THE HEAT OF OXYGENATION OF HEMOGLOBIN

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Measurements of the heat of the reaction of hemoglobin with oxygen have been carried out by a number of investigators. The most comprehensive work appears to be that of Roughton and his collaborators (1, 2) who have studied the problem both by a direct calorimetric method and by application of the van't Hoff equation to observations on the effect of temperature on the dissociation pressure of oxyhemoglobin. Their measurements were carried out at pH 6.8 and 9.5. At both pH values the heats obtained by the two methods agree to within the errors of the experiments. The directly measured heat of reaction of 1 mole of oxygen with a large amount of hemoglobin is found to be independent of the percentage saturation of the hemoglobin with oxygen. This accords with the fact that the oxygen dissociation curves obtained at a given pH but at different temperatures may all be made to coincide by proper choice of the scale of oxygen pressure for each. The heat liberated per mole of oxygen at pH 6.8 is 9350 calories; that at pH 9.5 about 13,000 calories. The difference is attributed to the heat of the dissociation of hydrogen ion which accompanies oxygenation at pH 6.8 but not at 9.5. The same results were obtained with solutions of purified hemoglobin as with whole blood and laked blood.

In the present paper we shall deal also with the problem of the heat of oxygenation of hemoglobin, but from a somewhat more general point of view and on the basis of additional data. We shall consider in particular how this heat varies with pH over the entire range from pH ~ 3 to pH ~ 11. From this we shall be led to questions involving the nature and number of the groups whose acid strength is affected by oxygenation. Finally we shall deal with free energy of oxygenation in its relation to pH. The
study will be confined to solutions of crystallized hemoglobin of the horse.

It is known from determinations of molecular weight that each molecule of hemoglobin is capable of combining in all with 4 molecules of oxygen. It follows from this that the most general expression for $y$, the percentage saturation of hemoglobin with oxygen, in terms of $p$, the partial pressure of the oxygen, is that given by Adair (3) in terms of four equilibrium constants $L_i$.

\[ \frac{y}{100} = \frac{L_1 p + 2L_2 p^2 + 3L_3 p^3 + 4L_4 p^4}{4(1 + L_1 p + L_2 p^2 + L_3 p^3 + L_4 p^4)} \]

In general, at any given partial pressure of oxygen, the hemoglobin will be present in five different forms distinguished by the number (0, 1, 2, 3, 4) of molecules of oxygen combined with each molecule of hemoglobin. If we refer to these by Hb, HbO$_2$, ..., Hb(O$_2$)$_4$, we may say that $L_1$, $L_2$, $L_3$, $L_4$ refer to the equilibria between Hb and HbO$_2$, ..., Hb(O$_2$)$_4$, respectively. Actually it is possible to fit the experimental results very exactly by Equation 1 (4). Since each $L_i$ is then found to be different from zero it follows that all five forms of hemoglobin are in fact present. Each $L_i$ is made up of the individual constants describing the equilibria of the four distinct oxygen-combining groups of the hemoglobin molecule, but this does not affect the generality of Equation 1 which holds whether or not the different groups have different constants and whether or not the constant of each group is affected by oxygenation of the other groups. It is known from experiment that, for a given value of $p$, $y$ varies with pH as well as with temperature. This means that there is an interaction between some at least of the groups which dissociate hydrogen ions and those which combine with oxygen, and shows that we must regard each $L_i$ as a function of pH as well as of the temperature.

There is a particular feature of the effect of temperature and of pH on the oxygen dissociation curves which leads to a very simple relation between $p$ and the $L_i$'s and is of the first importance for our problem. It is shown by the work of Ferry and Green (5) and by that of Roughton and his collaborators (2) that the oxygen dissociation curves obtained at a given temperature but at
different pH values, or at a given pH but different temperatures, may all be made to coincide if in each case the values of \( p \) are multiplied by a suitably chosen constant. It follows from this that

\[
\left( \frac{\partial \ln L_1}{\partial T} \right)_{\text{pH}} = \cdots = \left( \frac{\partial \ln L_4}{\partial T} \right)_{\text{pH}} = \left( \frac{\partial \ln 1/p}{\partial T} \right)_{\text{pH, y}}
\]

and

\[
\left( \frac{\partial \ln L_1}{\partial \text{pH}} \right)_T = \cdots = \left( \frac{\partial \ln L_4}{\partial \text{pH}} \right)_T = \left( \frac{\partial \ln 1/p}{\partial \text{pH}} \right)_{T, y}
\]

