The Effects of Deoxygenation of Adult and Fetal Hemoglobin on the Synthesis of Red Cell 2,3-Diphosphoglycerate and Its In Vivo Consequences

FRANK A. OSKI, ARLAN J. GOTTLIEB, WILLIAM W. MILLER, and MARIA DELIVORIA-PAPADOPOULOS

From the Departments of Pediatrics and Medicine, University of Pennsylvania School of Medicine, Department of Physiology, Graduate Division of the University of Pennsylvania School of Medicine, Hospital of the University of Pennsylvania and The Children's Hospital of Philadelphia, Philadelphia, Pennsylvania 19146

ABSTRACT Patients over 1 month of age with arterial oxygen pressures of less than 60 mm Hg were found to have elevated red cell 2,3-diphosphoglycerate (2,3-DPG) levels and blood with a decreased affinity for oxygen. The increase in 2,3-DPG was proportional to the degree of hypoxemia. In patients under 1 month of age this relationship was not observed. Red cells from adults, but not newborns, showed rapid increases in 2,3-DPG when incubated under nitrogen. Adult, but not fetal, deoxyhemoglobin was shown to facilitate in vitro synthesis of 2,3-DPG by binding this organic phosphate and relieving the product inhibition of 2,3-DPG mutase.

Throughout a wide range change in oxygen affinity as measured by the P_{50} is linear with respect to the 2,3-DPG concentration; a change of 430 mμmoles of 2,3-DPG/ml of red blood corpuscles (RBC) resulting in a change of the P_{50} of 1 mm Hg. It appears that the 2,3-DPG of the adult's red cells responds rapidly to metabolic and environmental influences and in turn effects metabolism and the cellular environment. Many of these effects are not shared by the red cells of the newborn.

A portion of this material was presented at the 39th Annual Meeting of The Society for Pediatrics Research, May 1969, Atlantic City, N. J.

Dr. Oski and Dr. Gottlieb are recipients of Career Development Awards K3-HD 38600 and 1-K04-AM 42392, respectively, and Dr. Delivoria-Papadopoulos of Special Fellowship 1F 3HD 40661.

Received for publication 18 September 1969 and in revised form 24 October 1969.

INTRODUCTION

In 1967 Benesch and Benesch (1) and Chanutin and Curnish (2) demonstrated that the affinity of a hemoglobin solution for oxygen may be decreased by its interaction with organic phosphates. The two principal organic phosphates of the human erythrocyte, 2,3-diphosphoglycerate (2,3-DPG) and adenosine triphosphate (ATP) combine reversibly with deoxyhemoglobin and shift the oxygen-hemoglobin dissociation curve to the right (3). Since 2,3-DPG comprises approximately 60% of the organic phosphate of the red cell, it is quantitatively the most important in this regard. The 2,3-DPG is produced in the red cell from 1,3-DPG by the action of 2,3-diphosphoglycerate mutase. Since 2,3-diphosphoglycerate mutase is quite sensitive to inhibition by its product 2,3-DPG (4), the binding of 2,3-DPG by deoxyhemoglobin should result in a relief of the product inhibition and allow for further synthesis of 2,3-DPG from 1,3-DPG. Thus in situations where hypoxemia is present it would be anticipated that levels of 2,3-DPG would be increased. Lenfant and associates (5) have demonstrated both a rapid increase in red cell 2,3-DPG levels in subjects moving to high altitudes and an associated decrease in the affinity of hemoglobin for oxygen. In a preliminary report we observed that patients with hypoxemia secondary to congenital heart disease or pulmonary insufficiency possessed red cells with increased 2,3-DPG levels (6).

It is the purpose of the present report to extend our original observations, correlate the changes in the red cell 2,3-DPG levels with the oxygen-hemoglobin dis-

sociation curve of whole blood, demonstrate the in vitro augmentation of synthesis of 2,3-DPG in the intact adult erythrocyte and in a cell-free system under conditions of deoxygenation, and call attention to the anomalous behavior of the erythrocytes from newborn infants.

METHODS

Patients. Arterial blood samples were obtained from 48 patients with congenital heart disease at the time of cardiac catheterization. The patients ranged in age from 1 day to 16 yr of life. None were judged clinically to be in congestive heart failure at the time of study. Arterial pH, Pa_{O_2} and Pa_{CO_2} were determined as previously described (6).

