*RECIPROCAL BINDING OF OXYGEN AND DIPHOSPHOGLYCERATE BY HUMAN HEMOGLOBIN**

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We have reported previously that organic phosphates have a profound effect on the reaction of hemoglobin with oxygen.[1] Organic polyphosphates such as 2,3-diphosphoglycerate (DPG) and adenosine triphosphate (ATP) in the millimolar concentrations at which they occur in the red cell can decrease the oxygen affinity of hemoglobin about 30-fold. These compounds therefore greatly facilitate the unloading of oxygen from hemoglobin. Some of these findings have recently been confirmed by Chanutin and Curnish.[2]

The displacement of oxygen from hemoglobin by DPG strongly suggested that the affinity of this compound for the deoxy form of hemoglobin is greater than for the oxy form. Direct measurements of the binding of DPG by oxy- and deoxyhemoglobin, described below, confirm this assumption. It will be shown that the difference in affinity can account for the observed displacement of the oxygen dissociation curve in the presence of DPG.

Preparation of Hemoglobin.—The method of Drabkin,[3] previously used by us and other workers, results in preparations which contain variable amounts of organic phosphate. A representative example of the DPG content at various stages of this procedure is shown in Table 1. It can be seen that the final preparation contains about 0.5 mole DPG per mole of hemoglobin. The presence of 0.6 atom of P per mole of human hemoglobin, also prepared by Drabkin's procedure, was already noted by Havinga.[4] Since DPG is a highly charged molecule (almost four of the five acid groups are ionized at neutral pH), it can only be displaced from the protein in the presence of sufficient concentrations of electrolyte.

The following procedure was finally adopted: Blood collected in neutral citrate (4 ml of 3.2% sodium citrate per 20 ml blood) was washed four times with ice-cold 0.9% sodium chloride, the cells were hemolyzed by shaking for 3 min with 1 vol of water and 0.4 vol of toluene followed by centrifugation. The clear hemoglobin solution was siphoned off and 5 ml (concentration approximately 15%) were placed on a 2.5 cm × 30-cm column of Sephadex G-25 equilibrated with 0.1 *M* sodium chloride. The protein was eluted with the same solvent at a rate of about 20 ml/hr.

Results.—By the above method, hemoglobin which is free of phosphate is obtained (Table 1); its oxygen dissociation curve in 0.01 *M* NaCl is shown in Figure 1, curve *A*. It is evident that this molecule still shows cooperative oxygen binding (Hill's coefficient n is 2.6–2.9).[5] By contrast, hemoglobin purified only by dialysis against distilled water, which still contains about 0.5 mole of DPG per mole, shows a flat, drawn-out curve (Fig. 1, curve *B*) which was previously reported to have an n of 1.1, i.e., to have lost all heme-heme interaction.[1] This erroneous conclusion must now be corrected by the realization that this oxygenation curve is really biphasic. It is the result of the successive oxygenation of two molecular species, present in roughly equal proportion, i.e., free or "stripped" hemoglobin with a high oxygen affinity (Fig. 1, curve *A*), followed by the DPG-complexed hemoglobin with a much lower oxygen affinity (Fig. 1, curve *C*). The

correctness of this assumption is confirmed by the oxygenation curve of "stripped" hemoglobin to which $^1/_2$ mole of DPG per mole of hemoglobin has been added (Fig. 1, curve *D*) which has a clear change in slope at 50 per cent oxygenation. Such biphasic curves are obtained whenever less than 1 mole of DPG per mole of hemoglobin is present and the salt concentration is low enough to shift the oxygenation curve of the "stripped" hemoglobin far to the left. The separation be-

TABLE 1. *DPG content of various hemoglobin preparations.*

Preparation	Moles DGP/moles Hb
1. Whole blood	0.92
2. Red cells washed four times with 0.9% NaCl	0.86
3. Hemolysate after destromatization with toluene	0.63
4. Destromatized hemolysate after dialysis for 24 hr against distilled water	0.63
5. Destromatized hemolysate after passage through Sephadex G-25 in 0.1 *M* NaCl	0.02*

The DPG content was determined on trichloracetic acid filtrates by the method of Bartlett.[22]
* Total phosphate.

tween the oxygenation regions of free and complexed hemoglobin is even greater when inositol hexaphosphate (IHP), the major organic phosphate of avian erythrocytes, is used as the cofactor in place of DPG (Fig. 2).