A formal proof of these almost self-evident relations is the following. Equation 1 shows that \( y \) is a zero order homogeneous function of \( L_1, \ldots, L_4 \) and \( 1/p \). Consequently

\[
\frac{\partial y}{\partial \ln L_1} + \cdots + \frac{\partial y}{\partial \ln L_4} + \frac{\partial y}{\partial \ln 1/p} = 0
\]

Consider now the effect of changing the temperature by a factor \( \alpha \) from \( T \) to \( \alpha T \) while pH is kept constant. Then we know that \( y \) remains unchanged if at the same time we multiply all the values of \( p \) by a factor \( \beta \) which depends only on \( T \) and \( \alpha \) but not on \( p \) (or \( y \)). Thus

\[
y(T, p) = y(\alpha T, \beta p)
\]

By differentiating \( y \) with respect to \( \alpha \) and then letting \( \alpha = 1 \), we obtain

\[
T \left( \frac{\partial y}{\partial T} \right)_p + p \left( \frac{\partial y}{\partial p} \right)_T \left( \frac{\partial \beta}{\partial \alpha} \right)_{\alpha=1} = 0
\]

\( (\partial \beta/\partial \alpha)_{\alpha=1} \) depends only on the temperature and may be written as \( Tf(T) \). Equation 6 may then also be written in the form

\[
\left( \frac{\partial y}{\partial \ln L_1} \right)_p \left( \frac{\partial \ln L_1}{\partial T} \right) + \cdots + \left( \frac{\partial y}{\partial \ln L_4} \right)_p \left( \frac{\partial \ln L_4}{\partial T} \right) + \frac{\partial y}{\partial \ln 1/p} + f(T) = 0
\]

Combination of this with Equation 4 gives

\[
\left( \frac{\partial y}{\partial \ln L_1} \right) \left( f(T) - \frac{\partial \ln L_1}{\partial T} \right) + \cdots + \left( \frac{\partial y}{\partial \ln L_4} \right) \left( f(T) - \frac{\partial \ln L_4}{\partial T} \right) = 0
\]
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Since this equation holds for all values of \( p \) and \( T \), while the expressions in parentheses depend only on \( T \), and since, as may be seen from Equation 1, each of the quantities \( \partial y/\partial \ln L_1, \ldots \partial y/\partial \ln L_4 \) is a function of \( p \) of higher degree than the preceding, it follows that each of the expressions in parentheses must vanish and therefore that

\[
\frac{\partial \ln L_1}{\partial T} = \frac{\partial \ln L_2}{\partial T} = \ldots = f(T)
\]

At the same time it follows from Equation 6 and the definition of \( f(T) \) that

\[
f(T) = -\left(\frac{\partial y}{\partial T}\right)_p \left(\frac{\partial \ln p}{\partial y}\right)_T = \left(\frac{\partial \ln p}{\partial T}\right)_v
\]

Consequently we have what we set out to prove; namely, Equation 2. Exactly the same procedure, in which \( pH \) is substituted for \( T \), leads to Equation 3.

Let us now introduce the symbols \( Q_1, Q_2, Q_3, Q_4 \) to refer to the amounts of heat absorbed due to the combination of 1, 2, 3, and 4 moles of oxygen respectively with 1 mole of hemoglobin at constant temperature and \( pH \). Then, since by the van't Hoff equation

\[
Q_1 = RT^2 \frac{\partial \ln L_1}{\partial T}, \quad Q_2 = RT^2 \frac{\partial \ln L_2}{\partial T'}, \quad \text{etc.}
\]

it follows from Equation 2, if we change from natural to Briggsian logarithms, that

\[
Q_i = \frac{Q_2}{2} = \frac{Q_3}{3} = \frac{Q_4}{4} = 2.303 RT^2 \left(\frac{\partial \log 1/p}{\partial T'}\right)_v pH = \frac{Q}{4}
\]

This means that the heat absorbed is the same for each stage of the process involving the combination of 1 mole of hemoglobin with 4 moles of oxygen and equal to one-quarter of the total heat \( Q \) absorbed in the whole process.