Measurement of red cell 2,3-DPG. 2 ml of heparinized blood from each subject were immediately placed in 4 ml of chilled 2 N perchloric acid, homogenized and extracted. The precipitate was reextracted with 0.5 N perchloric acid and the combined supernatants were neutralized with 5 N KOH in an ice bath and stored overnight at 4°C. The specimens were then centrifuged and the precipitate washed with 1 ml of water. The combined neutral extract was utilized for the determination of 2,3-DPG employing the Schroter and Heyden modification (7) of the method of Krimsky (8). In a final volume of 1 ml, the reaction mixture contained the following: Tris, pH 9.0, 50 μmoles; $MgCl_2$, 5 μmoles; phosphoenolpyruvate, 0.75 μmoles; enolase 100 μg; phosphoglycerate mutase, 10 μg; and 10 μl of a 1:5 or 1:10 dilution of the neutralized red cell extract. A standard curve was constructed using 2,3-DPG in final concentrations ranging from 0.1 to 0.8 μM and in this range the assay was found to be linear. Assays were performed at 27°C in a Gilford Model 2000 recording spectrophotometer at 240 mμ. All enzymes and substrates were obtained from the Boehringer Mannheim Corp., New York. The purity of the commercially obtained 2,3-DPG was determined by chromatography on Dowex 1 × 8-chloride (60–80 wet mesh) (Dow Chemical Co., Midland, Mich.) by the procedures described by Bartlett (9).

Oxygen-hemoglobin dissociation curves. 20 ml of whole heparinized blood was employed for the determination of the oxygen-hemoglobin dissociation curve. 3-ml samples of blood were equilibrated in tonometers at various oxygen tensions ranging from 10 to 50 mm Hg and at a constant partial pressure of carbon dioxide. The pH was maintained at 7.345. Oxygen tensions were measured with the oxygen electrode (10) of a gas analyzer (IL model 113-SI)[1] at 37°C. Oxygen saturations were measured spectrophotometrically (11). For each subject 4–5 blood samples, done in duplicate, were employed in order to obtain the points necessary to construct a regression line relating the oxygen tension to the hemoglobin saturation between a 20%–70% range of hemoglobin saturation. The P_{50} was then determined graphically as the whole blood oxygen tension at 50% oxygen saturation.

Anaerobic incubation of intact red cells. Freshly obtained venous samples from normal adults and 1 day old infants were collected in tubes containing dried sodium heparin.[2] The samples were washed free of leukocytes with buffered isotonic saline, pH 7.4, in a refrigerated centrifuge at 4°C and resuspended to a final hematocrit of approximately 33% in a 0.05 M Tris-HCl buffer, pH 7.4, containing phosphate 10 mM, sodium chloride 92 mM, potassium chloride 10 mM, magnesium chloride 1 mM, and glucose 10 mM. The sample was divided into two aliquots, one was incubated in an atmosphere of 100% carbon monoxide and the other incubated in 100% nitrogen. Gases were washed via a fritted glass dispersion tube immersed in distilled water before being delivered. At the start of the incubation, 2-ml aliquots were removed from each flask for 2,3-diphosphoglycerate detreminations and additional 0.2-ml samples were removed for the duplicate determination of glucose by the glucose oxidase method (12). After a 2 hr period of incubation, repeat determinations of 2,3-diphosphoglycerate and glucose were performed. In several studies adenosine triphosphate was also measured on the neutralized extract (13). The pH of the incubation mixture was found to range from 7.36 to 7.47 during the period of study.

Hemoglobin binding and 2,3-DPG synthesis. For the studies of hemoglobin binding of 2,3-diphosphoglycerate, hemoglobin and a source of red cell diphosphoglycerate mutase were prepared. Hemoglobin was prepared from washed erythrocytes by freezing-thawing three times in a dry ice–acetone bath. Stroma was removed by centrifugation at 100,000 *g* for 30 min. The red cell nonheme proteins were absorbed from the hemolysate by mixing the hemolysate with an equal volume of diethylaminoethyl-suspension (DEAE) (10% DEAE by dry weight) which had been equilibrated with 0.003 M phosphate buffer, pH 7.0, for 4 hr at 4°C. The resin was separated by centrifugation, washed free of hemoglobin with cold phosphate buffer, and the enzymes eluted in two washes with minimal volumes of 0.5 M NaCl. The hemoglobin-free, diphosphoglycerate mutase–rich eluate was stored in small vials at —20°C until immediately before use. The absorbed hemoglobin solution was dialyzed against 0.15 M NaCl and then concentrated, if necessary, by dialysis against 98% glycerol. The hemoglobin solution was reabsorbed with DEAE if it was found to contain residual diphosphoglycerate mutase activity. The hemoglobin solutions contained neither measurable 2,3-diphosphoglycerate nor diphosphoglycerate mutase in the concentrations employed experimentally.

Fetal hemoglobin was prepared chromatographically from umbilical cord blood on columns of Amberlite IRC-50 (CG-50) type II resin (Rohm & Haas Co., Philadelphia, Pa.) (14). Fetal hemoglobin was eluted from the columns by a sodium phosphate developer which was 0.1 M in sodium at pH 6.7, dialyzed free of phosphate, and concentrated by dialysis against glycerol. Cyanide was omitted from all solutions. Since significant diphosphoglycerate mutase activity was found in column effluent associated with the fetal hemoglobin, this fraction was also absorbed with DEAE and was free of enzyme activity before use. Purity of the fetal hemoglobin preparation was determined by Microzone[3] electrophoresis and alkali denaturation (15).

