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Direct Calorimetric Studies on the Heats of Ionization of Oxygenated and Deoxygenated Hemoglobin*

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SUMMARY

The total heats of ionization, $\bar{Q}_O$ and $\bar{Q}_R$, of bovine, human, and horse oxygenated and deoxygenated hemoglobin (O_2Hb and Hb) have been directly measured by the rapid calorimetric method over the pH range from 5.7 to 9.0, at 12–28°.

The most extensive determinations have been those on bovine hemoglobin: above about pH 6.6 the thermal titration curve for Hb lies systematically above that for O_2Hb by about 600 cal, this difference persisting practically unchanged up to the most alkaline pH (8.7) studied. The two thermal titration curves cross at approximately pH 6.3, below which the O_2Hb curve lies above the Hb curve by an increasing amount (up to 1,000 cal).

The fact that $\bar{Q}_R$ remains greater than $\bar{Q}_O$ at pH 8.7, at which the absolute value of $\bar{Q}_R$ is about 11,000 cal, implies that the heme-linked group, which ionizes in this pH range in the case of Hb, must have a heat of ionization, Q_R, of around 11,000 cal. This figure, which was confirmed by an approximate method of calculation, lies outside the range usually attributed to the heat of ionization of imidazole or its derivatives. There is some indication, from a comparison of the difference between the two thermal titration curves for human Hb and O_2Hb at approximately pH 7.3, that ($Q_R - Q_O$) is of the order of 4,000 cal, Q_O being the heat of ionization of the corresponding heme-linked group in O_2Hb. The results thus support the conclusions reached in the adjoining paper by Rossi-Bernardi and Roughton on the effect of temperature on the oxygen-linked ionizations of hemoglobin.

The relation of the present studies to the cognate effects of pH on the heat of oxygenation of hemoglobin is briefly indicated.

The heats of ionization of the protonic groups in hemoglobin have been extensively calculated from the effect of temperature on their ionization constants (*e.g.* Wyman (1)), but have, on the contrary, been relatively little studied by the methods of direct calorimetry. Some isolated observations by the latter technique were reported by Brown and Hill (2) and by Roughton (3, 4), but no extended investigation has, to our knowledge, been hitherto carried out or described. The aim of the present paper is to fill this gap in our knowledge, and, *inter alia*, to compare the values of the heats of ionization, as obtained directly, with those calculated indirectly by means of the van't Hoff isochore, as reported in the adjoining paper by Rossi-Bernardi and Roughton (5).

The present determinations have all been carried out by means of a modernized version (see Roughton (6) and Chipperfield (7)) of the rapid calorimetric method originally described by one of us 37 years ago (3). A solution of hemoglobin and a solution of acid (usually HCl) or of alkali (usually KOH) are driven into a mixing chamber, from which the emerging fluid travels down an observation tube in which one junction of a thermocouple is placed, the reference "cold" junction being situated in a constant temperature container. The "live" thermojunction was usually located at a distance from the mixing chamber corresponding to a lapsed time of 10 to 15 msec after mixture. Such a period is thousands of times longer than that required for the completion of the primary ionic reactions, but, on the other hand, is much shorter than the tempo of the slow secondary changes which may follow, after acidification of the hemoglobin, especially at acid pH. The rapid thermal method is thus at an advantage for this work, as compared with the slow methods of classical calorimetry, and, although less precise, is nevertheless accurate enough for the present purposes. The temperature of the streaming fluid could in fact usually be measured to within ±0.00015°, which, at the hemoglobin and acid (or alkali) concentrations generally used, corresponded to a precision of ±100 cal in the determination of the heat of the reactions. Experimentally it was found that duplicates usually agreed to within 200 cal or better, the results with hemoglobin solutions being, in fact, of about the same accuracy as in the numerous previous studies by this method on reactions of simpler molecules.

Hemoglobin solutions from three different mammalian species, *viz.* horse, man, and ox, have been studied. The majority of the present calorimetric experiments were done on bovine hemoglobin because of (*a*) the conveniently large amounts of blood (3 to 4 liters) obtainable from a single individual animal at the time of

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slaughter, and (*b*) the possible application of the results to a reappraisal both of the data of Roughton (4) on the effect of pH on the heat of oxygenation of bovine hemoglobin, and of the data by Ferguson and Roughton (8) and by Stadie and O'Brien (9) on the carbamino reactions of bovine hemoglobin (oxygenated and deoxygenated). The main results by direct calorimetry were also checked on human and horse hemoglobin solutions.

