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# Studies on the Relations between Molecular and Functional Properties of Hemoglobin

## V. THE INFLUENCE OF TEMPERATURE ON THE BOHR EFFECT IN HUMAN AND IN HORSE HEMOGLOBIN*

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The oxygen Bohr effect in the mammalian hemoglobins is the classical case of interaction between different ligands in a protein. It is familiar in two phenomena, which are, of course, only different aspects of the same thing, namely the variation in oxygen affinity with pH and the change of proton binding that accompanies oxygenation. The total free energy involved in the effect, as estimated either from the amplitude of the change of log $p_{\frac{1}{2}}$ realized over the pH range or the difference in the dissociation constants of the oxygen-linked groups in oxy- and deoxyhemoglobin, is in the neighborhood of 1500 calories per heme. It is of interest to know how much of these 1500 calories represents a heat effect and how much an entropy effect; also, to establish the identity of the oxygen-linked acid groups which are involved. Experiments bearing on both these questions were performed on horse hemoglobin over 25 years ago with the resources then available (1, 2). These dealt with the effect of temperature, in one case on the titration curves of the oxygenated protein, in another on differential titrations in which the pH shift accompanying oxygenation was measured. The former set of experiments showed that the average apparent heat of the acid groups which ionize in the pH range from about 6.5 to 8, the range in which a considerable part of the Bohr effect is realized, is in the neighborhood of 6500 calories ($\Delta H$ = 6500 calories), a value characteristic of imidazole. The latter set indicated that the differential titration curves, in which $\Delta\bar{H}^+$ (the difference in proton bound per heme between deoxy- and oxyhemoglobin) is plotted against pH, are invariant in shape for changes of temperature. This would imply that the Bohr effect curves (log $p_{\frac{1}{2}}$ *versus* pH) are also invariant with temperature and that the proton-oxygen interaction free energy results wholly from entropy effects. From the magnitude of the pH displacement of the differential titration curves with temperature (that of the Bohr effect curves is necessarily the same), it was calculated that the heat of ionizaton of the oxygen-linked acid groups, both those responsible for the normal and for the reversed Bohr effect, is about 6500 calories, as if, like the bulk of the groups active over the middle range of the titration curve, they were all imidazole.

It is important to observe that the invariance property of the Bohr effect curves requires that the heats of ionization of the various groups involved in the effect be all the same, and the same in the oxygenated and deoxygenated forms of the protein; also, and this is only another aspect of the same thing, that the inherent heat of oxygenation, *i.e.* the total heat corrected for the heat of any accompanying ionization, be independent of pH The inherent heat of oxygenation was estimated, both from calorimetric and thermodynamic data, as −13,500, with an estimated uncertainty of about 500. (It is given by the displacement of the Bohr effect curves parallel to the log $p_{\frac{1}{2}}$ axis.)

In the original interpretation of these results, it was assumed that the oxygen-linked acid groups must be spacially contiguous to the oxygen-combining sites. On the basis of this, the identification of these groups as histidine was taken to mean that it was a histidine residue which served as a primary point of attachment of each heme to the globin, and that there was a second histidine which interacted with the heme on the opposite side of its plane and accounted for the reversed Bohr effect. Recently, the idea that the heme-globin linkage is through a histidine residue in both myoglobin and hemoglobin has been confirmed by x-ray studies (3, 4); paradoxically enough, however, the old argument on which it was based is no longer tenable. It was pointed out some years after the original interpretation was given that it is not necessary, nor indeed perhaps even plausible, to suppose that the oxygen-linked acid groups are spacially contiguous to the hemes; it is not unlikely that the effect of oxygenation on these groups results from conformational changes affecting the molecule as a whole (5). The concept of allosteric effects which have their origin in conformational changes is now fairly well established for a number of enzymes (6).

In a recent note, Rossi, Chipperfield, and Roughton (7) have reported results on the titration of the oxy and deoxy forms of human hemoglobin between pH 7 and 8 at three temperatures, from which they calculate apparent heats of ionization. For oxyhemoglobin, they obtain a value of $\Delta H$ = 6000 calories at pH 7 and $\Delta H$ = 6800 calories at pH 8. The mean is about

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the same as that obtained for horse oxyhemoglobin in the earlier studies. However, in deoxyhemoglobin, the values appear to be systematically about 1000 calories greater than in oxyhemoglobin. This difference Rossi, Chipperfield, and Roughton attribute to a difference between the heats of ionization of the oxygen-linked acid groups in the oxygenated and deoxygenated forms of the protein. If this interpretation is correct, it has important implications. The differential titration curves and the Bohr effect curves cannot be invariant in shape for changes of temperature, the inherent heat of oxygenation cannot be independent of pH, and the free energy of interaction of oxygen and proton cannot be wholly, or even largely, an entropy effect. In the pH range of 7 to 8, about 5 equivalents of proton per heme are titratable. A difference of 1000 calories in the average heat of ionization would therefore mean a difference of 5000 calories between the total heats of protonating oxy and deoxyhemoglobin between these two pH limits. Since total heat is a point function of the state of the system, this implies an equal difference between the inherent heats of oxygenation of hemoglobin at the two pH values, and this difference should give the interaction heat corresponding to the interaction free energy realized between these pH values. In view of the fact that the total interaction free energy is only ~1500 calories, such a difference would be surprising. Moreover, if, as seems likely, only one acid group per heme is involved in the alkaline part of the Bohr effect, its heat of ionization would have to be roughly 5000 calories greater (*i.e.* nearly 100% greater) in deoxy- than in oxyhemoglobin in order to account for the observed 1000-calorie difference in the average ionization (the other nonheme-linked groups active in the range having a kind of dilution effect). This also would be surprising. In any case, the picture is quite different from that previously drawn for horse hemoglobin (1, 2). The difference might, of course, represent a genuine species difference or it might result from lack of precision in earlier work. As was originally pointed out, the results on horse hemoglobin, particularly at low pH, are subject to considerable error, and the reported invariance of shape of the differential titration curves cannot be taken too literally. In view of the interest of the subject, involving as it does the interpretation of the type case of a basic phenomenon, we decided to reinvestigate the situation as completely as possible in both horse and human hemoglobin.