The rate of 2,3-diphosphoglycerate formation from fructose-1,6-diphosphate was determined in a 10 ml suspension containing fructose-1,6-diphosphate 10 mM, diphosphopyridine nucleotide (NAD) 6 mM, 3-phosphoglyceric acid 20 mM, Ethylenediaminetetraacetic acid (EDTA) 5 mM, triethanolamine buffer 50 mM (pH 7.6), potassium disodium phosphate 5mM, aldolase 9 U, and glyceraldehyde-3-phosphate dehydrogenase 36 U. The incubation system contained approximately 0.06 U of diphosphoglycerate mutase/ml of reaction mixture supplied from the extracted erythrocytes. The reaction mixture was supplemented with varying quantities of either adult or fetal hemoglobin. Incubation studies were conducted in atmospheres of either room air, carbon monoxide, or nitrogen. Gases were delivered through a

[1] Instrumentation Laboratory Inc., Watertown, Mass.

[2] Vacutainer, Becton, Dickinson & Co., Rutherford, N. J.

[3] Microzone, Beckman Instruments Inc., Fullerton, Calif.

TABLE I

Red Cell 2, 3-Diphosphoglycerate Levels in Patients with Cogenital Heart Disease

Subjects	Arterial oxygen tension Pao_2	2, 3-Diphospho-glycerate
	mm Hg	*mμmoles/ml RBC's*
Normals, 1–40 yr (20)	>96*	4082 ±515
Congenital heart disease		
A. Older than 1 month of age		
Cyanotic (32)	<60	6279 ±1181
Noncyanotic (6)	>60	3905 ±387
B. Less than 1 month of age		
Cyanotic (12)	<60	5429 ±1360
Normals, 1–4 wk (8)	>60	4820 ±422

* Assumed and not determined.

fritted glass gas dispersion tube immersed in distilled water. Samples of 1.0 ml were removed periodically from the reaction mixture for the determination of 2,3-diphosphoglycerate. These samples were briefly aerated on a Vortex mixer (Lawrence Pump & Engine Co., Lawrence, Mass.) and then 0.5 ml of 2 N perchloric acid was added to the 1-ml aliquots. The samples were mixed and centrifuged. The precipitate was reextracted with 0.5 ml of 0.5 N perchloric acid, mixed, and recentrifuged. The pooled supernatants were brought to a final volume of 5.0 ml with 0.01 M triethanolamine buffer, pH 7.4. 50-μl aliquots of this extract were then assayed for 2,3-DPG.

RESULTS

Red cell 2,3-diphosphoglycerate levels in congenital heart disease. Patients over 1 month of age with a PaO_2 of less than 60 mm Hg were found to have elevations in their red cell 2,3-DPG levels (Table I, Fig. 1). The mean value for this group of 32 subjects was 6,280 mμmoles of 2,3-DPG/ml of red cells (normal 4080 ±515). The mean red cell 2,3-DPG in the six patients with PaO_2 greater than 60 mm Hg did not differ from that of normals. The 2,3-DPG level was found to be inversely related to the level of arterial oxygen tension. The correlation coefficient was 0.67 ($P < 0.001$).

In the 12 patients less than 1 month of age, the mean 2,3-DPG value was 5430/ml of red cells. Although some infants demonstrated elevated red cell 2,3-DPG levels, there was no significant correlation between the individual values and the arterial oxygen tension (Fig. 1). In this group the correlation coefficient was 0.32 ($P > 0.5$). Unfortunately, fetal hemoglobin concentrations were not measured in all these infants and thus the reason for the variability remains undetermined.

Red cell response to nitrogen in vitro. In 14 experiments in which the fresh red cells from adults were incubated in an atmosphere of carbon monoxide or nitrogen, it was found that glucose consumption was 55.9% higher in the nitrogen atmosphere (Table II). This increase in glycolysis under nitrogen was accompanied by a 41.3% increase in the red cell 2,3-DPG level during the 2 hr of incubation. No significant

