

## INTRAERYTHROCYTIC ADAPTATION TO ANEMIA\*

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**Abstract** The role of erythrocyte 2,3-diphosphoglycerate (2,3-DPG) in increasing the availability of hemoglobin oxygen in anemia was investigated. Measurements of 2,3-DPG and of oxygen dissociation ( $P_{50}$ ) were carried out on 57 normal subjects and 114 subjects with anemia. Twenty normal non-smoking males had a mean hemoglobin of 15.3 g per 100 ml, a mean DPG of 4.83 mM and a mean  $P_{50}$  of 27.1 mm of mercury. Twenty normal non-smoking females had a mean hemoglobin lower by 2.6 g per 100 ml, a DPG higher by 0.5 mM and a  $P_{50}$  increased by 0.4 mm of mercury DPG.  $P_{50}$  rose

progressively with decreasing hemoglobin concentrations. For each gram of hemoglobin fall, there was a DPG increase of about 0.23 mM and a  $P_{50}$  increase of about 0.30 mm of mercury. Increases in adenosine triphosphate also occurred but, because of the smaller amount involved, had less effect on the oxygen dissociation curve. A rise in inorganic phosphate level had no demonstrable effect, but in vivo pH changes appear of considerable importance. It was calculated that DPG-induced changes in hemoglobin affinity for oxygen may compensate for up to half the oxygen deficit in anemia.

**A**NEMIA, by lowering the oxygen-carrying capacity of the blood, reduces the supply of oxygen to tissues. However, the fact that symptoms resulting from anemia are often minimal or absent suggests the existence of mechanisms that compensate for the decrease in arterial blood oxygen content. One of these mechanisms involves a reduced affinity of hemoglobin for oxygen, mediated by an increase in red-cell 2,3-diphosphoglycerate (2,3-DPG).<sup>1,2</sup> The role of this mechanism in the adaptation to anemia is the topic of this report.

## METHODS

A total of 171 subjects were studied. 119 at sea level in Seattle, Washington, and 52 at 1550 meters in Medellin, Colombia. Medellin subjects were studied because local ecological conditions led to more severe anemia than in the North American patients; Medellin subjects consisted of seven normal males, 22 anemic males and 23 anemic females. The Seattle subjects consisted of 69 subjects with and 50 without anemia, the latter including 20 nonsmoking females and 30 male subjects — 20 nonsmokers and 10 who smoked 10 or more cigarettes daily. In all the anemic patients, the red-cell production rate was below twice the normal level as judged by the reticulocyte index. Of the Seattle patients with anemia, two subgroups of 20 each, all with hemoglobins less than 10 g per 100 ml, were selected for further comparison. One group had hypoproliferative anemia without renal disease and the other had chronic renal disease, all but two patients being under dialysis therapy.

Venous blood was collected and transferred into

vacutubes containing 1 mg of heparin per milliliter of blood. In patients undergoing hemodialysis, samples were obtained immediately before dialysis. Hemoglobin concentration was determined as cyanmethemoglobin. A microhematocrit was read from capillary tubes after centrifugation on the Clay-Adams autocrit for 15 minutes at  $14,490 \times g$ . Reticulocytes were counted as the percentage of 2000 red cells. For estimates of the rate of erythropoiesis, the reticulocyte count was corrected on the basis of red-cell count and bone-marrow maturation time,<sup>4</sup> except that in patients with renal disease, a normal maturation time was assumed.

## Abbreviations Used

ATP:	adenosine triphosphate
2,3-DPG:	2,3-diphosphoglycerate
$O_2$ -Hb:	oxygen hemoglobin
$P_{50}$ :	oxygen dissociation
$P_{O_2}$ :	oxygen tension
TCA:	trichloroacetic acid

Red-cell extraction for phosphate partition was carried out within one hour and usually within 20 minutes after bleeding. For this purpose, 5 ml of ice-cold 20 per cent trichloroacetic acid (TCA) was added to 2 ml of whole blood, and after centrifugation, 5 ml of supernatant fluid was taken for analysis. The TCA was removed by five extractions with 2 vol of diethyl ether, and the excess ether was evaporated with a stream of nitrogen; the extracts were then stored at  $-20^\circ\text{C}$ . A modification of the procedure described by Robinson, Loder and de Gruchy<sup>5</sup> was used for separation of the extract into various fractions. The extract was diluted to twice its volume with distilled water and adjusted to a pH of 7 to 11 with dilute ammonia. The solution was then washed into a column of Dowex 1  $\times$  8, 1 by 5 cm. The stepwise solutions shown in Table 1 were used to obtain their respective fractions. The total phosphate content of each fraction was determined by the method of Bartlett<sup>6</sup> and gave values of DPG

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Table 1. Separation of Extract into Fractions.