The experiments reported here comprise a study of the effect of temperature on the binding of proton by both the oxy and the deoxy forms of the two proteins and on the effect of temperature on the Bohr effect curves in which log $p_{\frac{1}{2}}$ is plotted as a function of pH. They were extended over the whole pH range in which the hemoglobins are stable. As will be seen here, the results on human hemoglobin are in general accord with the observations of Rossi, Chipperfield, and Roughton insofar as they show a flattening of the Bohr effect curves at the higher temperatures. On the other hand, observed more closely, they suggest a different interpretation from theirs, according to which the free energy of the oxygen-proton interaction would still be, at least primarily, a matter of entropy, the flattening of the curves being simply due to a difference between the heats of ionization of the groups responsible for the normal and reversed Bohr effects, oxygenation having no effect on these heats. The observations are consistent with the old view that the groups responsible for the "normal" part of the effect (the alkaline effect) are imidazole groups of histidine, but they suggest that the groups responsible for the "reversed" or acid part of the effect are carboxyl groups. Essentially, the same picture is obtained with horse hemoglobin.

## EXPERIMENTAL PROCEDURE AND RESULTS

*Titration Data for Oxyhemoglobins*—Tables I and II summarize all the titration data for horse and human hemoglobins obtained in the present study at four different temperatures under the conditions of ionic strength and protein concentration indicated in the headings. The results for the oxy forms were obtained by direct titrations performed according to the routine procedure already described (8). They are based on the convention that the amount of proton bound at 20° at pH 6.8 is 0 for the oxy forms. At each of the four temperatures, several titration curves were determined; the data obtained in different experiments were plotted on the same graph, and a smooth curve was drawn through the points, the scatter of which was always less than 5% in $\bar{H}^+$ over the whole curve. To relate the titration data obtained at different temperatures, we made measurements of the pH change accompanying change of temperature at constant $\bar{H}^+$ at several tie points. This is equivalent to determining the apparent heat of ionization at each such point. These measurements were then used to establish the position of the titration curves at the various temperatures in relation to the 20° curve and also to provide an internal check of the consistency of the titration results. The titration data obtained from the tie point measurements agreed to within better than 5% with those directly determined at the same temperature. A number of tie point measurements were made for human oxyhemoglobin at various values of $\bar{H}^+$, obtained in each case from the value of the pH at 20° on the basis of the titration curve at that temperature. In horse hemoglobin, two principal measurements of this kind were made, one at $\bar{H}^+ = -0.23$ (pH 6.88 a 20°) and one at $\bar{H}^+ = -1.4$ (pH 7.24 at 20°). These gave for the heats of ionization the two values $\Delta H =$ 6200 and $\Delta H = 6700$ calories, respectively, which are essentially identical with those for human hemoglobin.

*Heats of Ionization of Oxyhemoglobins*—Fig. 1 shows the apparent heats of ionization of the oxygenated forms of both proteins corresponding to the data listed in Tables I and II. They were obtained from the relation

$$\Delta H = \frac{-2.303RT_1T_2(\mathrm{pH}_{T_1} - \mathrm{pH}_{T_2})}{T_1 - T_2} \qquad (1)$$

and apply to the mean temperature of the measurements, namely 25°, as do the values of $\bar{H}^+$ against which they are plotted. For any value of $\bar{H}^+$, the pH values at the different temperatures were obtained from the titration curves constructed as described above and were therefore largely based on the tie point measurements. These pH values were then plotted against $1/T$ and the values of $\Delta H$ were calculated from the slope of the lines drawn through the points. The uncertainty involved in the $\Delta H$ values so calculated was estimated to be 300 calories or less. It will be seen that the heats for the two proteins are essentially the same over the whole range of $\bar{H}^+$ covered, probably the same within experimental error. They are also close to those originally reported by Wyman (2), as a function of pH, although the plateau in the middle range of pH, where $\Delta H =$ 6500 calories, is a little less well defined in the present results

TABLE I

*Proton bound per heme by human oxy- and deoxyhemoglobin**

All measurements were made in 0.25 M sodium chloride. Protein concentration, 0.5 to 1.0%.

| pH | 10° | | 20° | | 30° | | 40° | |
|---|---|---|---|---|---|---|---|---|
| | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ |
| 5.2 | | | +4.07 | +3.70 | +4.15 | +3.89 | | |
| 5.4 | | | +3.60 | +3.26 | +3.59 | +3.33 | +3.55 | +3.36 |
| 5.6 | +3.18 | +2.93 | +3.11 | +2.83 | +3.02 | +2.79 | +2.93 | +2.82 |
| 5.8 | +2.74 | +2.49 | +2.60 | +2.40 | +2.48 | +2.36 | +2.32 | +2.29 |
| 6.0 | +2.31 | +2.13 | +2.12 | +2.02 | +1.94 | +1.93 | +1.71 | +1.77 |
| 6.2 | +1.88 | +1.80 | +1.63 | +1.63 | +1.39 | +1.48 | +1.10 | +1.27 |
| 6.4 | +1.42 | +1.46 | +1.12 | +1.27 | +0.81 | +1.03 | +0.44 | +0.71 |
| 6.6 | +0.93 | +1.09 | +0.57 | +0.84 | +0.21 | +0.53 | −0.23 | +0.12 |
| 6.8 | +0.39 | +0.69 | 0 | +0.39 | −0.42 | −0.01 | −0.89 | −0.48 |
| 7.0 | −0.13 | +0.21 | −0.57 | −0.11 | −1.01 | −0.53 | −1.53 | −1.10 |
| 7.2 | −0.73 | −0.29 | −1.18 | −0.67 | −1.65 | −1.14 | −2.13 | −1.71 |
| 7.4 | −1.30 | −0.77 | −1.77 | −1.24 | −2.22 | −1.72 | −2.66 | −2.27 |
| 7.6 | −1.87 | −1.32 | −2.33 | −1.80 | −2.73 | −2.27 | −3.13 | −2.80 |
| 7.8 | −2.40 | −1.86 | −2.82 | −2.33 | −3.20 | −2.79 | −3.54 | −3.28 |
| 8.0 | −2.89 | −2.37 | −3.23 | −2.79 | −3.60 | −3.28 | −3.88 | −3.68 |
| 8.2 | −3.33 | −2.86 | −3.62 | −3.24 | −3.95 | −3.73 | −4.19 | −4.04 |
| 8.4 | −3.68 | −3.29 | −3.95 | −3.63 | −4.22 | −4.06 | −4.46 | −4.36 |
| 8.6 | −3.97 | −3.67 | −4.20 | −3.96 | −4.45 | −4.34 | −4.72 | −4.66 |
| 8.8 | −4.20 | −4.00 | −4.42 | −4.25 | −4.72 | −4.63 | −5.00 | −4.96 |
| 9.0 | −4.43 | −4.27 | −4.63 | −4.52 | −4.98 | −4.93 | −5.38 | −5.36 |
| 9.2 | −4.62 | −4.53 | −4.84 | −4.79 | −5.30 | −5.26 | | |
| 9.4 | −4.81 | −4.75 | −5.08 | −5.06 | | | | |
| 9.6 | −5.05 | −5.06 | −5.48 | −5.47 | | | | |
| 9.8 | −5.36 | −5.36 | −6.06 | −6.06 | | | | |
| 10.0 | −5.76 | −5.76 | −6.87 | −6.87 | | | | |
| 10.2 | | | −7.91 | −7.91 | | | | |
| 10.4 | | | −9.36 | −9.36 | | | | |

