isoionic α_S-casein correspond to the liberation of a small fraction of one hydrogen ion per molecule even though several potassium ions are ligated. Hence, a rise in pH should not be taken as proof that more anions than cations are bound by chymotrypsin.

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The Oxygenation of Hemoglobin in the Presence of 2,3-Diphosphoglycerate. Effect of Temperature, pH, Ionic Strength, and Hemoglobin Concentration*

Ruth E. Benesch, Reinhold Benesch,† and Chi Ing Yu

ABSTRACT: The interaction of 2,3-diphosphoglycerate with hemoglobin is markedly influenced by environmental factors. The strength and extent of binding is inversely proportional to pH, as would be expected for electrostatic interaction with a polyanion. The effect of 2,3-diphosphoglycerate in lowering the oxygen affinity of hemoglobin can be regarded as a special salt effect, since neutral salts act similarly, albeit in concentrations 1000 times greater than 2,3-diphosphoglycerate. This molecule is thus capable of regulating oxygen affinity at the millimolar concentrations in which it occurs in the erythrocyte without disturbing the osmotic equilibrium. Both oxygen and 2,3-diphosphoglycerate binding by hemoglobin are exothermic.

Since oxygenation involves displacement of 2,3-diphosphoglycerate, the temperature dependence of the oxygenation reaction is lowered in the presence of the phosphate cofactor, permitting correct oxygen release over a wider range of temperature. The reaction of 2,3-diphosphoglycerate with deoxyhemoglobin is accompanied by a decrease in entropy, which, together with other evidence, bears out the previously proposed model for the interaction of 2,3-diphosphoglycerate with hemoglobin.

We first demonstrated in 1967 that D-2,3-diphosphoglycerate and certain other organic phosphates of the human red cell substantially decrease the oxygen affinity of hemoglobin (Benesch and Benesch, 1967). This effect was quickly confirmed in other laboratories (Chanutin and Curnish, 1967; Tyuma and Shimizu, 1969). It subsequently became clear that D-2,3-diphosphoglycerate only affects the over-all affinity for the ligand but has little if any influence on the other allosteric properties of hemoglobin, *i.e.*, the cooperativity of the oxygen binding and the Bohr effect (Benesch *et al.*, 1968b). The general subject of intracellular organic phosphates as regulators of oxygen release by hemoglobin has been reviewed recently (Benesch and Benesch, 1969).

This report deals with the effect of temperature, pH, ionic strength, and the hemoglobin concentration on the reciprocal binding of D-2,3-diphosphoglycerate and oxygen by hemoglobin in buffered solutions. Information on the energetics of the reaction between D-2,3-diphosphoglycerate and hemoglobin could be expected to lead to a better understanding of the mechanism of the interaction as well as to certain extrapolations on the influence of D-2,3-diphosphoglycerate on oxygen release by hemoglobin in the red cell.

* From the Department of Biochemistry, College of Physicians & Surgeons of Columbia University. *Received February 5, 1969.* This work was supported by U. S. Public Health Service Grants HE-05791 and HE-06787 from the National Heart Institute and Grant GB-4530 from the National Science Foundation and by the American Heart Association.

† Recipient of a U. S. Public Health Research career award from the National Heart Institute.

Experimental Section

Hemoglobin was prepared from the blood of healthy donors at weekly intervals as described previously (Benesch *et al.*, 1968b). It was "stripped," *i.e.*, rendered phosphate free by dialysis against 8 l. of 0.1 M NaCl flowing at the rate of 500 ml/hr at 4°. The dialyzing membrane was two-dimensionally

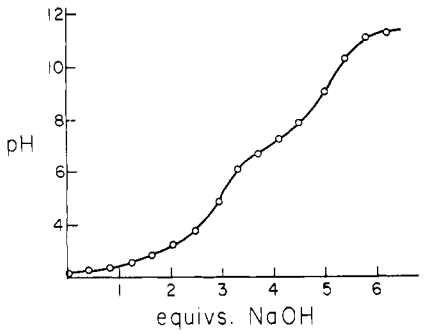

FIGURE 1: Titration curve of 2,3-diphosphoglycerate. 5×10^{-3} M diphosphoglycerate in 0.1 N NaCl was titrated with 0.5 N NaOH.

