduction is a result of diminished activity of the $NADH_2$-methemoglobin reductase system in the erythrocytes of cord blood. Neonatal deficiency in the activity of this enzyme system may contribute significantly to the increased susceptibility of infants to toxic methemoglobinemia.

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Received July 15, 1966. P.S.E.B.M., 1967, v124.