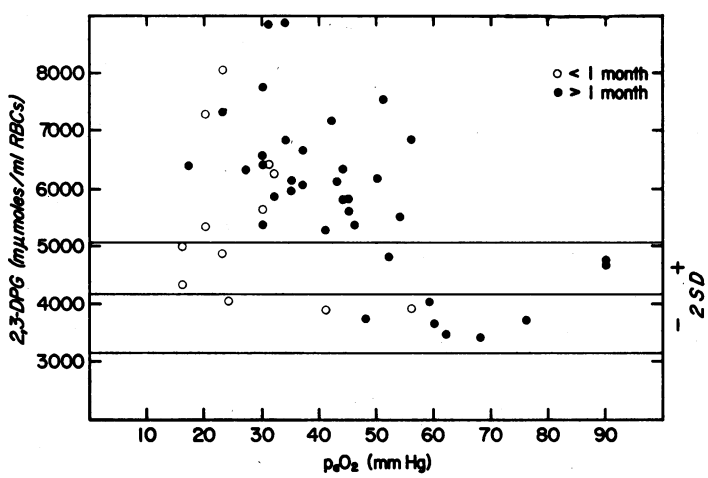

FIGURE 1 The relationship between red cell 2,3-DPG level and arterial oxygen pressure in patients with congenital heart disease. Patients are divided into those younger or older than 1 month of age. The normal mean value of 4082 mμmoles/ml RBC's is indicated by the solid line as is the range encompassing 2 SD on either side of the mean. The correlation coefficient for the relationship of red cell 2,3-DPG to Pao_2 in subjects over 1 month of age is 0.67, $P < 0.001$, while in subjects under 1 month of age the correlation coefficient was 0.32, $P > 0.5$.

TABLE II

Effect of Incubation in Nitrogen or Carbon Monoxide on Red Cell 2, 3-DPG, ATP, and Glucose

Subjects	Glucose consumption		2, 3-DPG			ATP*		
				2 hr			2 hr	
	CO	N	0 time	CO	N	0 time	CO	N
	μmoles/ml per hr		*mμmoles/ml RBC's*			*mμmoles/ml RBC's*		
Normal adults (14)								
Mean (±1 SD)	1.63	2.54	4470	4590	6230	1360	1380	1220
	±0.30	±0.59	±590	±690	±1060	±96	±81	±104
Per cent change (±1 SD)		+55.9		+3.1	+41.3		+1.3	−10.7
		±20.7		±11.7	±30.0		±4.7	±3.6
P		<0.01			<0.01		<0.02;	>0.01
Newborn infants (10)								
Mean (±1 SD)	1.98	3.23	5940	4820	5480	1390	1340	1260
	±0.17	±0.91	±1120	±830	±940	±135	±213	±95
Per cent change (±1 SD)		+63.1		−16.8	−6.8		−4.2	−9.7
		±18.2		±17.3	±11.9		±6.7	±3.0
P		<0.01		<0.2;	>0.1		<0.4;	>0.3

* ATP measurements performed in five adult and three infant experiments.

alteration was observed in the 2,3-DPG levels of cells incubated in carbon monoxide during a similar interval.

Measurement of red cell ATP levels were performed in five studies. A modest decrease in ATP levels was observed during the period of augmented 2,3-DPG synthesis. In these five studies the mean 2,3-DPG level increased by 20.5% while ATP fell by 10.7% (Table II). No significant change was noted in either 2,3-DPG or ATP levels during incubation under carbon monoxide.

In 10 experiments employing the red cells from newborn infants, an increase in the rate of glucose consumption was also noted in the flasks incubated in an atmosphere of nitrogen (Table II). In these experiments the mean 2,3-DPG level declined under nitrogen. The decrease in 2,3-DPG was not as great, however, as that observed in the flasks maintained under carbon monoxide.

In three studies in which ATP levels were also measured, a slight decline in these levels was found in cells incubated under nitrogen despite the lack of a mean increase in 2,3-DPG content.

Effect of adult and fetal hemoglobin on the rate of 2,3-DPG synthesis. In the absence of hemoglobin the rate of 2,3-DPG synthesis was similar in incubations conducted under carbon monoxide or nitrogen. The addition of carboxyhemoglobin (226 mμmoles/ml) had no effect on the generation of 2,3-DPG. When an equal quantity of adult hemoglobin in the deoxy form was added to the incubation system the net synthesis of 2,3-DPG rose from 132 mμmoles/ml to 183 mμmoles/ml during the 2 hr incubation (Fig. 2).

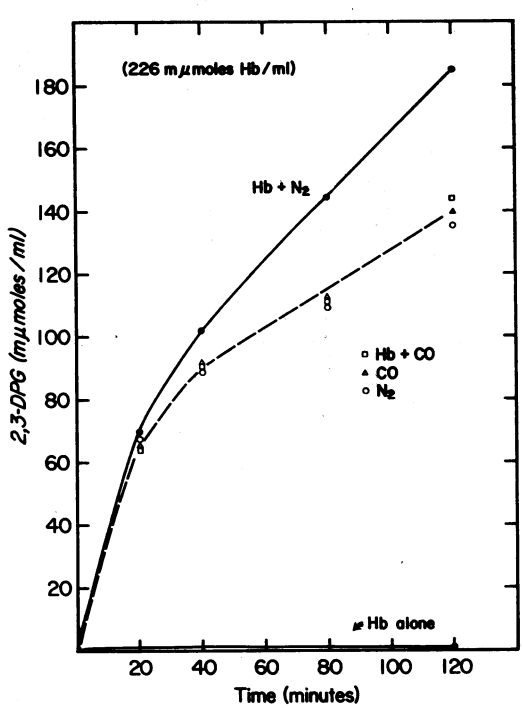

FIGURE 2 Effect of nitrogen (N_2) or carbon monoxide (CO) with and without adult hemoglobin (226 mμmoles/ml of reaction mix) on the rate of 2,3-DPG synthesis. All flasks had no 2,3-DPG at zero time. (See text for additional experimental details.) Incubation in nitrogen alone, carbon monoxide alone, or carbon monoxide plus hemoglobin resulted in similar rates of 2,3-DPG synthesis, while the addition of hemoglobin incubated under nitrogen facilitated 2,3-DPG synthesis.