It is therefore likely that the biphasic oxygenation curves of hemoglobin, which have been reported previously in solutions of low ionic strength,[6, 7] were due to incomplete removal of organic phosphates, rather than to negative interaction between the hemes as recently proposed by Guidotti.[8]

Binding of DPG by hemoglobin: Phosphate-free hemoglobin, prepared as described above, was used for these experiments. D-2,3-diphosphoglycerate was purchased as the cyclohexylammonium salt (Calbiochem) and converted to the free acid by shaking a solution with an excess of Dowex 50WX8 in the H form.

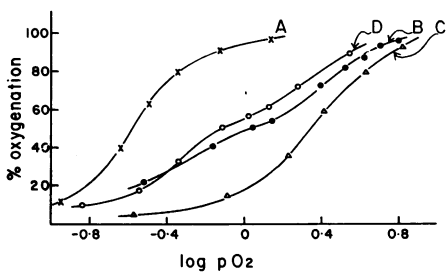

FIG. 1.—Oxygenation curves of 0.3% hemoglobin in 0.01 *M* NaCl at 10°C and pH 7.0 (before deoxygenation). ×——×, Curve *A:* "stripped" hemoglobin; ●——●, curve *B:* dialyzed, destromatized hemolysate (prep. 4, Table 1); Δ——Δ, curve *C:* "stripped hemoglobin + 1 mole DPG per mole; O——O, curve *D:* "stripped" hemoglobin + 0.5 mole DPG per mole.

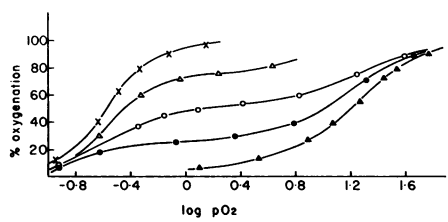

FIG. 2.—Effect of IHP on the oxygenation of hemoglobin. Hb concentration: 0.3% (4.6×10^{-5} *M*) in 0.01 *M* NaCl, pH 7.0 (before deoxygenation), temperature 10°C. ×——×, No IHP; Δ——Δ, 1.2×10^{-5} *M* IHP (0.26 mole/mole); O——O, 2.3×10^{-5} *M* IHP (0.5 mole/mole); ●——●, 3.5×10^{-5} *M* IHP (0.76 mole/mole); ▲——▲, 4.6×10^{-5}*M* IHP (1.0 mole/mole).

Solutions were neutralized to pH 7 and stored at 4°C. For the binding experiments known concentrations of hemoglobin and DPG were mixed and brought to a final sodium chloride concentration of 0.1 *M*. The pH was adjusted to pH 7.3, except in the case of deoxyhemoglobin where the initial pH was 7.0 to allow for the increase to pH 7.3 on deoxygenation. Seven-ml aliquots were placed in cellophane bags (Visking size 8/32) and 0.5 ml of ultrafiltrate was collected in an apparatus similar to that described by Craig.[9] For the experiments with deoxyhemoglobin, the solutions were deoxygenated as described previously[10, 11] and transferred anaerobically to the ultrafiltration apparatus which was kept under nitrogen. The phosphate concentration in the ultrafiltrate was determined by the method of Ames and Dubin.[12] The DPG concentration in the ultrafiltrate was corrected for slight losses of DPG on the membrane in control experiments without the protein. The protein concentration was varied from 0.3 per cent to 1.8 per cent with increasing DPG concentration so that at least 50 per cent of the added DPG was always present in the bound form.