FRACTION	ELUANT	FRACTION CONTENT
1	50 ml of water & then 50 ml of 0.2% hydrochloric acid	Inorganic phosphate from plasma & red cells, hexose monophosphate, adenosine monophosphate, reduced nicotine adenine dinucleotide & monophosphoglycerate
2	100 ml of 0.1 N ammonium chloride	Hexose diphosphate & adenosine diphosphate
3	200 ml of 0.2 N ammonium chloride	2,3-DPG
4	200 ml of 0.5 N ammonium chloride	ATP
5	100 ml of N hydrochloric acid	Unidentified phosphorus compounds

and adenosine triphosphate (ATP) similar to those reported by him.\*

The oxygen-hemoglobin ( $O_2$ -Hb) dissociation curve was determined by the mixing technic,\* in which oxygen saturation is predetermined by mixture of known amounts of fully oxygenated and fully

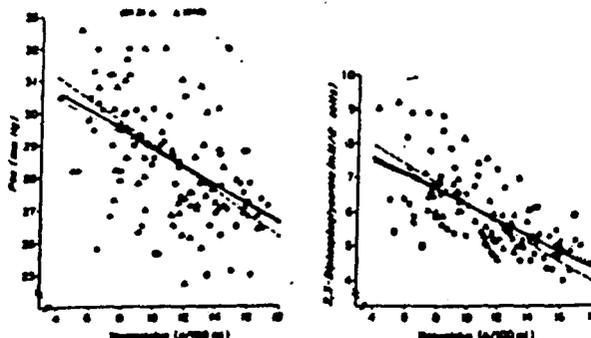


Figure 1. Relation between Hemoglobin Level and Affinity for Oxygen ( $P_{50}$ ) (A) and between Hemoglobin Level and 2,3-DPG (B), in Seattle Subjects.

In this and in Figures 2 and 3 the triangles represent females, and the open circles males. The solid regression line is for the entire group, the dotted line for males, and the dashed line for females.

DPG was higher by 0.5 mM, and the  $P_{50}$  was increased by 0.4 mm of mercury. Ten smoking males showed a slight but insignificant increase in hemo-

Table 2. Changes in Red-Cell Organic Phosphate Compounds in Normal and Anemic Subjects.

SUBJECTS	No. OF SUBJECTS STUDIED	HEMOGLOBIN*	DPG*	ATP*	$P_{50}$ *	FRACTION 2*	FRACTION 5*
		g/100 ml	mM/liter of cells	mM/liter of cells	mm Hg	mM/liter of cells	mM/liter of cells
<b>Seattle:</b>							
Normal subjects:							
Male nonsmokers	20	15.32 ± 1.02	4.83 ± 0.33	0.87 ± 0.20	27.1 ± 0.8	1.12 ± 0.17	1.26 ± 0.26
Female nonsmokers	20	12.68 ± 0.96	5.28 ± 0.40	0.80 ± 0.17	27.5 ± 0.9	1.15 ± 0.16	1.22 ± 0.14
Male smokers	10	15.50 ± 0.94	5.14 ± 0.59	0.89 ± 0.19	27.0 ± 1.3	1.30 ± 0.10	1.33 ± 0.24
Anemic patients:							
With uremia	20	7.62 ± 1.50	6.62 ± 0.86	1.68 ± 0.40	29.1 ± 1.6	1.75 ± 0.53	2.06 ± 0.83
Without uremia	20	7.88 ± 1.38	6.77 ± 1.19	1.48 ± 0.45	30.2 ± 1.9	1.79 ± 0.71	1.95 ± 0.82
<b>Medellin:</b>							
Normal males							
Normal males	7	16.04 ± 1.18	5.48 ± 0.27	0.94 ± 0.09	26.5 ± 0.2	1.13 ± 0.13	0.96 ± 0.12
Anemic patients:							
Males	22	8.33 ± 2.35	7.45 ± 1.22	1.32 ± 0.37	28.9 ± 1.4	1.59 ± 0.46	1.37 ± 0.35
Females	23	7.04 ± 2.81	7.45 ± 1.64	1.32 ± 0.45	29.2 ± 2.3	1.64 ± 0.39	1.52 ± 0.56

\* ± 1 SD.

deoxygenated blood and the oxygen tension ( $P_{O_2}$ ) of the mixture is directly measured by means of a  $P_{O_2}$  electrode. Hemoglobin affinity for oxygen taken from the  $O_2$ -Hb dissociation curve at pH 7.4 was expressed as the  $P_{50}$  (7.4) — that is,  $P_{O_2}$  in millimeters of mercury required for 50 per cent saturation of the blood with oxygen at pH = 7.4.