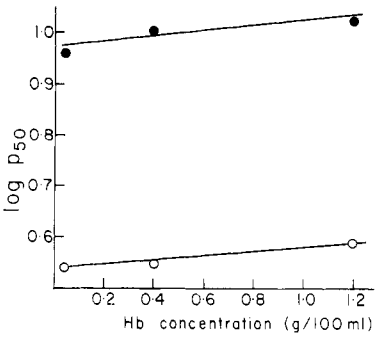

FIGURE 3: Effect of hemoglobin concentration on the oxygen affinity. Temperature 20°, bis-Tris buffer 0.05 M, pH 7.30, total chloride 0.1 M. Open circles, no diphosphoglycerate; filled circles, 1×10^{-3} M diphosphoglycerate.

stretched Visking sausage casing (Craig, 1965) supported in the rapid dialysis apparatus described by Englander and Crowe (1965). This procedure replaced the previously described phosphate removal with Sephadex G-25.

Oxygen dissociation curves were determined as before (Benesch *et al.*, 1965).

2,3-Diphospho-D-glyceric acid was purchased from Calbiochem as the pentacyclohexylammonium salt tetrahydrate and converted into the free acid with Dowex 50. The titration curve of this compound in 0.1 M NaCl (Figure 1) shows two buffering regions, the first involving three groups with an average pK of 2.8 and the second two groups with an average pK of 7.1. A similar titration curve was reported by Kiessling (1934) in the absence of salt. The concentration of the D-2,3-diphosphoglycerate solutions was estimated both by titration with alkali to the second end point and by total phosphate assay (Ames and Dubin, 1960), with excellent agreement between the two methods.

The L isomer was kindly synthesized by Mr. Hans-Helmut Flehmig in the laboratory of Professor Erich Baer as the barium salt. It was also converted to the free acid with Dowex 50 before use.

Bis-Tris, *i.e.*, bis(2-hydroxyethyl)iminotris(hydroxymethyl)-methane, was obtained from General Biochemicals, Chagrin Falls, Ohio. The buffers were prepared by mixing the base with HCl. The heat of ionization of the imino group was found to be 7.3 kcal. It was, therefore, necessary to adjust the composition of the buffers accordingly in order to maintain identical pH values at different temperatures.

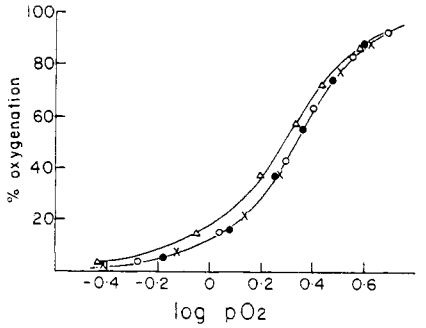

FIGURE 2: Effect of bis-Tris on the oxygenation of "stripped" hemoglobin. Temperature 10°, pH 7.20, total chloride 0.1 M, hemoglobin concentration 0.4%. Triangles, no buffer; open circles, 0.01 M bis-Tris; crosses, 0.05 M bis-Tris, and filled circles, 0.10 M bis-Tris.

Results and Discussion

In our original studies of the relation of D-2,3-diphosphoglycerate to oxygen binding, the use of buffers was avoided, since the anions used for this purpose in the neutral range (notably inorganic phosphate) compete with D-2,3-diphosphoglycerate. A search for a buffer which would not interfere with D-2,3-diphosphoglycerate binding resulted in the choice of bis-Tris, suggested to us by Dr. E. Shooter. This amine has a pK of 6.5 and is therefore predominantly in the uncharged form above pH 7. The oxygenation curves in Figure 2 show that at pH 7.3 this buffer has no effect on the p_{50} over a tenfold concentration range. In the presence of the buffer the slope of the curves is increased since a constant pH is maintained during the oxygenation and thus the flattening due to the Bohr effect is prevented.