In an experiment conducted under nitrogen in which the final concentration of adult hemoglobin added was varied from 0 to 512 mμmoles/ml, the net synthesis of 2,3-DPG increased from 23.0 mμmoles/hr per ml reaction mixture in the absence of hemoglobin to 169.0 mμmoles/ml of reaction mixture in the flask containing the highest concentration of deoxyhemoglobin A (Fig. 3). No 2,3-DPG was measurable at zero time indicating that the hemoglobin added in each case was free of 2,3-DPG. Similarly 2,3-DPG was not formed in flasks in which hemoglobin was maintained under nitrogen without enzyme. The hemoglobin solution was therefore enzyme-free and was not itself responsible for the augmentation of the synthetic rates observed. In flasks in which 2,3-DPG was initially present and enzyme alone was added in the absence of cofactors and substrates, the level of 2,3-DPG remained constant. Measurable phosphatase activity was thus absent and was not influencing the synthetic rates observed.

In experiments in which fetal hemoglobin (217 mμmoles/ml) was substituted for adult hemoglobin, the rate of 2,3-DPG synthesis was unaffected by the presence of hemoglobin, either as carboxy or deoxy fetal hemoglobin (Fig. 4).

Studies performed in a room air atmosphere did not differ from those performed under carbon monoxide for either the adult or fetal hemoglobin preparations.

Relationship between red cell 2,3-DPG level and the oxygen-hemoglobin dissociation curve. The P_{50} in nine normal nonsmoking adults was at 26.4 ±1.1 mm Hg (Table III). In 11 subjects with congenital heart disease, hypoxemia, and elevated red cell 2,3-DPG levels, the P_{50} ranged from 29.0 to 32.5 mm Hg. When the data from seven other subjects with inherited disorders of red cell metabolism having wide variations in their 2,3-DPG and P_{50}'s were tabulated and included in the formulation, a regression line having a slope of 430

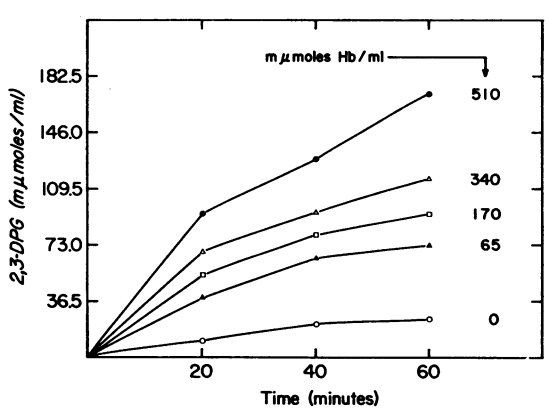

FIGURE 3 Effect of increasing concentrations of adult deoxy hemoglobin on the rate of 2,3-diphosphoglycerate synthesis.

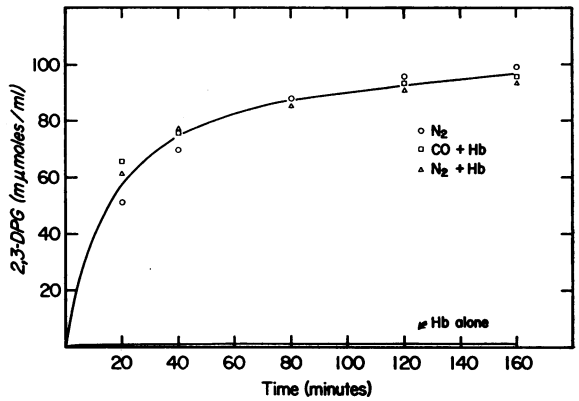

FIGURE 4 Effect of nitrogen, deoxy fetal hemoglobin, and carboxy fetal hemoglobin on the rate of 2,3-diphosphoglycerate synthesis. Fetal hemoglobin in a concentration of 217 mμmoles/ml was employed.

mμmoles of 2,3-DPG/mm Hg P_{O_2} was derived. The correlation between P_{50} and red cell 2,3-DPG in this group was 0.97 ($P < 0.001$).

