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THE INTERACTION OF 2,3-DIPHOSPHOGLYCERATE ON

HUMAN DEOXY AND OXYHEMOGLOBIN

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SUMMARY: A very accurate pH stat method was used to measure the number of protons which are taken up when 2,3-diphosphoglycerate (DPG) reacts with deoxyhemoglobin (Hb) and oxyhemoglobin (HbO_2). As judged by this method it appeared that the effect of DPG on both Hb and HbO_2 is pH dependent with this difference that similar results are obtained at different pH ranges. The data showed that the additional Bohr effect and therefore the oxygen affinity of hemoglobin in the presence of DPG is not only due to the binding of DPG to Hb, but also due to the interaction of DPG with HbO_2.

It has been known for some time that DPG strongly decreases the ligand affinity of human hemoglobin (1,2) and enhances the alkaline Bohr effect above pH=7 (3,7). Recently we have presented evidence that below pH=7 DPG decreases the alkaline Bohr effect and increases the acid Bohr effect (6). From a phenomenological point of view the effect of DPG on the Bohr effect can be described by saying that due to the presence of DPG some additional alkaline and acid Bohr groups are introduced in the hemoglobin molecule approximately equal in number and in magnitude of their pK shifts. On a molecular level we explained (7) the change in Bohr effect by assuming that at high pH upon dissociation of DPG from Hb protons are released due to a shift in pK to lower values of the positively charged groups on the protein involved in the binding of DPG; these groups are located at the entrance of the central cavity (8,9). The other assumption we made was that at low pH protons are consumed when completely ionized DPG molecules bound to Hb get adjusted to their proper degree of ionization as soon as they dissociate from Hb.

In the mathematical description (7) proposed for this extra Bohr effect we made the assumption that the binding site for DPG was the same in HbO_2 and Hb. Based on this assumption we reached the conclusion that the binding of DPG to Hb was much stronger than to HbO_2. This conclusion was consistent with the observations of Benesch et al. (1) and Caldwell et al. (10) at least as far as the binding above pH 7 was concerned, even though not in keeping with other
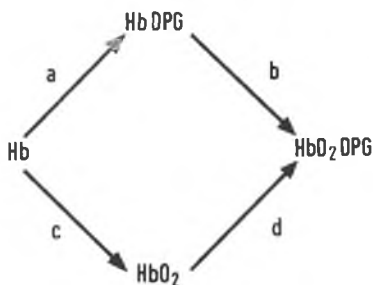
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Fig. 1.   Reaction scheme representing the several pathways as outlined in
          the text.

observations (11,12).

Since the extra Bohr effect is a net result of the reaction of DPG with
both Hb and HbO_2, we wish to report in this paper on a study about the number
of protons bound at constant pH upon a) mixing a solution of Hb and a solution
of DPG, b) oxygenation of Hb in the presence of DPG, c) oxygenation of Hb in
the absence of DPG, d) mixing a solution of HbO_2 and a solution of DPG. The
interrelation between these four processes is schematically produced in fig. 1.
HbDPG and HbO_2DPG stand for the complexes of DPG with Hb and HbO_2 respectively.
We will indicate the number of protons bound (on tetramer basis) along the
several pathways by ΔZ_a, ΔZ_b, ΔZ_c and ΔZ_d respectively. These quantities are
correlated by the equation:

$$\Delta Z_a + \Delta Z_b = \Delta Z_c + \Delta Z_d \tag{1}$$

The extra Bohr effect we reported on (6) is equal to $\Delta Z_b - \Delta Z_c$ and therefore
to $\Delta Z_d - \Delta Z_a$. In our analysis (7) we assumed that for the entire range of pH
levels ΔZ_d could be neglected as compared with ΔZ_a. The results obtained du-
ring our investigation proved this last assumption to be invalid at least for
the data below pH 7.5.

In order to be able to measure the several proton exchange processes we
developed a very accurate pH stat equipment as an extension to the titration
equipment we described earlier (13). It would go beyond the scope of this
paper to describe this pH stat equipment in detail; suffice it to say that
the rate of revolution of the burette motor is linearly prportional to devia-
tions from any particular fixed pH value. The sensitivity is such that acid
or base is added as soon as this deviation amounts to only a few tenthou-
sandths of a pH unit.