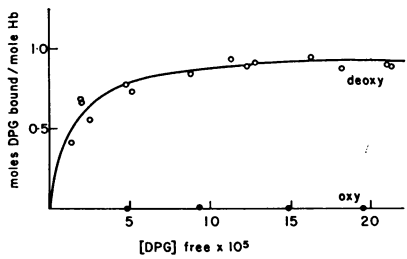

FIG. 3.—Binding of DPG by human hemoglobin in 0.1 *M* NaCl at pH 7.30, temperature 22° ± 2°C.

A plot of the moles of DPG bound per mole of hemoglobin as a function of the free DPG concentration is shown in Figure 3. It is clear that under the conditions of this experiment only deoxy- but not oxyhemoglobin binds DPG and that the binding levels off at 1 mole of DPG per hemoglobin tetramer. The reversibility of the reaction was demonstrated by reoxygenation of deoxygenated samples, which resulted in the immediate release of all the bound DPG. The association constant calculated from these data is 4.8×10^4 liters/mole in 0.1 *M* sodium chloride. This salt concentration is sufficient to suppress the less specific binding of the highly charged DPG molecule on both forms of the protein which occurs at lower ionic strength. It is also noteworthy that, under the conditions of the experiment in Figure 3, neither methemoglobin nor cyanomethemoglobin binds DPG.

If the decrease in the affinity of hemoglobin for oxygen in the presence of DPG is entirely due to the specific binding of this compound on deoxyhemoglobin, then the reaction of the two small molecules with hemoglobin can be regarded as competitive, although they undoubtedly occupy different sites.

The over-all reaction can thus be written as follows:

$$\mathrm{HbDPG} + n\mathrm{O_2} \rightleftarrows \mathrm{Hb(O_2)}_n + \mathrm{DPG} \qquad K_3 = \frac{[\mathrm{Hb(O_2)}_n][\mathrm{DPG}]}{[\mathrm{HbDPG}][\mathrm{O_2}]^n}.$$

It is the sum of the two separate reactions:

$$\mathrm{Hb} + n\mathrm{O_2} \rightleftarrows \mathrm{Hb(O_2)}_n \qquad K_1 = \frac{[\mathrm{Hb(O_2)}_n]}{[\mathrm{Hb}][\mathrm{O_2}]^n}$$

and

$$HbDPG \rightleftarrows Hb + DPG \qquad K_2 = \frac{[Hb][DPG]}{[HbDPG]}$$

so that $\log K_3 = \log K_1 + \log K_2$.

In order to compare the value of K_3 determined from the oxygenation curves in the presence of DPG (Figs. 4 and 5) with that obtained by combining the separately measured affinities of hemoglobin for DPG and for oxygen, a numerical value of $n = 2$ was used in the above equations. This is based on the assumption that the binding of two oxygen molecules is completely concerted[8] and results in

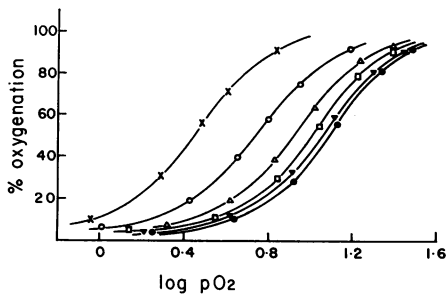

FIG. 4.—Effect of DPG on the oxygenation curve of human hemoglobin. Hemoglobin concentration: 0.3% (4.6×10^{-5} *M*) in 0.1 *M* NaCl, pH 7.0 (before deoxygenation), temperature 20°C. ×——×, No DPG; O——O, 2×10^{-4} *M* DPG; Δ——Δ, 4×10^{-4} *M* DPG; □——□, 6×10^{-4} *M* DPG; Δ——Δ, 8×10^{-4} *M* DPG; ●——●, 10×10^{-4} *M* DPG.

the displacement of the DPG molecule from the hemoglobin tetramer. This reaction would, on the average, be half completed at 25 per cent oxygenation.

As can be seen from Table 2, the average value of $\log K_3$ of −4.8 remains almost constant over a tenfold range of DPG concentration. This is not the case when $n = 4$ is substituted, since $\log K_3$ then varies systematically from −6.6 to −7.4 over the same concentration range.