Effect of Hemoglobin Concentration. For most of the experiments reported here a hemoglobin concentration about 100 times more dilute than that in the red cell was used. However, the remarkable insensitivity of the reaction of hemoglobin with oxygen to the total protein concentration (Wyman, 1964; Drake *et al.*, 1963) makes it probable that the influence of environmental factors will not be materially altered at higher hemoglobin concentrations including those of the red cell. The curves in Figure 3 confirm the constancy of the oxygen dissociation curve with hemoglobin concentration and show that this is not affected by D-2,3-diphosphoglycerate.

Effect of Salt. The profound influence of salt on the oxygenation of hemoglobin has been recognized for a long time (Barcroft and Camis, 1909; Barcroft and Roberts, 1909; Rossi-Fanelli *et al.*, 1961). In general the oxygen affinity decreases with increasing salt concentration. This is illustrated for NaCl in Figure 4 (top) and undoubtedly reflects preferential binding of salt by the deoxy as against the oxy form of hemoglobin. Corresponding oxygenation curves in the presence of D-2,3-diphosphoglycerate show that this anion with four negative charges per mole at pH 7.3 (Figure 1) acts in a similar manner, except that its efficacy is about three orders of magnitude greater than that of sodium chloride (Figure 4, bottom).

It is apparent from Figure 5 that in the presence of a suffi-

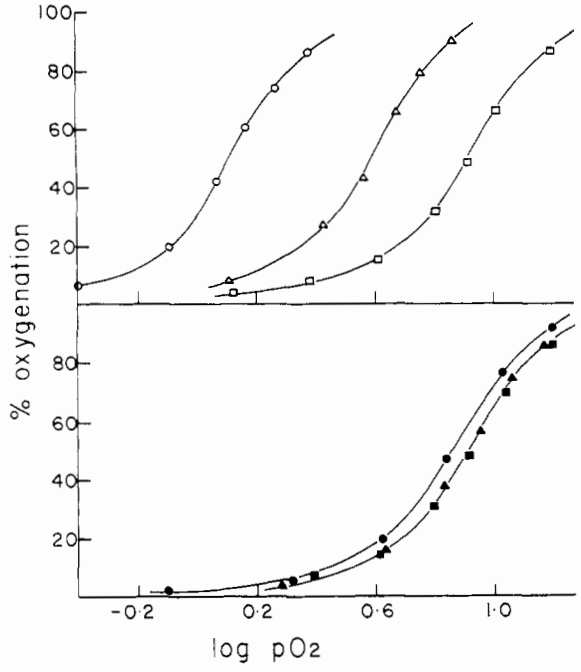

FIGURE 4: Influence of NaCl concentration on the oxygenation of hemoglobin. Temperature 20°, bis-Tris buffer 0.05 M, pH 7.30, hemoglobin concentration 0.4%. Circles, 0.01 M total chloride; triangles, 0.10 M total chloride; squares, 0.50 M total chloride; open symbols, no diphosphoglycerate; and, filled symbols, 2.5×10^{-4} M diphosphoglycerate.

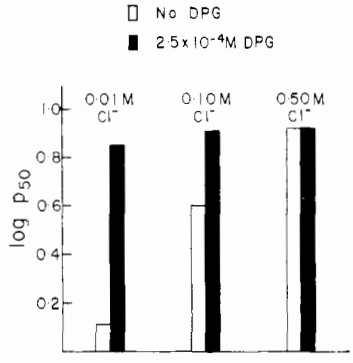

FIGURE 5: Comparison of the effect of NaCl and of diphosphoglycerate on the oxygen affinity of hemoglobin. Conditions as in Figure 4.

ciently high concentration of NaCl (0.5 M), the effect of D-2,3-diphosphoglycerate on the oxygen affinity disappears. Conversely, 2.5×10^{-4} M D-2,3-diphosphoglycerate is sufficient to make the oxygenation curve essentially independent of the salt concentration. D-2,3-diphosphoglycerate can therefore be regarded as a specially effective case of the general salt effect on hemoglobin oxygenation. This realization has made it possible to interpret the oxygenation curves obtained when less than 1 mole of D-2,3-diphosphoglycerate/mole of hemoglobin is present. Under these conditions the curves are biphasic, but only if the neutral salt concentration is sufficiently low, *e.g.*, 0.01 M (Benesch *et al.*, 1968b). A comparison of the log p_{50}'s in Figure 5 shows that the difference in log p_{50} with and without D-2,3-diphosphoglycerate is 0.74 in 0.01 M NaCl, but only 0.31 in 0.1 M NaCl. It is therefore much more difficult to discern a break in the curve of a mixture of "stripped" and D-2,3-diphosphoglycerate complexed hemoglobin at the higher salt concentration. Thus it is not surprising that this discontinuity was previously overlooked and misinterpreted as a continuous oxygenation curve with a flatter slope (Benesch and Benesch, 1967; Rossi-Fanelli *et al.*, 1961; Enoki and Tyuma, 1964).