Let us next introduce the symbols \( B_r, B_1, \ldots B_4 \) to denote the number of equivalents of base bound at any given temperature.
and pH per mole of Hb, HbO₂, ... Hb(O₂)₄ respectively. We have shown in a previous paper (6) that

\[
\frac{\partial \log L_i}{\partial \text{pH}} = B_1 - B_r, \quad \frac{\partial \log L_2}{\partial \text{pH}} = \frac{B_2 - B_r}{2}, \quad \text{etc.}
\]

Consequently it follows from Equation 3 that

\[
B_1 - B_r - \ldots - \frac{B_4 - B_1}{4} = \left( \frac{\partial \log 1/p}{\partial \text{pH}} \right)_{\nu T} = \frac{\Delta B}{4}
\]

This shows that the shift in base bound per mole of hemoglobin produced by combination with each successive mole of oxygen is the same and equal to one-quarter of the total shift \( \Delta B \) produced by complete oxygenation.

We are now in a position to write a general expression for the variation of the heat of oxygenation with pH. Since

\[
\frac{\partial}{\partial \text{pH}} \left( \frac{\partial \log 1/p}{\partial T} \right) - \frac{\partial}{\partial T} \left( \frac{\partial \log 1/p}{\partial \text{pH}} \right)
\]

it follows from Equations 11 and 13 that

\[
\frac{\partial Q}{\partial \text{pH}} = 2.303RT^2 \left( \frac{\partial \Delta B}{\partial T} \right)_{\text{pH}}
\]

We shall make use of this equation, together with experiments to be described presently, in order to determine the variation in the heat of oxygenation from pH \( \sim 4 \) to \( \sim 11 \), and in particular to answer the question whether this variation can be accounted for by the heat of hydrogen ion dissociation which is known to be coupled with oxygenation within this range. For this purpose, however, we shall employ Equation 14 in an integral form. Let \( Q_0 \) be the heat of oxygenation at some strongly acid pH = pHₐ, where the acid dissociation of the protein is unaffected by oxygenation and \( \Delta B = 0 \), and \( Q_z \) the heat at some other pH = pHₚ where \( \Delta B = \Delta B_z \), then

\[
Q_z - Q_0 = 2.303RT^2 \int_{\text{pH}_{\text{a}}}^{\text{pH}_{\text{p}}} \left( \frac{\partial \Delta B}{\partial T} \right)_{\text{pH}} d\text{pH}
\]

\[
\frac{\partial \log L_i}{\partial \text{pH}} = B_1 - B_r, \quad \frac{\partial \log L_2}{\partial \text{pH}} = \frac{B_2 - B_r}{2}, \quad \text{etc.}
\]
If we make use of the identity
\[
\left( \frac{\partial \Delta B}{\partial T} \right)_{pH} = -\left( \frac{\partial \Delta B}{\partial pH} \right)_{T} \left( \frac{\partial pH}{\partial T} \right)_{\Delta B}
\]
and integrate by parts, we obtain
\[
(16) \quad \frac{Q_s - Q_0}{\Delta B_s} = -2.303 RT^2 \left[ \left( \frac{\partial pH}{\partial T} \right)_{\Delta B} - \frac{1}{\Delta B_s} \int_{pH_s}^{pH_o} \Delta B \frac{\partial pH}{\partial T} \left( \frac{\partial pH}{\partial T} \right)_{\Delta B} dpH \right]
\]

In order to determine the effect of temperature on $\Delta B$, titration curves of oxygenated and reduced hemoglobin of the horse have been made at three temperatures: 7°, 25°, and 38°. Crystalline protein was prepared from red blood cells kindly furnished by the Massachusetts Antitoxin and Vaccine Laboratory by the same procedure as in earlier studies (6, 7). This was dissolved in 0.3 M NaCl to give the stock solutions used for the titrations. These solutions were always kept in the cold and aliquots were withdrawn for titration as the experiments proceeded. The total concentration of protein in each solution was determined by nitrogen analysis. It varied between 74 and 101 gm. per liter. The percentage of inactive hemoglobin was determined at the end of each experiment on the basis of a determination of oxygen capacity with a Van Slyke apparatus, as in earlier studies (6, 7). In one experiment this amounted to 10 per cent. In all the other experiments it was 2 per cent or less. No attempt was made to allow for it in calculating the amount of base bound by oxyhemoglobin, since the corrections involved are less than the experimental error.