* Results for carbon monoxide hemoglobin were indistinguishable from those for oxyhemoglobin. Those given here for oxyhemoglobin at 20° are in places slightly different from the ones previously reported (8) for oxyhemoglobin at 20° in 0.3 M sodium chloride. The differences are greatest at acid pH and represent the experimental error in establishing the absolute, as opposed to the differential, titration curves.

*Titration Data for Deoxyhemoglobins*—The data for the deoxy forms of the protein listed in Tables I and II were obtained indirectly from the results on the oxy forms together with differential titration results. The differential titrations were made in two different ways, as described in a previous paper (8). In one of these, the change of pH accompanying the oxygenation of an initially completely deoxygenated solution of hemoglobin was measured and tabulated as a function of the final pH, *i.e.* the pH of the oxygenated solution. In the other, back titrations were carried out which gave the amount of acid or base required to restore the pH of the oxygenated solution to its initial value, *i.e.* the pH of the deoxygenated solution. This second procedure gives directly the difference in proton bound between oxy- and deoxyhemoglobin at any given pH (or $\bar{H}^+$). By the first procedure, the difference is only obtained indirectly, in connection with the titration curves for oxyhemoglobin.

Differential measurements of these two types were made at each of the four temperatures. The results are shown in Figs. 2 to 5. Figs. 2 and 3 give the directly measured change of pH accompanying oxygenation, *i.e.* $\Delta pH = pH_{Hb} - pH_{HbO_2}$, as a function of $pH_{HbO_2}$. Figs. 4 and 5 give $\Delta\bar{H}^+ = \bar{H}_{Hb} - \bar{H}_{HbO_2}$ as a function of pH and include results obtained by both methods.

*Oxygen Equilibrium Data*—The only remaining data to be considered are those on the Bohr effect as obtained from the oxygen equilibrium curves measured in buffered solutions at different, essentially constant pH values (9). These are shown in Figs. 6 and 7.

## DISCUSSION

The first point to be considered is the phenomenological consistency of the measurements of the two aspects of the Bohr effect, *i.e.* the titration results and the results obtained from oxygen equilibrium studies which give log $p_{\frac{1}{2}}$ as a function of pH. If the difference between the "binding" of other ions by hemoglobin and oxyhemoglobin is negligible in comparison with that of proton, then

TABLE II

*Proton bound per heme by horse oxy- and deoxyhemoglobin**

All measurements were made in 0.25 M sodium chloride. Protein concentration, 0.5 to 1.0%.

| pH | 10° | | 20° | | 30° | | 40° | |
|---|---|---|---|---|---|---|---|---|
| | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ | $\bar{H}^+_{(HbO_2)}$ | $\bar{H}^+_{(Hb)}$ |
| 5.2 | | | +4.35 | +3.90 | | | | |
| 5.4 | | | +3.80 | +3.40 | | | | |
| 5.6 | | | +3.30 | +2.97 | | | | |
| 5.8 | | | +2.80 | +2.53 | +2.70 | +2.51 | +2.55 | +2.48 |
| 6.0 | +2.40 | +2.17 | +2.27 | +2.12 | +2.10 | +2.04 | +1.89 | +1.95 |
| 6.2 | +1.95 | +1.84 | +1.75 | +1.73 | +1.50 | +1.56 | +1.20 | +1.42 |
| 6.4 | +1.45 | +1.45 | +1.20 | +1.32 | +0.85 | +1.09 | +0.49 | +0.81 |
| 6.6 | +0.95 | +1.11 | +0.60 | +0.85 | +0.20 | +0.55 | −0.25 | +0.18 |
| 6.8 | +0.40 | +0.71 | 0 | +0.37 | −0.45 | −0.03 | −1.00 | −0.49 |
| 7.0 | −0.15 | +0.27 | −0.65 | −0.17 | −1.15 | −0.60 | −1.70 | −1.15 |
| 7.2 | −0.75 | −0.19 | −1.29 | −0.73 | −1.80 | −1.24 | −2.40 | −1.81 |
| 7.4 | −1.40 | −0.78 | −1.95 | −1.31 | −2.50 | −1.87 | −3.05 | −2.47 |
| 7.6 | −2.00 | −1.36 | −2.59 | −1.91 | −3.15 | −2.54 | −3.65 | −3.14 |
| 7.8 | −2.60 | −1.95 | −3.15 | −2.55 | −3.65 | −3.20 | −4.10 | −3.70 |
| 8.0 | −3.15 | −2.55 | −3.65 | −3.17 | −4.15 | −3.75 | −4.55 | −4.19 |
| 8.2 | −3.65 | −3.13 | −4.10 | −3.68 | −4.50 | −4.18 | −4.95 | −4.69 |
| 8.4 | −4.05 | −3.65 | −4.43 | −4.08 | −4.85 | −4.60 | −5.30 | −5.13 |
| 8.6 | −4.35 | −4.07 | −4.75 | −4.50 | −5.15 | −4.98 | | |
| 8.8 | −4.65 | −4.41 | −5.00 | −4.85 | −5.45 | −5.33 | | |
| 9.0 | −4.90 | −4.74 | −5.20 | −5.10 | | | | |
| 9.2 | −5.05 | −4.99 | −5.40 | −5.34 | | | | |
| 9.4 | | | −5.70 | | | | | |

* Results on carbon monoxide hemoglobin were indistinguishable from those for oxyhemoglobin.