METHODS

Preparation of Hemoglobin Solutions

Blood from a single individual was collected from the appropriate source (slaughterhouse or Blood Transfusion Centre), with heparin used as an anticoagulant. The blood was centrifuged, the plasma was removed, and the remaining red cells were washed twice with 0.9% NaCl solution. The hemoglobin solution was prepared from the red cells by one of two methods.

Method A—Diethyl ether (10 ml/100 ml of cells) was slowly added to the red cells with stirring. Then NaCl (10 g/100 ml of cells) was slowly added with stirring. The solution was then centrifuged, and the clear hemoglobin solution was removed from under the rubbery layer of stroma. This method is essentially that described long ago by Adair and Adair (10).

Method B—The red cells were hemolyzed by addition of distilled water (40 ml/100 ml of cells). The hemoglobin solution from either A or B was then dialyzed against running tap water for 6 hours, followed by dialysis against distilled water at 4° for 48 hours. The ionic strength of the hemoglobin solution was then adjusted to that required by addition of KCl, and the solution was centrifuged in a high speed centrifuge to remove any cell debris.

The hemoglobin was then deoxygenated by placing it in a large bottle and shaking it under vacuum for periods of 10 min, the solution being shaken with O_2-free nitrogen between evacuations. This stock solution, if stored under nitrogen at 0–4°, remained stable for up to 1 week from the time of drawing of the blood. In the case of bovine or horse hemoglobin, it was thus possible to carry out, on one individual sample, as many as 10 experiments on the heat of ionization both of oxygenated and of deoxygenated hemoglobin. Owing to the much smaller samples obtainable from human donors, only about three measurements could be made on any one individual sample.

Hemoglobin solutions prepared in this way usually had concentrations of 8 to 10 meq of iron per liter; their nitrogen content (as estimated by the Kjeldahl method) had been found, in previous work, to tally to within 1% with their carbon monoxide capacity, as measured gasometrically, thus indicating no significant presence of other proteins. No difference in properties in this work was detected between solutions prepared by Methods A or B, and as the latter method proved easier it was used in the majority of experiments.

Measurement of Heat of Buffering of Hemoglobin Solutions

The heats of combination of hydrogen ions with hemoglobin solutions were measured directly in the rapid calorimeter referred to in the introductory section. Hemoglobin solutions of the required initial pH were prepared by addition of oxygen-free HCl or KOH solutions to the hemoglobin solution. The ionic strength was kept constant with KCl (usually 0.2 M). The hemoglobin solution was placed in one of the reservoirs of the rapid calorimeter together with 1 drop of octyl alcohol. In the other reservoir was placed an oxygen-free solution of dilute hydrochloric acid (2.5 to 5.0 mM) of the same ionic strength as the hemoglobin. The heat of reaction of these two solutions was determined by measuring the temperature rise of their mixture 10 to 15 msec after mixing, and subtracting therefrom the temperature rise, θ, due to heat of dilution, viscosity, and other physical factors.

The hemoglobin solution was then oxygenated by bubbling oxygen through the solution for 10 min, and the heat of reaction was measured again. The total time taken for such a pair of experiments was from 3 to 4 hours.

The value of θ was determined by "blank" experiments in which the hemoglobin solutions were mixed in the rapid reaction apparatus with KCl solutions of the same ionic strength but containing no added HCl or KOH. For Hb solutions of the concentrations used in this work, $\theta = +0.00095 \pm 0.00018°$ (average of six experiments, over the range from pH 7.5 to 9.5, at 25°). For the same Hb solutions at 25° after oxygenation, $\theta = +0.00063 \pm 0.00025°$ (also over the range from pH 7.5 to 9.5). Further, and more precise, work would be needed to decide whether this small difference between the values of θ for O_2Hb and Hb is significant.

The pH values of the following solutions were measured: Hb + an equal volume of KCl = pH_1; Hb + an equal volume of HCl = pH_2; O_2Hb + an equal volume of KCl = pH_3; O_2Hb + an equal volume of HCl = pH_4.

In the rapid calorimeter the viscous hemoglobin solution and the HCl solution were not mixed in equal proportions, but in the ratio of 1 part of Hb to x parts of HCl, the value of x being obtained by measurement of the flow rates of the hemoglobin and HCl solutions at the same time as the temperature of the mixed solutions was recorded (for details of the technique for this purpose, see Chipperfield (7)). The mean pH of the hemoglobin solution was taken equal to $pH_1 - x(pH_1 - pH_2)/2$, and similarly, for oxyhemoglobin, the mean pH is $pH_3 - x(pH_3 - pH_4)/2$. Usually x was in the range of 1 to 1.4, and $(pH_1 - pH_2)$ or $(pH_3 - pH_4)$ was around 0.3 unit. Within such pH ranges, the variation of pH with the amount of acid added is closely linear except at points of inflection of the titration curve (*e.g.* approximately pH 6.5), where there is a departure of about 10% from linearity.