Titrated aliquots of solution were divided into two parts, one of which was reduced, the other oxygenated, before injection into the electrode, in accordance with a procedure already described (6, 7). During titration enough water was always added to each aliquot in addition to acid or base so that the total dilution was always the same. This involved an increase of volume to 132 per cent of the initial volume. The pH was measured with a glass electrode. The details of the measurements, including the

1 The experiment at 7°, represented by squares in Fig. 1.
temperature control and the calibration of the electrode with standard buffers, were the same as in an earlier study (7).

The results of seven experiments are shown in Fig. 1. The quantity which is directly measured in these experiments is pH and the significant feature of the results is the difference in pH.
of a given titrated aliquot in the reduced and oxygenated conditions. In combining the results of different experiments at the same temperature a complication arises due to the fact that there are nearly always minor differences of shape in the titration curves, whether of oxygenated or reduced hemoglobin, obtained with different preparations. For this reason, in the case of the measurements at 7° and at 38°, which involve different stock solutions and preparations, we have constructed a composite titration curve for oxyhemoglobin at each of the two temperatures by drawing in free-hand a curve based on all the observations on oxyhemoglobin at that temperature. In Fig. 1 therefore no points are shown for oxyhemoglobin at 7° and 38°. The only points which are plotted are for reduced hemoglobin. They are located with reference to the composite curve for oxyhemoglobin at each temperature from the observed difference of pH of the oxygenated and reduced forms. The ordinate of each is of course fixed by the amount of acid or base present. This procedure serves to bring together the different sets of measurements at each temperature in a satisfactory way and introduces no appreciable error. The pH shift produced by oxygenation is the only significant factor in the determination of $\Delta B$, for small differences in the shape of the titration curve of oxyhemoglobin have no appreciable effect. The results given for 38° do not extend below pH 5.5, since at this temperature hemoglobin undergoes an irreversible change very rapidly at acid reactions, which renders the data unreliable. The procedure of constructing a composite curve was unnecessary for the data at 25° which were obtained from a single experiment. Only one experiment was made at 25°, because of the extensive earlier work at this temperature. In Fig. 1 the smooth curves for 25° are drawn to give the values of $\Delta B$ obtained in the earlier work (6), and show that the present results are in good agreement with it.

Careful measurement of these results shows that the only effect of changing the temperature is to displace by a constant amount the pH values at which given values of $\Delta B$ occur. The smooth curves for reduced hemoglobin at 7° and 38° are drawn so that the displacement amounts to $+0.30$ pH unit for the change from 25° to 7° and to $-0.20$ unit for the change from 25° to 38°, on the basis of the data for $\Delta B$ as a function of pH at 25° given by earlier
work. The $\Delta B$-pH curves used for this purpose are shown in the inset in Fig. 1.

If we revert now to Equation 16, we see, on the basis of these results, that of the two expressions in brackets the first is constant and equal to $-0.016$ and the second is, therefore, equal to zero. Consequently, at $25^\circ$,\[Q_z - Q_0 \over \Delta B_z = 6500 \text{ calories}\]