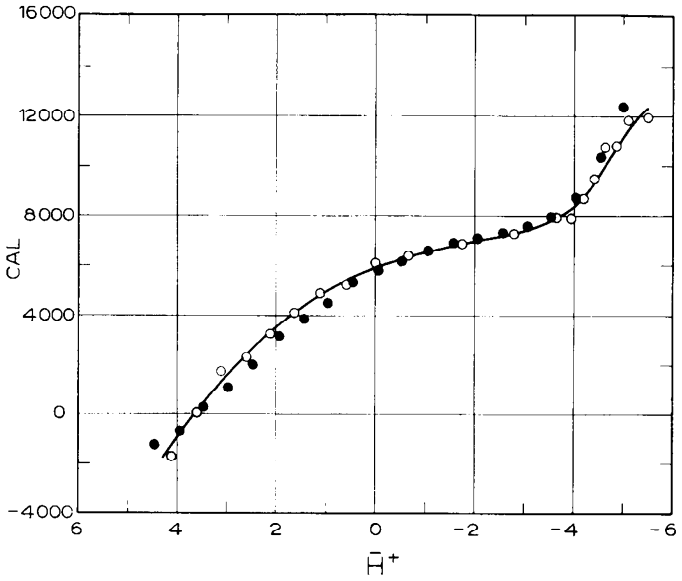


FIG. 1. Apparent heats of ionization of human and horse oxyhemoglobin in relation to proton bound, $\bar{H}^+$. These heats correspond to data shown in Tables I and II. ○, values for human hemoglobin. ●, values for horse hemoglobin. They were obtained from a plot of the values of pH *versus* $1/T$ at 10°, 20°, 30°, and 40°, and apply to the mean temperature of the measurements, 25°.

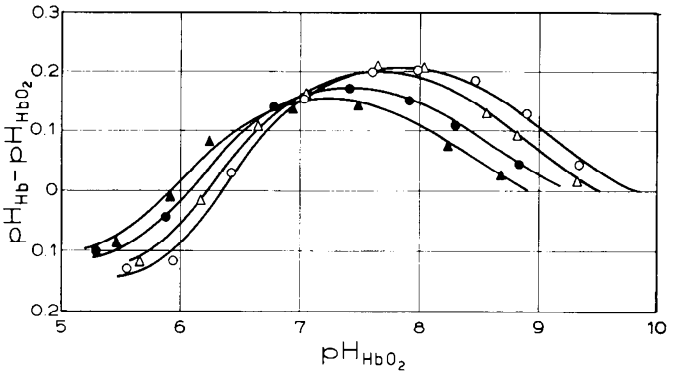


FIG. 2. Observed values of $\Delta pH = pH_{Hb} - pH_{HbO_2}$ *versus* pH of the oxygenated solution for human hemoglobin. ○——○, 10°; △——△, 20°; ●——●, 30°; ▲——▲, 40°.

$$\frac{d \log p_m}{d\text{pH}} = -\Delta\bar{H}^+ \qquad (2)$$

Here, $p_m$ is the median oxygen pressure[1] obtained from an equilibrium curve measured at constant pH, and $\Delta\bar{H}^+$ is the difference in proton bound per heme between hemoglobin and oxyhemoglobin, *i.e.* the quantity shown in Figs. 4 and 5. Owing to the invariance, or at least very near invariance, of the oxygen

[1] For a definition of this, see Wyman (10).

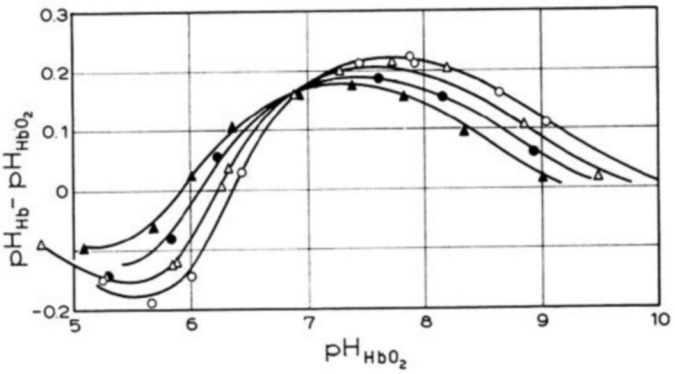


FIG. 3. Observed values of $\Delta pH = pH_{Hb} - pH_{HbO_2}$ *versus* pH of the oxygenated solution for horse hemoglobin. ○—○, 10°; △—△, 20°; ●—●, 30 °; ▲—▲, 40°.