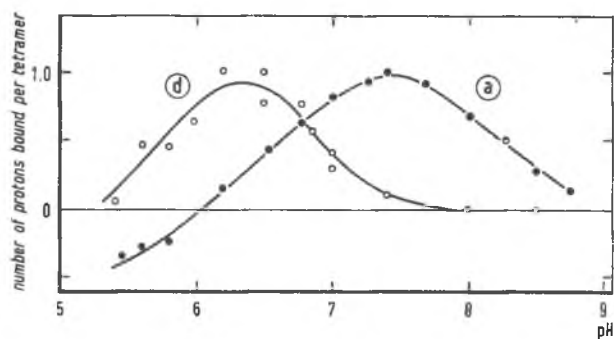Solutions of human HbO_2 were prepared according to the toluene procedure

Fig. 2.   Number of protons bound upon mixing DPG with deoxyhemoglobin
(curve a, pathway a) and oxyhemoglobin (curve b, pathway b).
Ionic strength 0.1 (KCl), hemoglobin concentration 1.2×10^{-3} M
in heme; DPG concentration 4×10^{-4} M.

(14); after dialysis the hemoglobin solutions were deionized and freed from
DPG as described (6). Solutions of Hb (2×10^{-3} M in heme; 0.1 M in KCl) were
prepared by flushing argon through a rotating cylindrical tonometer. The time
needed for complete deoxygenation was about 5 minutes. 4 ml of the solution
were anaerobically transferred to a thermostated (25°) reaction vessel and
adjusted to a particular pH. When pathway d was studied 4 ml of a solution
of HbO_2 were added. DPG (Calbiochem) was converted into the acid form by
repeated passing through Amberlite IR 120 and subsequently neutralized with
NaOH; KCl was added to adjust the solution to a 0.1 M concentration. For each
experiment 2.7 ml of the DPG solution (10^{-3} M) was added to a vessel thermo-
stated in line with the reaction vessel and adjusted to the pH of the hemo-
globin solution. When pathway a was studied the DPG solution was carefully
freed from oxygen by bubbling argon through. The DPG solutions were anaero-
bically transferred to the reaction vessel through a thin PVC tubing under
argon pressure, and the amount of acid or base needed to keep the pH con-
stant was recorded.

        In order to ensure that the pH readings recorded did in fact correspond
to the actual pH values, blanks were carried out in which 2.7 ml of a DPG
solution (10^{-3} M) were mixed with solutions of phosphate buffer having the
same buffer capacity as the hemoglobin solutions actually used. In all cases
the amounts of acid or base needed to correct for possible misadjustments
could be neglected.

        For pathway b a solution containing HbO_2 (2×10^{-3} M in heme), KCl (0.1
M) and DPG (6.5×10^{-4} M) was deoxygenated using the above mentioned tono-
meter. For each experiment 4 ml of this solution was transferred to the
reaction vessel and subsequently oxygenated. The pH was kept constant by
adding acid or base. Pathway c was carried out like b except for the absence
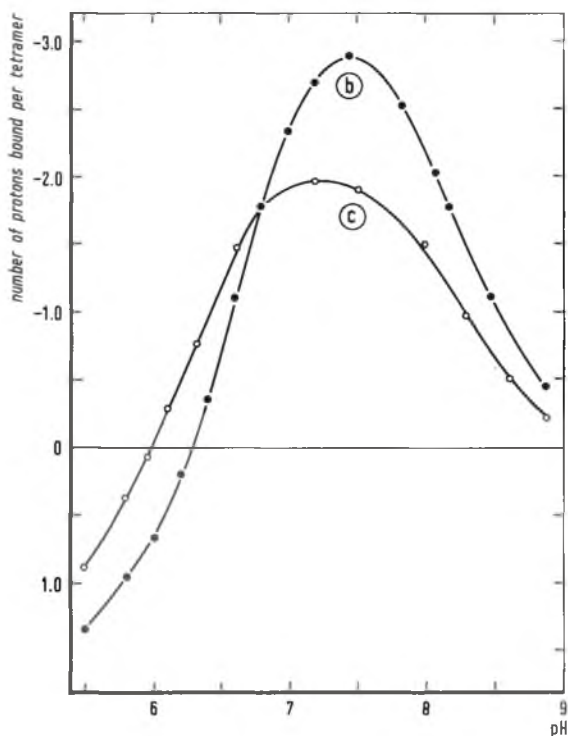
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Fig. 3. The number of protons bound on oxygenation of deoxyhemoglobin
(Bohr effect) in the presence (curve b) and absence (curve c) of
DPG. Experimental conditions as mentioned in the legend to figure 2.

of DPG. In all our experiments n the mole to mole ratio of DPG to hemoglobin
was 1.3. The hemoglobin concentrations were determined by drying in air at
105°C to constant weight, the DPG concentrations were measured by hydrogen
ion titration.

In Figure 2 ΔZ_a and ΔZ_b are plotted vs. pH. The results are very sur-
prising, for it can unambiguously be said that, judging by the results we
obtained, the interaction between DPG and Hb is similar to the one between
DPG and HbO_2. The main difference consists of a shift of the two curves with
respect to each other amounting to about one pH unit. When pathway d was
studied the data show more scattering than was seen in the study of pathway
a; the curve, however, is well defined.