The average value of $\log K_3$ of −4.8 may be compared with the sum of $\log K_1 + \log K_2$, which is −5.1. This difference may reflect the obvious approximations which are involved in the model used for these calculations.

TABLE 2. *Variation of the oxygen affinity of hemoglobin with DPG concentration.*

Total DPG (moles/liter)	Log[DPG]	Log p_{25}	$-\text{Log } K_3$ $(2 \log p_{25} - \log[DPG])$
0	—	0.23	—
2×10^{-4}	−3.82	0.50	4.8
3×10^{-4}	−3.60	0.64	4.9
4×10^{-4}	−3.46	0.69	4.8
6×10^{-4}	−3.26	0.79	4.8
8×10^{-4}	−3.12	0.84	4.8
10×10^{-4}	−3.02	0.88	4.8
25×10^{-4}	−2.60	0.98	4.6

Measurements in 0.1 *M* NaCl at 20°C, pH 7.0 (before deoxygenation). Hemoglobin concentration 0.3%, i.e., 4.5×10^{-5} *M*.

DPG constitutes the major portion of the organic phosphates of most mammalian red cells, including human erythrocytes. It is replaced by IHP in the bloods of birds and turtles,[13] which are known to have a lower oxygen affinity than human blood.[14] It can be seen from Table 3 that the oxygen affinities of "*stripped*"

TABLE 3. *Effect of DPG and IHP on human and chicken hemoglobin.*

	Log p_{50}	
	Human	Chicken*
"Stripped" Hb in 0.01 *M* NaCl	−0.64	−0.59
"Stripped" Hb in 2.5×10^{-3} *M* DPG + 0.01 *M* NaCl	+0.87	+1.11
"Stripped" Hb in 2.5×10^{-3} *M* IHP + 0.01 *M* NaCl	+1.35	+1.35

Concentration of Hb = 0.3%, temperature 10°C, pH = 7.0 (before deoxygenation).
* Chicken hemoglobin was freed of IHP by passage through DEAE-Sephadex in 0.05 *M* phosphate buffer pH 7.0, followed by removal of inorganic phosphate with G-25 Sephadex in 0.1 *M* NaCl. The final preparation was phosphate-free.

human and chicken hemoglobin are very similar, but that IPH is more effective than DPG in lowering the oxygen affinity of both hemoglobins. In fact, in the presence of excess IHP, the oxygenation curves of chicken and human hemoglobin become identical. It is therefore probable that the lower oxygen affinity of chicken blood can be accounted for in terms of a more powerful cofactor rather than any differences in the hemoglobins themselves.

A very different situation is found in sheep, which, in common with some other ungulates, contain little organic phosphate and only traces of DPG in their erythrocytes.[13] Here the oxygen affinity of the "stripped" hemoglobin was found to be about ten times lower than that of "stripped" human hemoglobin (Table 4). Furthermore, stripping scarcely altered the oxygen affinity of sheep

TABLE 4. *Effect of DPG on sheep and human hemoglobin.*

	Log p_{50}	
	Human	Sheep
Unpurified hemolysate in 0.02 *M* NaCl	—	+0.71
"Stripped" Hb in 0.02 *M* NaCl	−0.30	+0.68
"Stripped" Hb in 2.5×10^{-3} *M* DPG + 0.02 *M* NaCl	+0.90	+1.28

Concentration of Hb = 0.3%, temperature 10°C, pH = 7.0 (before deoxygenation).

hemoglobin, which is consistent with the low organic phosphate content of sheep blood. It therefore seems that sheep hemoglobin can have a sufficiently low oxygen affinity without the intervention of cofactors. Nevertheless, the oxygen affinity can be further lowered by the addition of DPG, as is evident from Table 4.