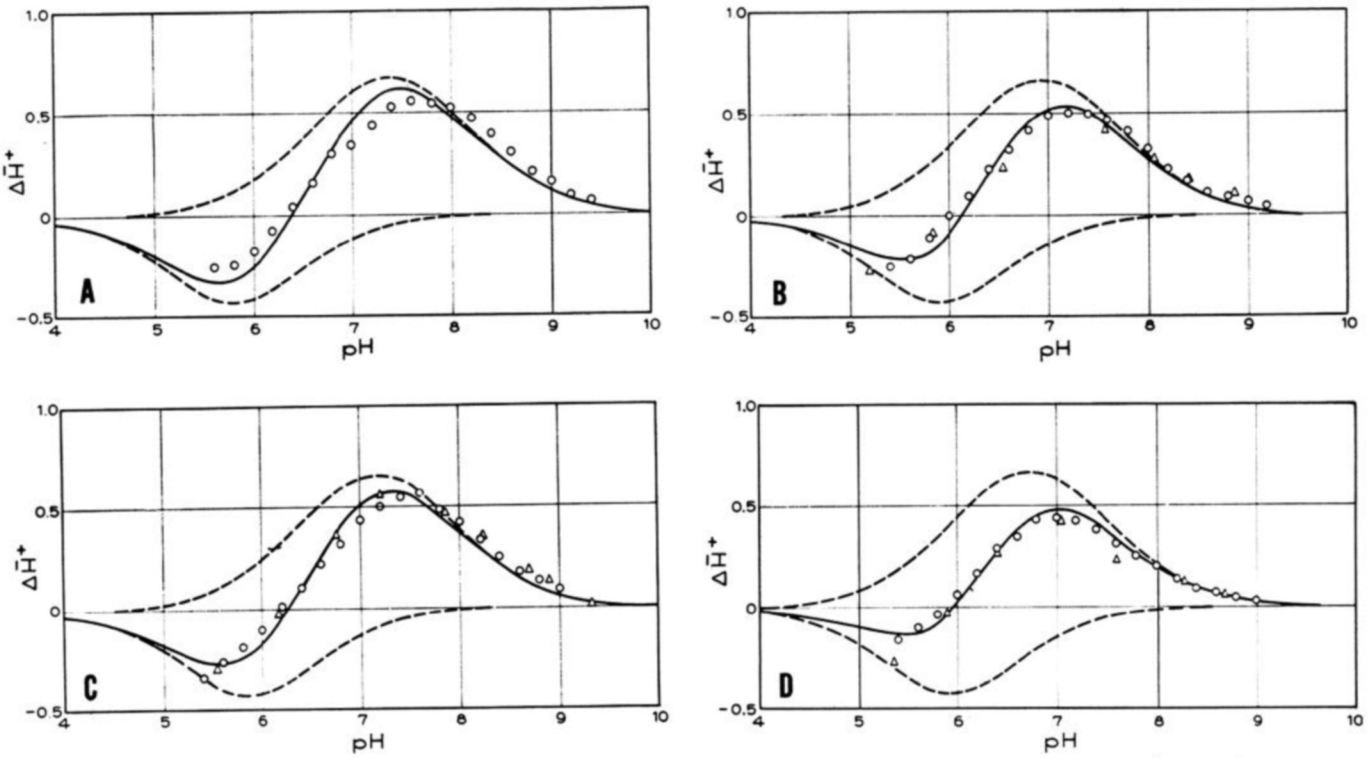


FIG. 4. Difference in proton bound per heme by human hemoglobin in the deoxy and oxy forms, *i.e.* $\Delta\bar{H}^+ = \bar{H}^+_{Hb} - \bar{H}^+_{HbO_2}$. ○ obtained from measurements of ΔpH. △ obtained directly by back titration. *Curves* are calculated from constants shown in Table III. *Dashed curves* are for each of the two groups separately. *Full curves* give net values. *A*, 10°; *B*, 30°; *C*, 20°; *D*, 40°.

equilibrium curves with pH, $p_m$ may, with good approximation, be replaced by $p_{\frac{1}{2}}$. Integration of any one of the sets of data shown in Figs. 4 and 5 with respect to pH should therefore give log $p_{\frac{1}{2}}$ as a function of pH at the corresponding temperature, subject to an arbitrary constant of integration. The *dashed curves* shown in Figs. 6 and 7 were obtained from the data plotted in Figs. 4 and 5 by graphical integration with a planimeter. They are based on the actual observations and not on the full curves shown in those figures, which are calculated curves based on assumptions to be explained presently. It will be seen that the agreement between the *dashed curves* of Figs. 6 and 7 and the experimental values of log $p_{\frac{1}{2}}$ is reasonably good, although there is some indication that specific effects of ions other than $H^+$, derived from buffers used in the oxygen equilibrium experiments, may be just appreciable.[2] All the titration measurements were of course made in the absence of buffer.

The second question to be considered is whether it is possible to fit these more extensive data, like the earlier ones on horse hemoglobin, on the assumption that the ionization heats of the oxygen-linked acid groups are the same in hemoglobin and in oxyhemoglobin. It will be recalled that the earlier data on horse hemoglobin were satisfactorily accounted for by postulating just two oxygen-linked groups per heme, each of which had an ionization heat of about 6500 calories in both oxy- and deoxyhemoglobin and was presumed to be an imidazole group. The question of the equality of the heats in the two forms of the protein is fundamental. If the groups have the same ionization heats in the two forms it means that the free energy of interaction of each group with the oxygen sites is purely an entropy effect. It implies that the heat of oxygenation of the hemoglobin is the same at pH values above and below the two ends of the region covered by the Bohr effect. It should be noted however that within this region the heat of oxygenation, even that measured at constant $\bar{H}^+$, need not be constant. If the heats of ionization of different oxygen-linked groups are different from one another and different from that of other groups which share in the buffering, then there will be heat effects accompanying oxygenation at constant $\bar{H}^+$ due to an internal redistribution of proton.

The *solid curves* in Figs. 6 and 7 are calculated on the assumption that there are just two oxygen-linked groups per heme, the

[2] This is particularly evident in the oxygen equilibrium data obtained in experiments in acetate buffers, which give systematically higher values of log $p_{\frac{1}{2}}$ than those predicted by the differential titration experiments.