### RESULTS

The 20 nonsmoking normal males at sea level had a mean hemoglobin of 15.32 g per 100 ml, a mean DPG of 4.83 mM and a mean  $P_{50}$  of 27.1 mm of mercury (Table 2). In 20 nonsmoking females the mean hemoglobin was lower by 2.6 g per 100 ml,

\*Whole blood rather than washed red cells was extracted because the previously used washing procedure\* caused a decrease in the recovery of DPG and ATP, and plasma does not contain organic phosphates.

globin and DPG as compared to nonsmoking males. The relation between hemoglobin level and  $P_{50}$  (Fig. 1A) and between hemoglobin level and DPG (Fig. 1B) was plotted for the Seattle subjects. The same relation for Medellin subjects is shown in Figures 2A and B. In both groups of subjects there was a progressive increase in  $P_{50}$  and DPG as the hemoglobin level fell. In the renal and nonrenal subgroups composed of Seattle subjects with a hemoglobin of less than 10 g per 100 ml, there were similar mean hemoglobin levels, similar elevations in red-cell  $P_{50}$  and organic phosphate content (Table 2). As shown in Figures 3A and 3B, there was a close relation between red-cell DPG and  $P_{50}$  in both Seattle and Medellin subjects.

Anemia was associated with a rise in red-cell organic phosphates other than DPG as indicated by the regression equations relating the various

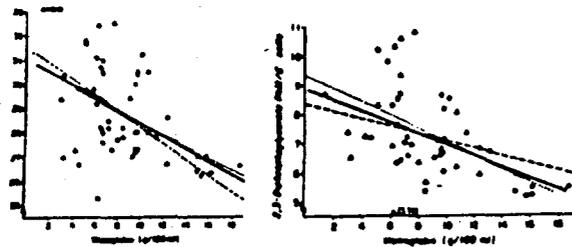


Figure 2. Data in Medellin Subjects.

phosphate-containing fractions to hemoglobin concentration (Table 3). All fractions showed a significant correlation ( $p$  less than 0.001) with  $P_{50}$ . More than 80 per cent of the increased phosphate in the four fractions listed was accounted for by DPG and ATP. Although the percentage increase in red-cell ATP was actually greater than that of DPG, the ATP rise on a molar basis was less than a third of the DPG increase.

The possible relation between arterial pH and changes in red-cell organic phosphate level was evaluated in the Medellin female patients. Any change in pH values in these patients was incidental since they had been selected on the basis of anemia alone. That a relation existed between pH and 2,3-DPG is shown in Figure 4A. In view of this, an attempt was made to show the effect of pH alone by correction of each DPG level for the expected change related to anemia (Fig. 4B). A similar correction is made in the  $P_{50}$  level for expected changes related to anemia (Fig. 4C). It is seen that

when the pH is 7.4, the changes in DPG and  $P_{50}$  are those expected from the degree of anemia; however, with acidosis, the changes in  $P_{50}$  and DPG failed to reach the level predicted whereas with alkalosis, the level of  $P_{50}$  and DPG exceeds the level predicted from anemia alone.

### DISCUSSION

There is convincing evidence that organic phosphate modifies the affinity of hemoglobin for oxy-

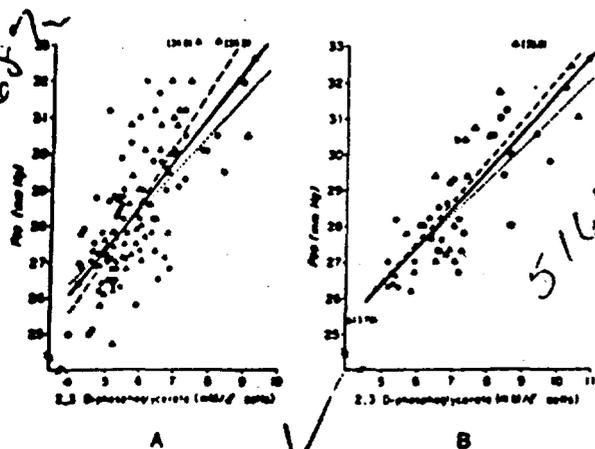


Figure 3. Relation between  $P_{50}$  and 2,3-DPG in Seattle Subjects (A) and Medellin Subjects (B).