This value is subject to an uncertainty of at least 10 per cent. The fact that $Q_z - Q_0$ is strictly proportional to $\Delta B_z$ shows that the variation in the heat of oxygenation with pH can be accounted for entirely on the basis of the heat of the dissociation of hydrogen ion with which oxygenation is coupled. Whenever $\Delta B_z = 0$, as at strongly basic reactions and at the pH at which the titration curves of oxygenated and reduced hemoglobin cross, $Q_z = Q_0$. The figure 6500 gives the heat of dissociation of the base-binding groups which interact with the oxygen-combining centers of the protein molecule. In a previous paper we have studied the apparent heat of dissociation of hydrogen ion by oxyhemoglobin at $25^\circ$ between pH 4 and 10. In the middle part of the range this is found to be 6200 calories per equivalent, which is the value characteristic of the imidazole group of histidine. We have interpreted this to mean that the middle portion of the titration curve of oxyhemoglobin is due to the imidazole groups of the thirty-three histidine residues which are known to occur in each molecule of hemoglobin. On the basis of a more detailed analysis we have concluded that in oxyhemoglobin the pK of the weakest imidazole group is certainly less than 7.5 and that of the strongest in the neighborhood of 6. These conclusions accord well with the results of the present study. The figures 6500 and 6200 agree better than might be expected from the experimental errors. This is strong evidence that the base-binding groups which interact with the oxygen-combining groups are all imidazole groups of histidine. This conclusion may be somewhat unexpected in view of the fact that some of these groups are rendered more acid, others more basic, by oxygenation, a fact which follows from the crossing of the titration curves of oxygenated and reduced protein.
The hemoglobin molecule is known to contain four hemes and it is generally believed that these are the four oxygen-combining groups. This of itself suggests a certain degree of symmetry of the hemoglobin molecule, as if it consisted of four quadrants, one for each heme. This suggestion receives support from two facts which have been dealt with above; namely, that the heat absorbed is the same for each stage in the process involving the combination of 1 mole of hemoglobin with 4 moles of oxygen, and that the shift in the amount of base bound by the protein caused by each stage of the process is also the same. At least these facts lead us to believe that each of the oxygen-combining groups is related in the same way to a certain number of histidine units with which there is an interaction. If so, there must be at least two such units for each heme, one of which is rendered more acid, the other more basic, by oxygenation.

Let us now assume that there are in fact just two histidine units which interact with each heme, and that the behavior of each heme and its associated histidines is identical with that of the others. Then it should be possible to fit the $\Delta B$-$pH$ data satisfactorily by a suitable choice of four apparent dissociation constants $k'$, one for each histidine when the associated heme is in the reduced condition, one for each histidine when the associated heme is in the oxygenated condition. If the data were perfect they should of course determine these four $k'$ values uniquely. The basis for determining the values of $k'$ from the observed values of $\Delta B$ is the following. The shift in base bound by a weak acid at any given hydrogen ion activity $H$ in consequence of a shift of its apparent dissociation constant from $k'_1$ to $k'_2$ is given by

$$\Delta B = \frac{x(x^2 - 1)}{(1 + xz)(x + z)}$$

in which $x = \sqrt{k'_1 k'_2} / H$ and $z = \sqrt{k'_2} / \sqrt{k'_1}$. The maximum value of $\Delta B$ is

$$\Delta B_{\text{max.}} = \frac{z - 1}{z + 1}$$

and occurs at $pH = (pk'_2 + pk'_1)/2$. The shift in the total amount of base bound by a complex molecule containing a num-
ber of acid radicals is the algebraic sum of the shifts in the amounts of base bound by each. On this basis we have determined values of $k'$ for the two histidines assumed to interact with each heme which account for the $\Delta B$-pH data for 25° in a very satisfactory way. The smooth curve of Fig. 2 is constructed for the case in which the $p k'$ of one histidine is changed from 7.81 to 6.80, that of the other from 5.25 to 5.75, as a result of oxygenation of the associated heme. The circles give the experimental data taken from an earlier study (6). Over most of the range the agreement is good. The discrepancy at the extreme alkaline

![Fig. 2. Difference in the number of equivalents of base bound per mole by oxygenated and reduced hemoglobin as a function of pH. The smooth curve is calculated on the basis of assumptions discussed in the text.](image)