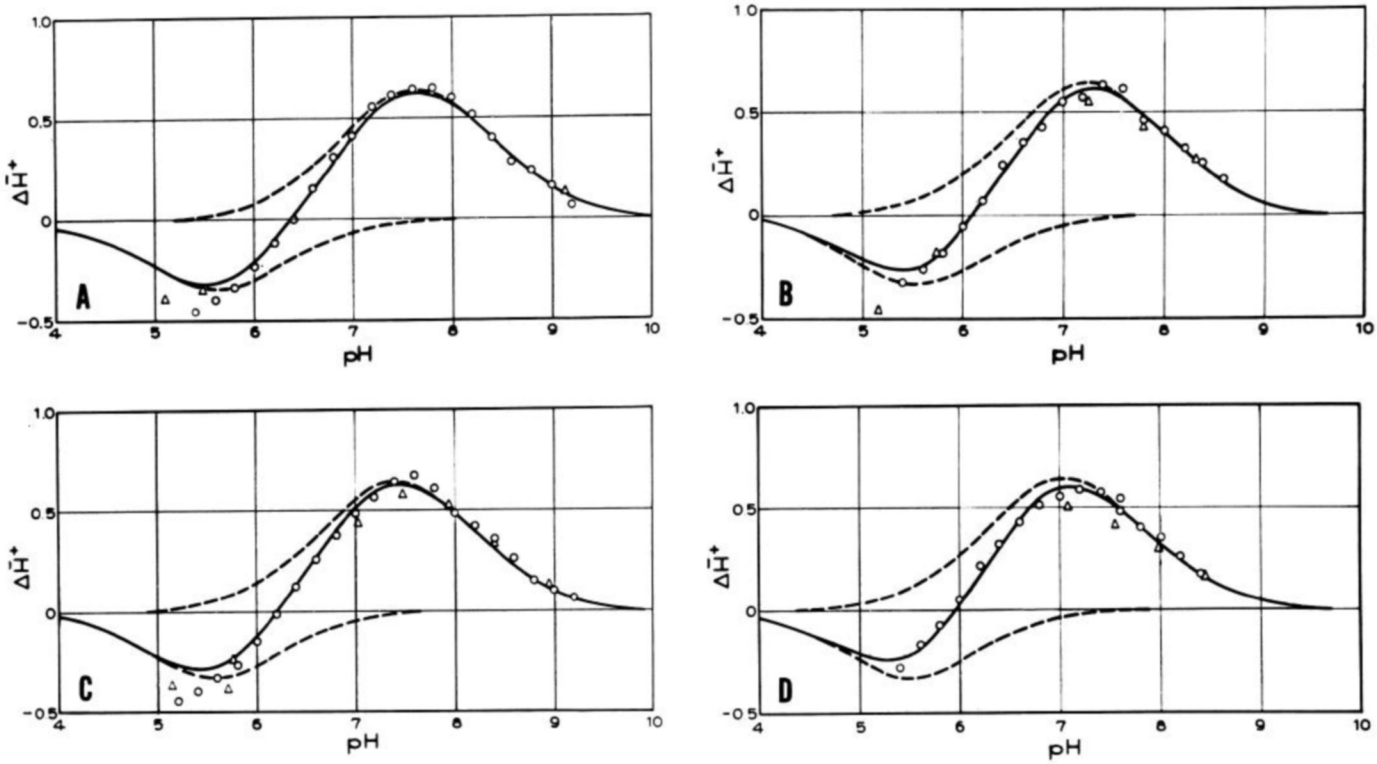


FIG. 5. Difference in proton bound per heme by horse hemoglobin in the deoxy and oxy forms, *i.e.* $\Delta\bar{H}^{+} = \bar{H}^{+}_{Hb} - \bar{H}^{+}_{HbO_2}$. ○ obtained from measurements of ΔpH. △ obtained directly by back titration. *Curves* are calculated from constants shown in Table IV. *Dashed curves* are for each of the two groups separately. *Full curves* give net values. *A*, 10°; *B*, 30°; *C*, 20°; *D*, 40°.

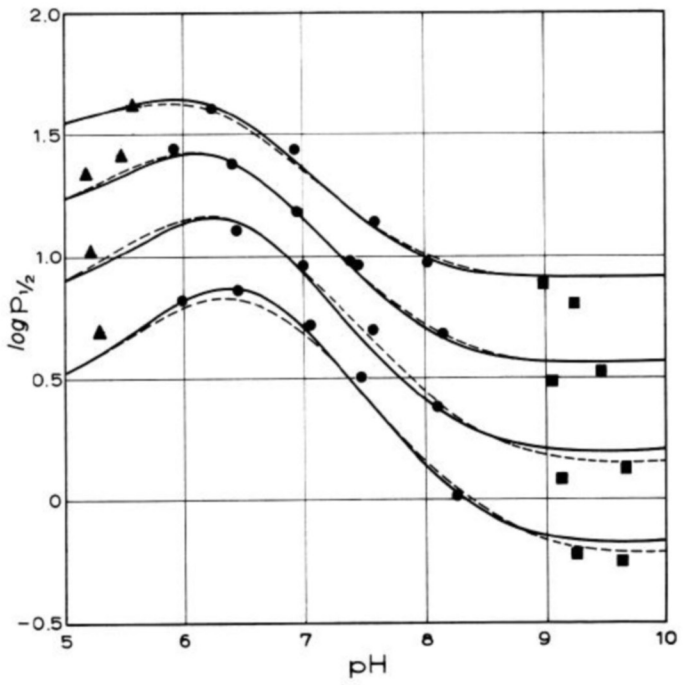


FIG. 6. Bohr effect in human hemoglobin at four temperatures, 40°, 30°, 20°, 10°, in descending order from top to bottom. *Points* show experimental values of log $p_{\frac{1}{2}}$ read from the oxygen equilibrium curves. ●, in 0.15 M phosphate buffer; ▲, in 0.4 M acetate buffer; ■, in 2% borate buffer. *Dashed lines* correspond to a graphical integration of titration data shown in Fig. 4 and involve an arbitrary constant of integration. *Solid lines* are calculated from the constants given in Table III.

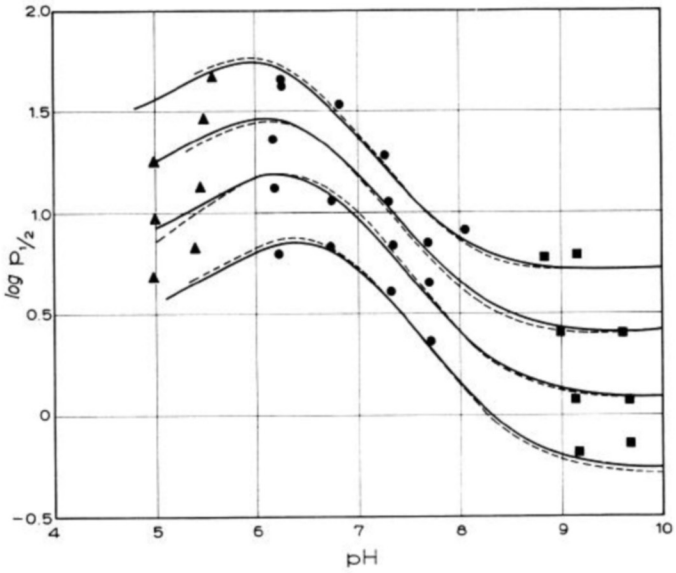


FIG. 7. Bohr effect in horse hemoglobin at four temperatures, 40°, 30°, 20°, 10°, in descending order from top to bottom. *Points* show experimental values of log $p_{\frac{1}{2}}$ read from the oxygen equilibrium curves. ●, in 0.15 M phosphate buffer; ▲, in 0.4 M acetate buffer; ■, in 2% borate buffer. *Dashed lines* correspond to a graphical integration of titration data shown in Fig. 5 and involve an arbitrary constant of integration. *Solid lines* are calculated from the constants given in Table IV.