DISCUSSION

The observation that organic phosphates influence the oxygen-hemoglobin dissociation curve has led to both a reexamination of previously puzzling physiologic observations and to an intense search for its clinical ramifications. Although it has been recognized since 1939 that 2,3-DPG is present in large quantitites within the human erythrocyte (16), its precise role in red cell metabolism has escaped elucidation. Equally puzzling has been the problem of how the red cell maintains such high levels of this compound when 2,3-diphosphoglycerate mutase, the enzyme responsible for its synthesis, is so sensitive to inhibition by its product, 2,3-DPG (4, 17, 18). The finding by Benesch, Benesch, and Yu (3) that deoxyhemoglobin preferentially binds 2,3-DPG immediately offered an explanation for these problems. When the cell is deoxygenated, 2,3-DPG is bound to deoxyhemoglobin, product inhibition is removed, and further 2,3-DPG should be synthesized. Studies reported in this paper confirm this thesis. When the red cells from normal adults are incubated under nitrogen a prompt increase in red cell 2,3-DPG occurs. When 2,3-DPG–free adult hemoglobin is added to a 2,3-DPG generating system, the synthesis of this organic phosphate is augmented in the presence of deoxyhemoglobin but not in the presence of liganded hemoglobin, be it carboxyhemoglobin or oxyhemoglobin. Asakura, Sato, Minakami, and Yoshikawa (19) first observed that while the rate of red cell glycolysis and the rate of 2,3-DPG synthesis was the same for human red cells incubated in either an oxygen or carbon monoxide atmosphere they both increased significantly in incuba-

TABLE III

Relationship of Red Cell 2, 3-DPG to Whole Blood Oxygen Tension Required for 50% Saturation of Hemoglobin (P_{50})

Subjects	Pao_2	pH	P_{50}	2, 3-DPG
	mm Hg		*mm Hg*	*mμmoles/ml RBC's*
Normals (9)	>96	7.41 ±0.12	26.4 +1.1	4102 ±476
Congenital heart disease				
P.	30	7.37	32.0	7792
Sn.	42	7.50	31.5	7173
Sm.	41	7.37	29.0	5278
M.	56	7.36	31.0	6875
W.	34	7.29	32.5	6857
R.	45	7.37	29.0	5817
E.	30	7.30	30.5	6415
St.	37	7.27	31.5	6076
We.	34	7.35	29.5	5983
F.	26	7.10	29.0	5579
S.	—	7.45	31.5	6925
Pyruvate kinase deficiency	—	7.42	36.5	8750
	—	7.40	38.0	10677
	—	7.42	40.5	11458
	—	7.42	38.0	10270
Glucose-6-phosphate dehydrogenase deficiency	—	7.43	26.0	4410
Hexokinase deficiency	—	7.41	19.0	2740
Glucose-phosphate isomerase deficiency	—	7.39	23.0	3810

tion performed under nitrogen. Our findings in the red cels of normal adults confirm those observations.

The regulatory role of deoxyhemoglobin A on 2,3-DPG synthesis is in sharp contrast to the lack of effect of fetal hemoglobin under similar experimental conditions. Our studies demonstrate that the red cells from newborn infants, despite the fact they contained only approximately 70% fetal hemoglobin, do not increase their 2,3-DPG levels when incubated under nitrogen and, furthermore, that neither deoxy nor liganded fetal hemoglobin can be shown to stimulate 2,3-DPG synthesis in a red cell–free system.

Employing different techniques, Bauer, Ludwig, and Ludwig (20) and Tyuma and Schimizu (21) have also found that the reaction of the organic phosphates, adenosine triphosphate and 2,3-diphosphoglycerate, with fetal hemoglobin differs from the reaction of these organic phosphate compounds with adult hemoglobin. These compounds have little effect on altering the oxygen affinity of fetal hemoglobin while their effect on the oxygen affinity of adult hemoglobin is profound. These observations help to explain the lack of correlation between hypoxemia and red cell 2,3-DPG observed in many of the infants. Apparently the affinity of 2,3-DPG for deoxy fetal hemoglobin is insufficient to facilitate the generation of additional 2,3-DPG.

Significant reversible conformational changes occur in the hemoglobin molecule upon deoxygenation (22). These changes have been thought to explain the higher affinity of 2,3-DPG for human adult hemoglobin in the unliganded as opposed to the liganded conformation. 1 mole of deoxyhemoglobin tetramer appears to bind 1 mole of 2,3-DPG (3). The central cavity of the hemoglobin has been suggested as the site of binding (23). The histidine residue at the 143rd position in the beta chain (21st position in the H helix) has also been suggested as an alternative binding site for 2,3-DPG (24). It is noted that the gamma chain of fetal hemoglobin contains an uncharged serine residue at this position rather than the basically charged histidine of the beta chain. Both proposed sites appear to be more accessible in the deoxy conformation.