Since proton uptake as a result of the reaction with DPG means that some
groups in hemoglobin change their pK to higher pK values, it is reasonable to
assume that these groups are those responsible for the binding of DPG in both
Hb and HbO_2. The binding site for DPG in Hb is well established $(8,9)$. Let us

suppose that DPG binds to the same site in HbO_2. In that case the pK shift
upon binding should start from the same pK values in both Hb and HbO_2, since
there is no proof that the groups making up the binding site in Hb have dif-
ferent pK values in HbO_2. Consequently curves a and d should have fallen in
the same pH range. This is evidently not the case, so that we are led to the
conclusion that the binding site for DPG in HbO_2 is different from the one
in Hb. We should point out, however, that this conclusion is entirely based
on the assumption that the binding groups in Hb do not show a pK shift upon
ligation. In case evidence would be produced which shows that, quite on the
contrary, DPG binds to the same site in Hb and HbO_2 our data would be an
indication for a possible role of these groups as the still missing normal
Bohr groups.

The data as presented in Figure 2 are accessible for an analysis in
terms of the equations for linked functions as described by Wyman (15).
Applying the general equation we can write:

$$\left(\frac{\delta \Delta Z}{\delta \nu}\right)_{a_H} = -\left(\frac{\delta \ln (DPG)}{\delta \ln a_H}\right)_{\nu} \tag{2}$$

where ν represents the number of DPG molecules bound by Hb or HbO_2, a_H
the proton activity and (DPG) the free DPG concentration. For $\nu = 0.5$ we
can write $(DPG) = K^{-1}$ where K is the association constant of the hemoglobin-
DPG complex. At constant pH ΔZ will be proportional to ν or $\Delta Z = \Delta Z_{\nu=1} \cdot \nu$;
$\Delta Z_{\nu=1}$ is the number of protons bound when $\nu=1$. For equation 2 we can write
therefore:

$$- \frac{d \log K}{d \ pH} = \Delta Z_{\nu=1}$$

ΔZ is always positive at least above pH=6; so that log K must increase with
decreasing pH. The result is consistent with the observation in direct bin-
ding studies of several authors (3,7,10,11). Integrating curve a between pH
7.8 and 7 an increase in log K of about 0.8 could be calculated while in
direct binding studies this increase in log K was in fact found to be about
1.0 (3). The agreement is satisfactory, especially when one bears in mind
that ν, calculated using the binding constants as given by Benesch et al.
(3), is only 0.7 to 0.8 on the average. The crossover point at pH=6 in the
curve for Hb is probably due to the fact that at low pH the change in de-
gree of ionization of the DPG molecule upon binding, accompanied by a re-
lease of protons, overcomes the proton uptake by the groups on hemoglobin
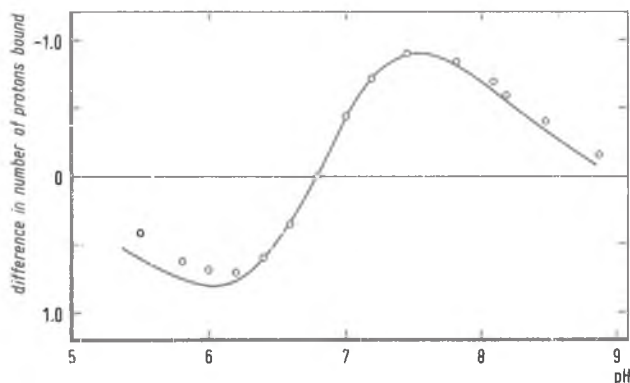involved in the binding of DPG.

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Fig. 4.   The additional DPG induced Bohr effect. The drawn line is obtained
by subtracting curve a from curve d as shown in Fig. 2. The points
are obtained by subtracting curve c from curve b as shown in Fig. 3.

In Fig. 3 ΔZ_b and ΔZ_c are plotted vs. pH. They represent the Bohr
effect in the presence and absence of DPG respectively. The curves are very
similar to those obtained from hydrogen ion titration curves (6).

In Fig. 4 $\Delta Z_d - \Delta Z_a$ and $\Delta Z_b - \Delta Z_c$ are plotted vs pH. As is seen the
agreement is satisfactory and lies within the expected experimental accuracy.
It must be emphasized here that the magnitude of ΔZ_a or ΔZ_b at a particular
pH does not give any information as to the affinity of DPG to either Hb or
HbO_2. To get information about the relative affinities of DPG to Hb and HbO_2
we have to study ΔZ of the several pathways at constant pH as a function of n;
these experiments are in progress.

In summary we can say that the additional Bohr effect and therefore the
oxygen affinity of hemoglobin in the presence of DPG is due to the interaction
of DPG with both Hb and HbO_2. In this respect the data presented seem to recon-
cile some conflicting reports on the binding of DPG to HbO_2 (3,10,11,12).

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