same for each heme but different from one another, which have the same heats of ionization in the deoxy and oxy forms of the proteins. The values of the constants are those shown in Tables III and IV. According to this choice, the ionization of the more acid of the oxygen-linked groups in human hemoglobin is −1,500 calories, that of the more alkaline one +9,000 calories. The corresponding ionization heats in the case of horse hemoglobin are +1,500 and +7,600 calories, respectively. In locating the *solid curves* in Figs. 6 and 7, there is the question of their absolute up or down position, which arises from the presence of a constant of integration in each case. The positions of the curves shown in Figs. 6 and 7 were chosen so that the vertical spacing of the asymptotes at very high or low pH should give

TABLE III

*Constants for oxygen-linked acid groups in human hemoglobin*

These constants correspond to an ionization heat of $\Delta H$ = −1500 calories for the acid group and $\Delta H$ = +9000 for the alkaline group. They give the full curves shown in Fig. 6.

| Temperature | $pK_{1(Hb)}$ | $pK_{1(HbO_2)}$ | $pK_{2(Hb)}$ | $pK_{2(HbO_2)}$ |
|---|---|---|---|---|
| 10° | 5.42 | 6.22 | 8.08 | 6.68 |
| 20 | 5.46 | 6.26 | 7.85 | 6.45 |
| 30 | 5.495 | 6.295 | 7.63 | 6.23 |
| 40 | 5.54 | 6.34 | 7.42 | 6.02 |

TABLE IV

*Constants for oxygen-linked acid groups in horse hemoglobin*

These constants correspond to an ionization heat of $\Delta H$ = +1500 calories for the acid group and $\Delta H$ = +7600 calories for the alkaline group. They give the full curves shown in Fig. 7.

| Temperature | $pK_{1(Hb)}$ | $pK_{1(HbO_2)}$ | $pK_{2(Hb)}$ | $pK_{2(HbO_2)}$ |
|---|---|---|---|---|
| 10° | 5.32 | 5.92 | 8.27 | 6.97 |
| 20 | 5.27 | 5.87 | 8.07 | 6.77 |
| 30 | 5.23 | 5.83 | 7.88 | 6.58 |
| 40 | 5.20 | 5.80 | 7.70 | 6.40 |

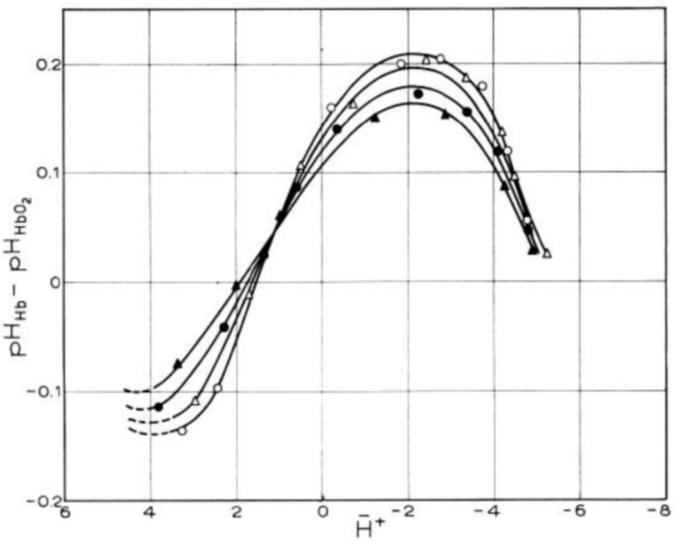


FIG. 8. Values of $\Delta pH = pH_{Hb} - pH_{HbO_2}$ as a function of proton bound, $\bar{H}^+$, for human hemoglobin. These values were read from the smooth curves in Fig. 2. ○, 10°; △, 20°; ●, 30°; ▲, 40°.

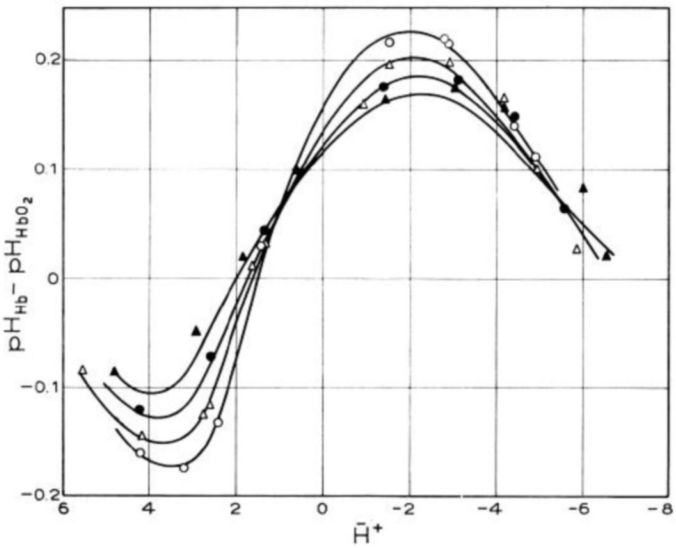


FIG. 9. Values of $\Delta pH = pH_{Hb} - pH_{HbO_2}$ as a function of proton bound, $\bar{H}^+$, for horse hemoglobin. These values were read from the smooth curves in Fig. 3. ○, 10°; △, 20°; ●, 30°; ▲, 40°.

the same inherent heat of oxygenation for each temperature interval.[3] For human hemoglobin the value of this is $\Delta H$ = −14,500 calories; for horse, −13,500, the same as that reported earlier (11).