end of the curve might be attributed to the difficulty of completely reducing hemoglobin in this region; that at the other end to the rapidity with which hemoglobin undergoes a reversible change involving loss of oxygen-combining power at strongly acid reactions. It is found that altering the value of $p k''_2 - p k'_{1}$ for either histidine unit by as much as 10 per cent very noticeably impairs the fit of the curves, as does altering the mean $p k'$ value of either group by 0.1 to 0.2 unit. Were we to choose different $p k'$ values for the histidines associated with different hemes, the effect would be to broaden the curve and decrease the fit. On the other hand,
very little improvement could be effected by assuming that more
than two histidines interact with each heme, although if the num-
ber were made sufficiently large some compression of the curves
without loss of amplitude could be achieved. On the whole,
therefore, it appears that our assumption is justified and that
just two histidine groups interact with each heme in accordance
with the pk' values given above. The elements of the picture
then fit together in a very satisfactory way, and a variety of facts
involving the heats of oxygenation and the amount of base bound
all find a common explanation in the view that the hemoglobin
molecule is composed of four identical histidine-heme complexes.
In particular it may be pointed out that the pk' values ascribable
to the two histidine groups in the oxygenated complex conform
to the limits predicted from the effect of temperature on the titra-
tion curve of oxyhemoglobin.

We have purposely analyzed the situation on the most general
basis. The result at which we have arrived, however, accords
closely with the model recently proposed by Pauling (4) on the
basis of which he has been able to account for the oxygen disso-
ciation curves of hemoglobin very exactly. In this model, to be
sure, histidine finds no place, but it is assumed that the four
hemes are located at the corners of a square and that they are all
identical in respect to their oxygen affinity except in so far as the
equilibrium constant of each is affected by the oxygenation of the
adjacent hemes. It is of interest to compare the free energy of
interaction of two adjacent hemes as given by this model with the
free energies of interaction of each heme with the two associated
histidine units. The value for the heme-heme interaction is
1470 calories; the values for the heme-histidine interactions, cal-
culated from the pk' values given above, are 680 and 1380 calories.
They are of the same general magnitude. Our results as they re-
late to the interaction of each heme with two histidine groups
also accord with Conant's (8) picture of the hemoglobin molecule,
although of course they do not of themselves imply anything so
specific. In this picture it is assumed that the ferrous iron of
each heme is coordinated with the 4 nitrogen atoms of the
porphyrin ring in one plane and with one group of the globin
part of the molecule above, and one below, this plane. When
the heme is oxygenated, it is supposed that one of the two globin
groups is displaced from the iron by a molecule of oxygen, although the group still remains attached to the hemoglobin molecule through the globin. Provided therefore we identify these two globin groups with two histidines, we have a physical picture which would account satisfactorily for our own conclusions. The dissociation of the imidazole groups of both histidines would certainly be affected by the introduction of oxygen into the heme. It is known that the dissociation constant of the imidazole group is very sensitive to substituents and may be either increased or decreased, depending on the nature of the substituent. Thus Kirby and Neuberger (9) have shown that the acidity constant of glyoxaline is raised from 6.95 to 7.86 by the introduction of a methyl group in the 2 position and still further to 8.36 by an additional methyl group in the 4 position. On the other hand the introduction of a phenyl group in the 4 (or 5) position lowers the constant to 6.00, which is about the value observed in histidine. Of the two constants which we have ascribed to the histidines in reduced (deoxygenated) hemoglobin, one (pk' = 5.25) is much lower, the other (pk' = 7.81) much higher, than that of free histidine. This is perhaps not surprising if we suppose that the two histidines are situated on opposite sides of the plane of the porphyrin ring. When oxygenation occurs, the two values are shifted in opposite directions, each towards the value characteristic of free histidine. It may be remarked that as compared with the interaction of the hemes and the histidines, the interaction of the different hemes, as required by Pauling's model, presents a more difficult problem in relation to the spatial arrangement of these large groups.

Let us return now to the problem of the heat oxygenation. We have seen that this heat changes over the pH range in accordance with the product ΔB X 6500 calories. Probably the best values of ΔB are those calculated from the four pk' values given above, and it is these values which we have employed in calculating $Q_z - Q_o$. From the results so obtained we may at once reckon the actual heat of oxygenation over the whole pH range from a knowledge of its value at any one pH. Of Roughton's two directly determined values for the heat absorbed by combination of hemoglobin with 1 mole of oxygen, that of -9350 calories for pH 6.8 is the more reliable. It appears to be subject to an
uncertainty of no more than a few per cent. We have used 4 times this value as a fixed point for locating Curve 1 in Fig. 3, which gives the heat absorbed due to the combination of 1 mole of hemoglobin with 4 moles of oxygen over the range from pH 3 to 11. This leads to a value of $-46,800$ calories for $Q_0$, the heat of the reaction as it occurs without an accompanying change in the amount of base bound. One-fourth of this quantity, or 11,700 calories, is to be compared with Roughton's figures for pH 9.5 where there is no appreciable shift in base bound due to oxygenation. Roughton gives results for two hemoglobin preparations. For the first the directly measured heat at 19° was 12,400 calories and the calculated heat $12,450 \pm 1200$ calories; for the second the corresponding values are 14,250 and 14,100 $\pm$ 1000 calories. At this alkaline reaction measurements are more difficult and
much less accurate than at pH 6.8, and Roughton's results do not appear to be at serious variance with our calculations.