Table 3. Regression Equations and Correlation Coefficients for All 171 Subjects Studied.

REGRESSION EQUATION	STANDARD ERROR OF ESTIMATE	CORRELATION COEFFICIENT*
Fraction 3 (DPG) = $-0.244 \text{ hemoglobin} + 8.88$	$\pm 1.03$	$-0.654$
Fraction 4 (ATP) = $-0.066 \text{ hemoglobin} + 1.92$	$\pm 0.37$	$-0.541$
Fraction 2 = $-0.072 \text{ hemoglobin} + 2.24$	$\pm 0.39$	$-0.555$
Fraction 5 = $-0.071 \text{ hemoglobin} + 2.29$	$\pm 0.56$	$-0.418$

\*  $p < 0.001$ .

gen.<sup>1,2</sup> This effect is evident when the  $P_{50}$  curve of hemoglobin in red-cell hemolysates is compared to that of hemoglobin stripped of its organic phosphates,<sup>9</sup> and in the  $P_{50}$  measurements on the organophosphate-depleted red cells of stored blood in vitro.<sup>10</sup> Of more physiologic importance is the demonstration that organic phosphates increase in vivo in response to hypoxia and that this rise is associated with a heightened availability of oxygen to tissues. A fall in blood oxygen content due to decreased oxygen loading at high altitudes was shown to be associated with a corresponding rise in both DPG and  $P_{50}$ .<sup>7,11</sup> In patients with cardiac disease associated with decreased cardiac output, compensatory increases in both DPG and  $P_{50}$  have been demonstrated.<sup>12</sup> The regulation of red-cell DPG content has thus come to be recognized as an important adaptive mechanism to combat tissue hypoxia.<sup>13,14</sup>

In the present study the effect of decreased concentrations of hemoglobin on DPG and  $P_{50}$  was examined over a wide range of hemoglobin concentration. The two groups investigated were considered separately, not because of the altitude differences, which had little effect, but because the determinations, although carried out by the same investigators, were under somewhat different laboratory conditions. Normal men and women provided a base line for comparison with anemic subjects. A higher mean DPG was found in females as compared with males in accord with two previous groups of subjects studied,<sup>15,16</sup> but at variance with one other.<sup>16</sup> Since the hemoglobin level in women



Figure 4. Effect of pH in Medellin Females.

In A DPG level is related to in vivo pH. In B the difference between actual DPG and DPG level predicted on the basis of the subject's hemoglobin concentration is related to in vivo pH.

In C the difference between actual  $P_{50}$  and that predicted from the DPG level is related to in vivo pH.

is lower than that in men, the reciprocal increase in DPG appeared consistent with the effect observed in subjects with greater degrees of anemia. The regression curves relating hemoglobin to DPG or  $P_{50}$  were similar in male and female anemic subjects.

Anemia is well known to be associated with a decreased affinity of hemoglobin for oxygen.<sup>17-21</sup> These increases are confirmed in the present study, and their interrelations with changes in red-cell organic phosphates are demonstrated. Thus, in the Seattle patients, for every gram per 100 ml of hemoglobin decrease, there is a DPG increase of about 0.23 mM and a  $P_{50}$  increase of about 0.30 mm of mercury. For each millimole increase in DPG, there was a corresponding rise of about 1.25 mm of mercury in the  $P_{50}$ . ATP was also increased, but on a molar basis was about a third of the DPG increase. Other organic phosphates in the red cell also increase with anemia, but since these increases are much smaller than those of DPG or ATP (Table 2), they probably have little effect in modifying hemoglobin affinity for oxygen.

The rise of these glycolytic intermediates is consistent with the fact that red-cell glycolysis is stimulated by deoxygenation.<sup>22-24</sup> It has been suggested that the Luebering-Rapoport shunt is an alternate to the conversion of 1,3-DPG to 3-phosphoglycerate and that reciprocal changes may occur between the levels of ATP and DPG.<sup>24-26</sup> However, our studies of normal subjects at high altitude<sup>7</sup> and of patients in cardiac failure<sup>24</sup> and the current report of people with anemia all demonstrate increases in both compounds and thus reflect an overall stimulation of glycolysis. This stimulation may be the result of a rise in deoxygenated hemoglobin, which, by binding more organic phosphate than oxyhemoglobin, lowers the free organic phosphate in the cell; alternately, it has been suggested that intracellular pH changes may affect the activity of red-cell enzymes and alter the DPG level.<sup>27</sup> However, there can be little doubt that when there is a rise in the relative amount of deoxygenated hemoglobin in venous blood, there is also a rise in red-cell organic phosphates.