Inspection shows that the analysis just given fits the facts to within the experimental error of the measurements for both proteins. If we accept it, we are led to the following conclusions. (*a*) The interaction of each oxygen-linked acid group with the oxygen-binding sites is an entropy effect. (*b*) The inherent heat of oxygenation, *i.e.* the heat of oxygenation corrected for any accompanying ionization or internal redistribution of proton, is constant. (*c*) The more alkaline of the oxygen-linked groups is probably an imidazole group of histidine, as has long been supposed. (*d*) The more acid of these groups, instead of being an imidazole group, is probably a carboxyl group. The last two conclusions are of course based on the very low ionization heats (−1500 and +1500) of the more acid groups and the much higher heats (9000 and 7600) for the more alkaline groups. The figures 9000 and 7600 calories both lie within the range characteristic of imidazole (12). Thus, Hammes[4] reports a value of roughly 9000 for a group identified as histidine in ribonuclease, and Chipperfield, Rossi, and Roughton[5] report a value of 8000 for free imidazole, which is very close to the value of 7700 reported by Edsall and Wyman (12).

The most critical test of the interpretation just given would lie in direct determinations of the heat of oxygenation at the two ends of the region occupied by the Bohr effect. Unfortunately, hemoglobin is too unstable at low pH to allow of this; however, another test results if we plot $\Delta pH$ against $\bar{H}^+$ at each of the four temperatures as in Figs. 8 and 9. Since $\Delta pH = pH_{Hb}$ −

[3] Based on

$$\Delta H = \frac{RT_1T_2\,(\ln p_{\frac{1}{2}T_1} - \ln p_{\frac{1}{2}T_2})}{T_1 - T_2}$$

[4] G. G. Hammes, personal communication.

[5] J. R. Chipperfield, L. Rossi, and F. J. W. Roughton, personal communication.

$pH_{HbO_2}$, it follows from Equation 1 that

$$2.303 \times RT_1T_2 \frac{(\Delta pH_{T_1} - \Delta pH_{T_2})}{T_1 - T_2} = \Delta H_{HbO_2} - \Delta H_{Hb} \quad (3)$$

in which $\Delta H_{Hb}$ and $\Delta H_{HbO_2}$ are the ionization heats of hemoglobin and oxyhemoglobin. If, therefore, our hypothesis is correct the integral of $(\Delta H_{HbO_2} - \Delta H_{Hb})\, d\bar{H}^+$ between the limits $\bar{H}^+ = 0$ and any value of $\bar{H}^+$ where the protein is saturated with proton must vanish. Since ΔpH is itself a second order quantity we are dealing here with a third order effect and our errors are correspondingly magnified. For this reason, in order to make the best of the data, the curves shown in Figs. 8 and 9 were constructed from smoothed values of ΔpH read from the curves drawn through the points in Figs. 2 and 3. It will be seen that in the case of both proteins the values of $d(\Delta pH)/dT$ have opposite signs in opposite halves of the Figs. 8 and 9. It *looks* as if they would essentially cancel in the integrals, but the data are too limited and the errors are too great for us to be sure. Calculations based on these figures show that in the alkaline portion of the Bohr effect, for which the data are most reliable, the value of $\Delta H_{Hb} - \Delta H_{HbO_2}$ is at no point greater than 600 to 800 calories. In the acid range, the figure is about the same.

It is reassuring that the results shown in Figs. 8 and 9 are what would be expected as to sign. In the acid range of pH, where the oxygen-linked groups are weakened as a result of oxygenation and where they have a negative or small heat of ionization (smaller than that of the background of groups responsible for the buffering) $\Delta H_{HbO_2} - \Delta H_{Hb}$ is positive as would be expected. In the alkaline range, the situation is reversed.[6]

In conclusion it should be pointed out that the exact values of the constants introduced in the discussion just given were obtained by trial and error and are somewhat arbitrary. No doubt with pains they could be improved on. Moreover, it is not certain that more than two ionizing groups per heme may not be involved in the Bohr effect. The important implications of our results are (*a*) that the heterotropic oxygen-proton interaction free energy, regardless of the groups involved, would seem to be predominantly a matter of entropy; (*b*) that the groups responsible for the acid part of the Bohr effect are probably carboxyl groups, those responsible for the alkaline part, imidazole as has been supposed in the past.

[6] The inherent heat of oxygenation of horse hemoglobin given by this analysis, namely, 13,500 calories, is the same as the observed heat of oxygenation of horse myoglobin, which is uncomplicated by the presence of oxygen-linked acid groups. This fact is suggestive and may be taken as additional evidence in favor of our interpretation, although it is of course far from conclusive. Recent unpublished experiments on horse myoglobin give a value of 13,500 calories for the heat of oxygenation.

SUMMARY

The titration curves of human and horse oxyhemoglobin have been determined at 10°, 20°, 30°, and 40°. Under the same conditions the pH change, and the change in proton bound, accompanying oxygenation have also been measured; from these data, the titration curves of deoxyhemoglobin were then obtained.

At the same temperatures, the oxygen equilibrium of human and horse hemoglobin was measured in buffered solutions at pH values from 5 to 9.

Both the differential titrations and the oxygen equilibrium data showed that in the two hemoglobins the Bohr effect decreased with the increase in temperature.

The results have been interpreted in terms of two oxygen-linked acid groups per heme, one responsible for the normal or alkaline Bohr effect, the other for the acid or reverse Bohr effect. The decrease of the Bohr effect with temperature can be explained, according to this model, if the two groups have different heats of ionization, which, however, for each group remain the same in both oxy- and deoxyhemoglobin.

Calculations based on this interpretation indicate that in both human and horse hemoglobin the heat of ionization of the group responsible for the normal Bohr effect is 7 to 9 kcal per mole and hence, in agreement with older conclusions, this group may be identified with an imidazole. The heat of ionization of the group involved in the acid or reverse Bohr effect appears to be very near to zero; the low value of the heat of ionization suggests that this group is a carboxyl group.

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