Several other important problems also remain. If the inhibition constant (K_i) for diphosphoglycerate mutase is 0.85 μM (4), it is difficult to understand how the cell is capable of synthesizing any 2,3-DPG in the absence of a compound or compounds capable of either binding the product and thus relieving inhibition, or altering the K_i within the cell. Since the content of 2,3-DPG in the erythrocytes of the newborn infant is very similar to that of normal adults (25, 26), although less than what would be expected from cells of a similar young mean age (27), it would appear that other factors, presently undefined, must operate in the absence of significant hemoglobin binding to facilitate 2,3-DPG synthesis.

The erythrocytes of both infants and adults demonstate an increase in glycolysis under nitrogen. This effect cannot be related to changes in red cell 2,3-DPG levels. The presence of other major intracellular alterations associated with deoxygenation is suggested.

The fall in ATP observed in association with the rise in 2,3-DPG levels in the adult cells incubated under nitrogen appears to be a consequence of the flow of 1,3-diphosphoglycerate through the diphosphoglycerate mutase pathway rather than through the phosphoglycerate kinase (PGK) step to lactic acid. By circumventing the phosphoglycerate kinase in this manner a decrease in ATP regeneration would result. A marked increase in PGK activity is found in the red cells of the newborn infant (*27*). As a result, when associated with elevated adenosine diphosphate levels, increased glycolysis by way of PGK rather than through the diphosphoglycerate cycle might occur. Under these circumstances the 2,3-DPG instability observed in these cells in the present study and previously by Schroter and Winter (25) would be explicable. The decrease in ATP levels noted in these experiments has been previously described (*27*) but not adequately explained.

The studies reported here together with the studies relating changes in 2,3-DPG levels to the P_{50} of blood in such diverse situations as anemia with and without intrinsic defects in red cell glycolysis (28, 29), altitude change (5, 30), and blood storage (31, 32), as well as the demonstrated ability of added 2,3-DPG to correct the differences between the oxygen affinity of whole blood and hemolysate (3) establish the important role of the organic phosphates in the regulation of oxygen release to the tissues. Throughout the physiologic range (2000–10,000 mμmoles of DPG/ml of RBC's), change in oxygen affinity as measured by the P_{50} was found to be linear with respect to the 2,3-DPG concentration; 430 mμmoles of 2,3-DPG changing the P_{50} by 1 mm Hg. These data were derived from subjects over 3 months of age.

It is now apparent that the position of the oxygen-hemoglobin dissociation curve is not only influenced by changes in pH, P_{CO_2}, and temperature, but also by the organic phosphate compounds within the red cell. The interrelationships that exist between these factors and those which regulate the synthesis of 2,3-DPG in the cells of the adult and newborn require further study.

ACKNOWLEDGMENTS

We wish to express our appreciation to Mrs. Tina Brigandi, Miss Patricia O'Neal, and Miss Natalie Hutchins for their valuable technical assistance, to Mrs. Judith Clark for her secretarial aid, and to Dr. William J. Williams for his review of the manuscript.

This work was supported in part by U. S. Public Health Service Grants HD 01919, AM 12255, and AM 11984.