We have postulated (Benesch *et al.*, 1968a) that D-2,3-diphosphoglycerate is bound in the central cavity on the dyad axis of deoxyhemoglobin. Apparently other anions, such as chloride can fill this cavity, too, with similar effects on the oxygen affinity, albeit at very much greater concentrations than D-2,3-diphosphoglycerate. The relatively low specificity of the D-2,3-diphosphoglycerate effect is also illustrated by the similar activity of certain other polyphosphates such as ATP and even pyrophosphate (Benesch and Benesch, 1967). Furthermore, we have now found that L-2,3-diphosphoglycerate is indistinguishable from the usual D isomer in its effect on the oxygen affinity.

The concentration of neutral salt also has a decisive influence on the *extent* of the D-2,3-diphosphoglycerate binding. It is only in the range of physiological salt concentrations, such as 0.1 M NaCl, that the specific binding of 1 mole of D-2,3-diphosphoglycerate/deoxyhemoglobin tetramer is observed and no reaction with oxyhemoglobin takes place. At lower salt concentrations binding of more than 1 mole of D-2,3-diphosphoglycerate/mole of hemoglobin occurs and even oxyhemoglobin begins to bind. Any such binding which does not discriminate between the oxy and deoxy form of hemoglobin is, of course, not relevant as far as the oxygen affinity is concerned. Reports on oxyhemoglobin–D-2,3-diphosphoglycerate complexes must be viewed in the light of this consideration. Thus the original demonstration by Sugita and Chanutin (1963) that various organic phosphates form complexes with oxyhemoglobin was performed under conditions which favor nonspecific binding, *i.e.*, low ionic strength and low pH.

By contrast, the early observations of Solomon *et al.* (1940) that organic phosphates resist ultrafiltration from hemolysates prepared by freezing are pertinent, since the experiments were done under anaerobic conditions and without diluting the physiological salt concentration. It is of further significance that the organic phosphates appeared more tightly bound to the "colloid" at 7° than at 37° (*Cf.* below).

Effect of pH. Since cationic groups on the protein undoubtedly play an important role in binding highly charged phosphate esters like D-2,3-diphosphoglycerate, it is not surprising that the affinity of hemoglobin for D-2,3-diphosphoglycerate decreases with increasing pH (Table I).

A much more unexpected result is the fact that the Bohr effect is identical in the absence and in the presence of a large excess of D-2,3-diphosphoglycerate (Figure 6). At interme-

TABLE I: Effect of pH on 2,3-Diphosphoglycerate Binding.

pH	$1/K_2$
7.0	1.3×10^5
7.3	6.3×10^4
7.8	1.2×10^4

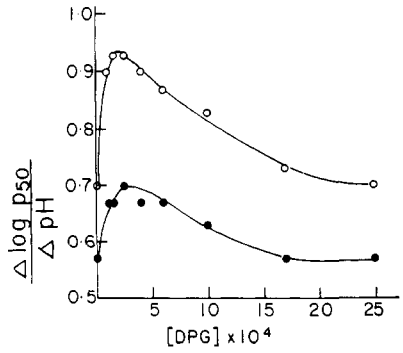

FIGURE 6: Relation between diphosphoglycerate concentration and the Bohr effect. Hemoglobin concentration 6×10^{-5} M, total chloride 0.1 M, bis-Tris buffer 0.05 M, temperature 20°. Open circles, pH interval 7.3–7.6; filled circles, pH interval 7.0–7.3.

TABLE II: Effect of Temperature on 2,3-Diphosphoglycerate Binding by Deoxyhemoglobin.