Discussion.—Perhaps the most surprising result of these investigations is the finding that completely "stripped" human hemoglobin dissolved in dilute sodium chloride (0.01–0.1 *M*) shows unimpaired heme-heme interaction. Previous reports by us and other workers[1, 6, 7] that this interaction decreases as the ionic strength is lowered must therefore be regarded as an artifact resulting from incomplete removal of organic phosphates. Moreover, judging from the pH change on deoxygenation, "stripped" hemoglobin also shows a Bohr effect within the normal range.

DPG and other organic polyphosphates therefore appear to influence only the over-all affinity of hemoglobin for oxygen and may be regarded as cofactors of oxygen unloading. In the case of DPG, the displacement of the oxygenation curve can be accounted for quantitatively by its preferential binding to deoxyhemoglobin.

Several facts must be considered in applying these results to oxygen transport by human erythrocytes:

(1) The hemoglobin concentration in the red cell is about 100 times that used in these experiments. However, the oxygen affinity is known not to vary significantly with hemoglobin concentration.[15]

(2) The DPG concentration in the red cell is comparable to that of the hemoglobin on a molar basis (approximately 5×10^{-3} *M*).[16] In addition, other organic phosphates with a comparable activity to DPG (principally ATP) are present at a concentration of $1\text{–}2 \times 10^{-3}$ *M*.[16] If 1 mole of organic phosphate compound per mole of hemoglobin is bound, then the free concentration would be in the range of $1\text{–}2 \times 10^{-3}$ *M*. On the basis of the results in Figure 5, this would correspond to a log p_{50} of 1.08–1.16. The comparable value for whole blood at 20°C calculated from the data of Astrup *et al.*[17] is 1.15. It should further be noted that the plot of log p_{50} versus DPG concentration (Fig. 5) becomes very flat above a concentration of 1×10^{-3} *M*. Variations in organic phosphate level in the physiological range would thus have little effect on the oxygen affinity. By the same token, a decrease to less than $^3/_4$ of the normal concentration should lead to a sharp decrease in the ability of hemoglobin to unload oxygen.

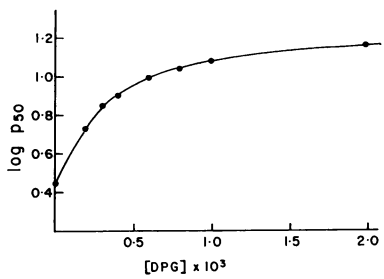

FIG. 5.—Change of p_{50} of human hemoglobin with DPG concentration: conditions as in Fig. 4.

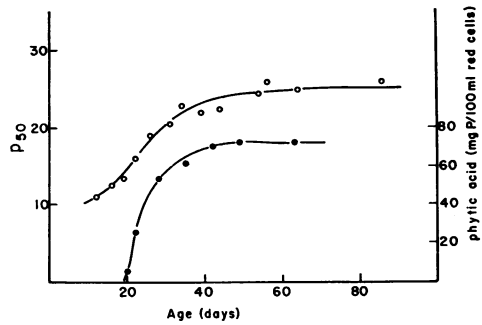

FIG. 6.—Variation of erythrocyte IHP content and oxygen affinity with age in the developing chick. ●——●, IHP content, from Oshima *et al.*[23] O——O, p_{50}, from Hall.[24]

Significant differences in the oxygen affinity of young and old erythrocytes are known to exist,[18] and Edwards and Rigas have recently suggested[19] that the increase in oxygen affinity during *in vivo* aging of red cells may be due to a decrease in their DPG content.

Storage of blood in acid-citrate-dextrose at 4°C leads to a progressive decline in the level or organic phosphates with concomitant loss of viability of the cells.[20] It is therefore very significant that Valtis and Kennedy[21] found a shift in oxyhemoglobin dissociation curves towards higher oxygen affinities after one week of storage in acid-citrate-dextrose at 4°C. This shift was still evident on transfusion of the blood to a recipient. The oxygenation curve returned only slowly to normal, presumably as a function of the resynthesis of organic phosphates in the depleted cells.

The red cells of the developing chicken provide a system with large physiological variations in organic phosphate content. As can be seen from Figure 6, there

is a striking inverse correlation between IHP content and oxygen affinity as the chicken matures.

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