Roughton's measurements were made on ox hemoglobin, ours on hemoglobin of the horse. It might be objected that it is unjustifiable to combine results on the two kinds of hemoglobin, as we have done in using Roughton's value for the heat at pH 6.8. In view of such a possible objection Dr. D. B. Dill of the Harvard Fatigue Laboratory was good enough to determine for us oxygen dissociation curves of one of our own preparations of horse hemoglobin at different temperatures. The crystallized protein was dissolved in a phosphate buffer of ionic strength 0.5 and pH 7.05 \(^2\) at 25° to give a solution containing 49.6 gm. of hemoglobin per liter. Oxygen dissociation curves were determined at 0.5°, 20°, and 40°. The percentages of total hemoglobin in the active form in the solutions studied at these three temperatures were 98, 96.5, and 85.5 respectively. The results of the measurements are shown in Fig. 4, in which the data for the three temperatures have been brought together by adding 0.615

\[^2\text{Total phosphate} = 0.212 \text{ mole per liter; mole fraction } \text{Na}_2\text{HPO}_4 = 0.680. \text{ See Green (10).}\]

![Graph showing oxygen dissociation curves of horse hemoglobin obtained by Dill at different temperatures: \(\bigcirc\), 20°; \(\bullet\), 40°; \(\bigcirc\), 0.5°. The smooth curve corresponds to Pauling's model. \(y\) represents percentage saturation of hemoglobin with oxygen.](http://www.jbc.org/)

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to the values of log p at 0.5° and subtracting 0.425 from the values at 40°. The smooth curve corresponds to Pauling's model (4). On the basis of Equation 2 these figures give for the heat absorbed due to the reaction of 1 mole of oxygen with 1 mole of hemoglobin at the mean temperatures 10° and 30° the values 11,500 and 9100 calories respectively. The average value for 20° may be taken as 10,300, with an uncertainty of about 1000 calories. Unfortunately, no measurements were made of the pH of the solutions, but since they were made by dissolving approximately isoelectric protein (pH 6.81 for reduced hemoglobin) the pH must have been somewhat acid to that of the buffer (7.05) and not far from that at which Roughton's value of 9350 was obtained. At pH 6.9 the change in the heat corresponding to a change in pH of 0.1 unit amounts to about 550 calories. These results are therefore in agreement with the more extensive studies of Roughton on ox hemoglobin.³

In Equation 1 the activity of the oxygen is taken as equal to the partial pressure of oxygen in the gas phase. The standard state is therefore that of unit partial pressure, and the heats that we have calculated are for the reaction of hemoglobin in solution with oxygen at unit partial pressure in the gas phase. These heats therefore include the heat of solution of oxygen in the liquid phase, which has nothing to do with the combination of oxygen with hemoglobin. It is of interest to consider how much of the calculated heat, e.g. 9350 calories at pH 6.8, is due to this heat of solution of oxygen. The heat of solution of oxygen in pure water may be calculated from the data on the solubility of oxygen in water as a function of temperature. On the basis of the data given in the handbooks we obtain the following values for the heat absorbed (in calories) due to the solution of 1 mole of oxygen: at 10°, -3620; at 20°, -3200; at 30°, -2940. These figures are