Temp (°C)	Log $(1/K_2)$	ΔF (kcal)	ΔH (kcal)	ΔS (cal/deg)
10	5.28	−6.81		−22.6
			−13.2	
15	5.02	−6.61		−22.9
			−13.2	
20	4.92	−6.59		−22.6
			−13.2	
25	4.80	−6.54		−22.5
			−13.2	
30	4.56	−6.32		−22.8

diate concentrations of D-2,3-diphosphoglycerate, however, an increase in the Δ log p_{50}/ΔpH is observed which disappears when enough D-2,3-diphosphoglycerate is present (2.5×10^{-3} M) to suppress the difference in affinity of D-2,3-diphosphoglycerate for hemoglobin at the two different pH values.

Effect of Temperature. Under conditions where 1 mole of D-2,3-diphosphoglycerate is bound specifically to 1 mole of deoxyhemoglobin, we have shown (Benesch *et al.*, 1968b) that the free energy of binding calculated from the shift in the oxygenation curve is in good agreement with the value obtained from direct binding measurements, *i.e.*, 6.4 kcal/mole at 20°. The energetics of the deoxyhemoglobin–D-2,3-diphosphoglycerate reaction were therefore determined from the oxygenation curves as a function of temperature.

As formulated previously (Benesch *et al.*, 1968b), log K_2 = log K_3 − log K_1 where K_2 is the dissociation constant of the deoxyhemoglobin–D-2,3-diphosphoglycerate complex and K_1 and K_3 are the equilibrium constants for the oxygenation reaction in the absence and the presence of D-2,3-diphosphoglycerate, respectively. Log K_1 was obtained from $-4 \log p_{50}$ and log K_3 from log [D-2-3-diphosphoglycerate] $- 4 \log p_{50}$. In these experiments at constant pH, it was found that more constant K_3 values were obtained when $-4 \log p_{50}$ was used rather than the value of $-2 \log p_{25}$ used before.

The variation of the association constant, $1/K_2$, of the hemoglobin–D-2,3-diphosphoglycerate complex with temperature and the relevant thermodynamic parameters are shown in Table II. It is evident that the association constant decreases with increasing temperature and that the reaction is therefore strongly exothermic. Since 13 kcal are liberated as heat when 1 mole of D-2,3-diphosphoglycerate combines with 1 mole of deoxyhemoglobin, but the corresponding free-energy decrease is much smaller, the binding process is accompanied by a decrease in entropy of about 23 eu. Interaction with D-2,3-diphosphoglycerate therefore imposes a constraint on the protein, which is consistent with the concept that D-2,3-diphosphoglycerate acts as a bifunctional cross-linking reagent between two halves of the macromolecule across the central cavity.

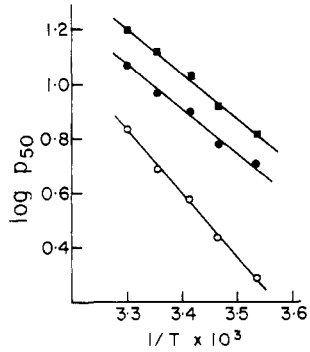

FIGURE 7: Effect of diphosphoglycerate on the temperature dependence of the oxygen affinity. Hemoglobin concentration 0.4%, bis-Tris buffer 0.05 M, pH 7.3, total chloride 0.1 M. Open circles, no diphosphoglycerate; filled circles, 2.5×10^{-4} M diphosphoglycerate; and filled squares, 1.0×10^{-3} M diphosphoglycerate.

Both oxygen and D-2,3-diphosphoglycerate binding are thus accompanied by an evolution of heat. Since oxygenation in the presence of D-2,3-diphosphoglycerate involves displacement of D-2,3-diphosphoglycerate and therefore absorption of heat, the temperature coefficient of the oxygenation reaction is decreased in the presence of the phosphate ester. This is illustrated by the plots in Figure 7.