REFERENCES

1. Benesch, R., and R. E. Benesch. 1967. The effect of organic phosphates from the human erythrocyte on the allosteric properties of hemoglobin. *Biochem. Biophys. Res. Commun.* **26**: 162.
2. Chanutin, A., and R. R. Curnish. 1967. Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. *Arch. Biochem. Biophys.* **121**: 96.
3. Benesch, R., R. E. Benesch, and C. I. Yu. 1968. Reciprocal binding of oxygen and diphosphoglycerate by human hemoglobin. *Proc. Natl. Acad. Sci. U. S. A.* **59**: 526.
4. Rose, Z. B. 1968. The purification and properties of diphosphoglycerate mutase from human erythrocytes. *J. Biol. Chem.* **243**: 4810.
5. Lenfant, C., J. Torrance, E. English, C. A. Finch, C. Reynafarje, J. Ramos, and J. Faura. 1968. Effect of altitude on oxygen binding by hemoglobin and on organic phosphate levels. *J. Clin. Invest.* **47**: 2652.
6. Oski, F. A., A. J. Gottlieb, M. Delivoria-Papadopoulos, and W. W. Miller. 1969. Red cell 2,3-diphosphoglycerate levels in subjects with chronic hypoxemia. *N. Engl. J. Med.* **280**: 1165.
7. Schröter, W., and H. Heyden. 1965. Kinetik des 2,3-diphosphoglyceratumsatzes in menschlichen erythrocyten. *Biochem. Z.* **341**: 387.
8. Krimsky, I. 1963. D-2,3-diphosphoglycerate. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 1st edition. 238–240.
9. Bartlett, G. R. 1959. Methods for the isolation of glycolytic intermediates by column chromatography with ion exchange resins. *J. Biol. Chem.* **234**: 459.
10. Severinghaus, J. W., and A. F. Bradley. 1958. Electrodes for blood pO_2 determination and pCO_2 determination. *J. Appl. Physiol.* **13**: 515.
11. Nahas, G. G. 1958. A simplified lucite cuvette for the spectrophotometric measurement of hemoglobin and oxyhemoglobin. *J. Appl. Physiol.* **13**: 147.
12. Huggett, A. S. G., and D. A. Nixon. 1957. Use of glucose oxidase, peroxidase, and *o*-dianisidine in the determination of blood and urinary glucose. *Lancet.* **2**: 368.
13. Lamprecht, W., and I. Trautschold. 1963. Determination with hexokinase and glucose-6-phosphate dehydrogenase. *In* Methods of Enzymatic Analysis. H. U. Bergmeyer, editor. Academic Press, Inc., New York. 543–545.
14. Allen, D. W., W. A. Schroeder, and J. Balog. 1958. Observations on the chromatographic heterogeneity of normal adult and fetal human hemoglobin: a study of the effects of crystallization and chromatography on the heterogeneity and isoleucine content. *J. Amer. Chem. Soc.* **80**: 1628.
15. Singer, K., A. I. Chernoff, and L. Singer. 1951. Studies on abnormal hemoglobins. I. Their demonstration in sickle cell anemia and other hematologic disorders by means of alkali denaturation. *Blood.* **6**: 413.
16. Guest, G. M., and S. Rapoport. 1939. Role of acid-soluble phosphorus compounds in red blood cells in experimental rickets, renal insufficiency, pyloric obstruction, gastroenteritis, ammonium chloride acidosis, and diabetic acidosis. *Amer. J. Dis. Child.* **58**: 1072.
17. Rapoport, S., and J. Luebering. 1952. An optical study of diphosphoglycerate mutase. *J. Biol. Chem.* **196**: 583.
18. Joyce, B. K., and S. Grisolia. 1959. The purification and properties of muscle diphosphoglycerate mutase. *J. Biol. Chem.* **234**: 1330.

19. Asakura, T., Y. Sato, S. Minakami, and H. Yoshiskawa. 1966. Effect of deoxygenation of intracellular hemoglobin on red cell glycolysis. *J. Biochem.* (*Tokyo*). **59**: 524.
20. Bauer, C., I. Ludwig, and M. Ludwig. 1968. Different effects of 2,3-diphosphoglycerate and adenosine triphosphate on the oxygen affinity of adult and foetal human hemoglobin. *Life Sci.* 7(Pt. I) : 1339.
21. Tyuma, I., and K. Shimizu. 1969. Different response to organic phosphates of human fetal and adult hemoglobins. *Arch. Biochem. Biophys.* **129**: 404.
22. Perutz, M. F., and H. Lehmann. 1969. Molecular pathology of human haemoglobin. *Nature* (*London*). **219**: 902.
23. Benesch, R., and R. E. Benesch. 1969. Intracellular organic phosphates as regulators of oxygen release by haemoglobin. *Nature* (*London*). **221**: 618.
24. deVerdier, C. H., and L. Garby. 1969. Low binding of 2,3-diphosphoglycerate to Haemoglobin F. *Scand. J. Clin. Lab. Invest.* 23: 149.
25. Schroter, W., and P. Winter. 1967. Der 2,3-diphsphoglyceratstoffwechsel in den erythrocyten neugeborener and erwachsener. *Klin. Wochenschr.* **45**: 255.
26. Zipursky, A., T. LaRue, L. G. Israels, and A. Fairbairn. 1960. The *in vitro* metabolism of erythrocytes from newborn infants. *Can. J. Biochem.* **38**: 727.
27. Oski, F. A. 1969. Red cell metabolism in the newborn infant. V. Glycolytic intermediates and glycolytic enzymes. *Pediatrics.* **44**: 84.
28. Mourdjinis, A., C. Walters, M. J. Edwards, R. D. Koler, B. Vanderheiden, and J. Metcalfe. 1969. Improved oxygen delivery in pyruvate kinase deficiency. *Clin. Res.* **17**: 153.
29. Delivoria-Papadopoulos, M., F. A. Oski, and A. J. Gottlieb. 1969. Oxygen-hemoglobin dissociation curves: effect of inherited enzyme defects of the red cell. *Science* (*Washington*). **165**: 601.
30. Eaton, J. W., G. J. Brewer, and R. F. Grover. 1969. Role of red cell 2,3-diphosphoglycerate in the adaptation of man to altitude. *J. Lab. Clin. Med.* **73**: 603.
31. Bunn, H. F., M. H. May, W. F. Kocholaty, and C E. Shields. 1969. Hemoglobin function in stored blood. *J. Clin. Invest.* **48**: 311.
32. Åkerblom, O., C. H. deVerdier, L. Garby, and C. Högman. 1968. Restoration of defective oxygen-transport function of stored red blood cells by addition of inosine. *Scand. J. Clin. Lab. Invest.* 21: 245.