Extracellular inorganic phosphate levels may also have a role in regulating red-cell glycolysis and thus in controlling the level of intermediate compounds such as ATP.<sup>28</sup> This effect was not demonstrable in the 20 patients with renal anemia (Table 2), in which, despite an average increase in plasma inorganic phosphate to 7.4 mM, no significant difference was found in ATP or DPG levels as compared to a similar group of patients with non-renal regenerative anemia whose plasma inorganic phosphate levels were normal.

Undoubtedly, many variables influence the correlation between hemoglobin and DPG or  $P_{50}$ . Smoking, which is known to affect the  $P_{50}$  curve, was examined in a group of otherwise normal subjects (Table 2). Since the mean values for DPG and

$P_{50}$  in these smokers did not differ significantly from those of the normal subjects, smoking was not taken into consideration in the study of anemic subjects. Another possible cause of variation is the age of the red-cell population. Edwards and Rigas<sup>29</sup> found that young cells separated by centrifugation had a  $P_{50}$  of 33.5 mm of mercury as compared with 27 mm in older cells. Because of this observation, subjects with hemolytic anemia as defined by a reticulocyte output of more than twice basal values were excluded from the present study. Likewise, patients with enzyme and hemoglobin abnormalities associated with altered  $P_{50}$  were excluded.

Changes in pH are known to affect the  $P_{50}$  curve, a shift toward the right accompanying acidosis, and one toward the left accompanying alkalosis (the Bohr effect). Previous workers have also shown that severe acidosis causes a decrease, and alkalosis an increase in DPG.<sup>30</sup> Studies demonstrated such effects in patients in whom pH changes had not been suspected on clinical grounds (Fig. 4). Even though the response to pH change occurs almost instantaneously whereas that to DPG has a half-time of about six hours, the degree of correlation observed in this study is impressive and supports the fact that DPG concentration is subject to the combined effect of hemoglobin concentration and in vivo pH. The regulating effect of DPG is thought to be the prime mechanism for modifying hemoglobin affinity in adjusting oxygen supply to tissue needs.

The importance of a shift in  $O_2$ -Hb dissociation curve in determining  $PO_2$  tension in the tissue capillaries is shown in Figure 5. For instance, a subject with a hemoglobin concentration of 7.5 g per 100

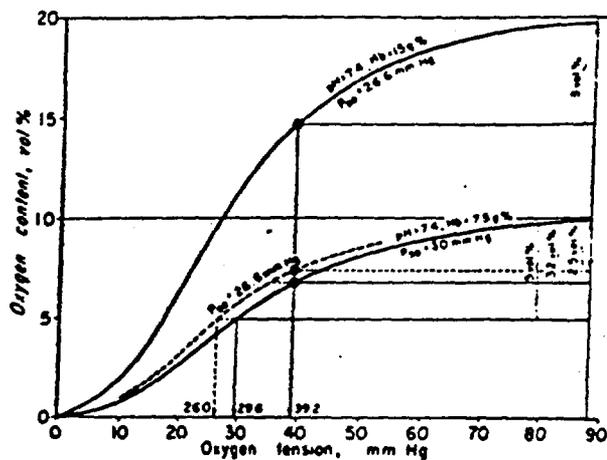


Figure 5. Effects of Anemia on  $P_{50}$ .

The normal curve is shown at the top, and the anemic curves below. The dashed line is the unshifted curve, and the solid line indicates the changes in affinity for oxygen observed. If venous oxygen remains at 39.2 mm of mercury, the shift in the curve permits an increased oxygen extraction from 2.5 to 3.5 vol per cent. If the required 5 vol per cent were extracted, oxygen tension would be at 29.6 vol per cent.

ml and an unshifted curve would release only 2.5 vol per cent of oxygen to maintain a normal end-tissue capillary  $P_{O_2}$  of 39 mm of mercury. A shifted curve, as observed in these studies, yields an oxygen release of 3.2 vol per cent. A normal release of 5 vol per cent either requires an increase in cardiac output of 55 per cent above normal or a lowering in end-tissue capillary  $P_{O_2}$  to 29.6 mm of mercury. In the absence of a shift, the release of 5 vol per cent of oxygen would necessitate doubling the cardiac output or lowering further  $P_{vO_2}$  to 26 mm of mercury. Thus, a shift of the  $O_2$ -Hb dissociation curve to the right explains the relatively slight increase in cardiac output and decrease in mixed venous  $P_{O_2}$  observed in anemic patients<sup>21</sup> as well as the minimal symptomatology of moderate anemia.

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