The heat of reaction calculated from the slopes of these lines is −10.7 kcal/mole of oxygen bound in the absence and −7.3 kcal in the presence of D-2,3-diphosphoglycerate. As pointed out by Wyman (1948) the former value is composed of the heat of oxygenation plus that due to the ionization of oxygen-linked acid groups. The latter value contains, in addition, the heat of dissociation of the D-2,3-diphosphoglycerate. If it can be assumed that the heats associated with proton movement are unaffected by the D-2,3-diphosphoglycerate reaction, which is supported by the data in Figure 6, then the enthalpy for the displacement of D-2,3-diphosphoglycerate is $\{-7.3 - (-10.7)\} \times 4$, *i.e.*, +13.6 kcal/mole of D-2,3-diphosphoglycerate displaced. This agrees well with the value of 13.2 kcal obtained in Table II.

It is therefore evident that the effect of D-2,3-diphosphoglycerate in lowering the oxygen affinity decreases with increasing temperature so that, *e.g.*, the shift in log p_{50} caused by 2.5×10^{-4} M D-2,3-diphosphoglycerate at 10° is 0.42 but is only 0.23 at 30°. The association constant for the calculation of free *vs.* bound D-2,3-diphosphoglycerate concentrations at the temperature of the red cell *in vivo*, *i.e.*, 37°, was calculated by extrapolation of the data in Table II to be 2.5×10^4.

The average temperature coefficients $\{\Delta \log p_{50}/\Delta T\}$ of the oxygenation of whole blood which have been reported range from 0.019 (Dill and Forbes, 1941) to 0.024 (Astrup *et al.*, 1965). The corresponding values for hemoglobin in the absence and presence of D-2,3-diphosphoglycerate are 0.029 and 0.018, respectively. D-2,3-Diphosphoglycerate therefore brings the temperature dependence of hemoglobin oxygenation closer to that of whole blood.

The results presented here clearly show that with the exception of the hemoglobin concentration, environmental factors can have a profound influence on the reciprocal binding of oxygen and D-2,3-diphosphoglycerate to hemoglobin. Oxygen release from hemoglobin is therefore not only regulated by the concentration of organic phosphates but the interaction of these compounds with hemoglobin in turn depends upon other factors such as temperature and ionic environment. As shown above, salt, in particular, can greatly affect the specificity of the hemoglobin–D-2,3-diphosphoglycerate reaction. Furthermore, certain ions present in significant concentrations in the erythrocyte such as magnesium, are likely to have even more specific effects, since they are known to complex organic phosphates very effectively (Rose, 1968). There is, moreover, some evidence that the free D-2,3-diphosphoglycerate concentration of the red cell plays a role in ion transport across the red cell membrane (Gardos, 1966; Benesch and Benesch, 1968) and could thus influence the composition of its own ionic environment.

Both oxygen and D-2,3-diphosphoglycerate binding were also seen to be highly temperature dependent. Since, however, the binding of oxygen involves the displacement of D-2,3-diphosphoglycerate, the temperature coefficient of the oxygen affinity is lowered in the presence of the organic phosphate. Therefore, correct oxygen unloading is made possible over a wider range of temperatures.

This recalls the curious but hitherto unexplained finding that the oxygenation curve of *Thunnus thynnus* hemoglobin is practically unaffected by temperature (Rossi-Fanelli and Antonini, 1960). It is possible that the hemoglobin in the blood of this poikilothermic species interacts with a cofactor which is bound with an enthalpy very close to that of the oxygenation.

The remarkable influence of D-2,3-diphosphoglycerate in stabilizing the deoxy conformation of hemoglobin has a striking parallel in the effects of certain anions which maintain the conformation of ribonuclease. Here too, these ions are embedded in a crevasse at a specific site (Kartha *et al.*, 1967). Furthermore, as Nelson *et al.* (1962) have shown, the binding is always in a ratio of 1 mole/mole of protein and the strength of binding is in the order pyrophosphate > 2′-cytidylate > phosphate > sulfate > chloride. These authors conclude that the anions secure the polypeptide chain in place by electrostatic binding to two or more cationic groups. Moreover, Markus *et al.* (1968) were able to show that ligands such as 2′-cytidylate decisively alter the susceptibility to proteolysis of peptide bonds well removed from the binding site. Thus both in the case of hemoglobin and ribonuclease, the binding of an appropriate anion "provides increased resistance against disruptive influences in regions of the molecule that are remote from the binding site."

Acknowledgment

We are deeply indebted to Professor Erich Baer for providing us with a generous sample of the barium salt of L-2,3-diphosphoglyceric acid.

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