³ In calculating the heat of oxygenation by the van't Hoff equation Roughton has for some reason reduced the dissociation pressures observed at different temperatures to 0° (273° absolute). The correct value of the heat is obtained by using the actual pressures observed at each temperature, since the standard state for the oxygen gas must be defined by the same hydrostatic pressure at each temperature. Actually the effect of Roughton's procedure does not appear to make a significant difference in the value for the heat at 25°. Values calculated directly from Roughton's scale factors (5), p. 2121) are 0-10°, 8680; 10-20°, 9600; 20-30°, 10,900; 30-40°, 8900; average 9500.
obtained by taking the activity of oxygen in solution as proportional to its molality; in other words by treating the solutions as ideal solutions. In so far as this is true the values should be independent both of the pressure of the saturating gas and the concentration of the oxygen in solution. They show that of the 9350 calories about 3000, or approximately one-third, are due simply to the heat of solution of oxygen at 25°. Curve 2 of Fig. 3 is for the heat of reaction of hemoglobin with oxygen in solution, obtained by taking account of this effect.

In contrast to this the free energy of the reaction is of course independent of whether the oxygen is in solution or in the gaseous phase at the corresponding partial pressure. Let us consider the free energy change in detail. If we denote by ΔF the increase of free energy accompanying the combination of 1 mole of hemoglobin with 4 moles of oxygen when the reactants are in their standard states, then

$$\Delta F = -RT \ln L_4$$

Equations 3 and 13 therefore make it possible, from a knowledge of ΔB in relation to pH, to reckon the change of ΔF with pH, just as we have reckoned the change in the heat of the reaction with pH. If ΔF₁ and ΔF₂ be the values of ΔF at pH₁ and pH₂ respectively, then

$$\Delta F_2 - \Delta F_1 = -RT \left( \ln L_4 \right)_{pH_2} + RT \left( \ln L_4 \right)_{pH_1} - 2.303 RT \int_{pH_1}^{pH_2} \Delta B \, dpH$$

If, as in reckoning the heat of the reaction, we use the values of ΔB given by the apparent dissociation constants discussed above, this equation may be integrated directly to give, for 25°

$$\Delta F_2 - \Delta F_1 = -4 \times 1365 \log \frac{H + k'_1 H + k'_4}{H + k'_1 H + k'_3}$$

in which the four k's are given by pk'₁ = 7.81, pk'₂ = 6.80, pk'₃ = 5.25, and pk'₄ = 5.75. From a knowledge of ΔF at any one pH, we may reckon it for the whole pH range. Pauling has shown that all the data of Ferry and Green at 25° when reduced to pH 8.30 may be accurately fitted by a choice of constants which leads to a value of ΔF = +2200 calories for this pH. Curve 3 in Fig. 3 is plotted from Equation 21 on the basis of this value.
The value 2200 is for the reaction when the oxygen is in the standard state; i.e., at a partial pressure of 1 mm. For any other pressure \( p \) mm. \( \Delta F \) will be \((2200 - 5450 \log p)\) calories. It follows from Equation 3 that the change with pH in the free energy of each stage of the oxygenation process is the same and equal to \( \frac{1}{2} \frac{\partial \Delta F}{\partial \text{pH}} \), although, as Pauling shows, the free energy itself is different for each stage of the process, owing to the interaction of the hemes.

**SUMMARY**

The variation in the heat of oxygenation of hemoglobin with pH has been studied from pH 3 to 11. The results can be accounted for on the basis of the dissociation of hydrogen ion which accompanies oxygenation if we assume a heat of dissociation of 6500 calories per equivalent. This is the heat of dissociation characteristic of the imidazole group of histidine, and indicates that it is groups of this kind which dissociate as a result of oxygenation of the hemoglobin molecule. From these results and Roughton's value for the heat of oxygenation at pH 6.8 we have calculated the heat of oxygenation of hemoglobin over the whole pH range from 3 to 11. It is pointed out that the heat is the same for each stage of the oxygenation process, as is the shift in the amount of base bound at constant pH. A further analysis based on the shift in the amount of base bound at 25° indicates that each molecule of hemoglobin contains four identical heme-histidine complexes, in which two histidine units interact with each heme, the energy of interaction amounting to 680 calories for one and to 1380 calories for the other. These conclusions accord with inferences based on the effect of temperature on the dissociation curves of oxyhemoglobin, and they also agree with a model recently proposed by Pauling. Finally on the basis of the shift in base bound as a function of pH we have calculated the free energy of oxygenation from pH 3 to 11.

**BIBLIOGRAPHY**