Fuller details as to the basis and operation of the rapid calorimetric method can be obtained from References 3, 4, 6, and 7.

Notation

The results will be interpreted in terms of the original scheme of German and Wyman (11) and Wyman (12), according to which the mammalian hemoglobin molecule contains two acid groups per heme, the ionization constants of which vary according to whether the molecule is oxygenated (O_2Hb) or deoxygenated (Hb). These two groups are called "O_2-linked" acid groups or "Bohr" groups. Hemoglobin, of course, also contains many other acid groups, but their ionization constants are, on the present theory, supposed to be independent of the state of oxygenation of the molecule. These groups have been called "oxystable" groups (Roughton (4)).

The notation adopted in the present paper and the adjoining one by Rossi-Bernardi and Roughton (5) is similar to that previously used in Reference 4, *viz*

K_O = the ionization constant of the O_2-linked acid groups of O_2Hb in the physiological pH range

K'_O = the ionization constant of the O_2-linked acid group of O_2Hb in the more acid pH range

K_R, K'_R = the ionization constants of the corresponding acid groups of Hb

K_S = generic term for the ionization constant of the oxystable groups

Q_O, Q'_O, Q_R, Q'_R, Q_S = the corresponding heats of ionization per mole of HCl added per equivalent of iron[1]

β_O, β'_O, β_R, β'_R, β_S = the buffer powers of the corresponding groups = $-dA/d\text{pH}$, where A = equivalents of acid added per eq of iron

$\bar{Q}_O$ = total heat of buffering of O_2Hb at any given pH per mole of HCl added per eq of iron

$\bar{Q}_R$ = the total heat of buffering of Hb

$\bar{\beta}_O$, $\bar{\beta}_R$ = the corresponding total buffer powers of O_2Hb and Hb as determined experimentally from the respective titration curves

Some explanation in regard to β_S is desirable. The oxystable titration curve of hemoglobin (see Reference 4) is obtained by subtracting from the ordinary Hb titration curve the proportion of Hb present in oxylabile ionized forms at each pH (as calculated from the ionization constants, K_R and K'_R) or, similarly, by subtracting from the ordinary O_2Hb titration curve the proportion of Hb present in oxylabile ionized forms (as calculated from K_O and K'_O). The value of β_S is derived from the tangent of the angle made with the pH axis by the tangent to the oxystable titration curve at each pH (see also Equation 4 below).

This procedure for obtaining β_S thus requires a knowledge of K_R and K'_R or of K_O and K'_O, and in practice only gives reasonably dependable results above pH values of about 7.5 and if the Hb titration curve, rather than the O_2Hb titration curve, is used. The reasons therefor are that (*a*) pK_R is known with much greater certainty than pK_O (compare References 5 and 13) and (*b*) since pK'_R is less than 5.5, the ionization of this group is at least 99% complete at pH 7.5, and errors due to lack of exact knowledge of pK'_R become unimportant.

EXPERIMENTAL RESULTS

Comparison of Values of $\bar{Q}_O$ and $\bar{Q}_R$ by Direct Calorimetry with Those Calculated from Effect of Temperature on Titration Curves of O_2Hb and Hb

Table I shows the results in a set of experiments in which the heats of buffering of bovine O_2Hb and Hb were measured at several pH values within the range from 6.8 to 8.1 at 15° and 28°, the titration curves of the same O_2Hb and Hb solutions also being determined over the same pH and temperature ranges. The arithmetic mean of the directly measured values of $\bar{Q}_O$ or $\bar{Q}_R$ at 15° and 28° was then compared with the value of $\bar{Q}_O$ or $\bar{Q}_R$ calculated from the titration curves at 15° and 28° by means of the integrated form of the van't Hoff isochore equation, *viz.*

$$\bar{Q} = 2.303\,R\,(\text{pH}_{15} - \text{pH}_{28})\,\frac{(273 + 28) \times (273 + 15)}{(273 + 28) - (273 + 15)} \qquad (1)$$

[1] Q is used in place of the conventional symbol ΔH, as the latter would be apt to be confused with the changes in hydrogen ion concentration which are a prominent feature in the present work.

TABLE I

Comparison of direct and indirect values of $\bar{Q}_O$ and $\bar{Q}_R$ for bovine hemoglobin, pH 6.8 to 8.1, at 15° and 28°

Oxyhemoglobin				Deoxyhemoglobin			
pH	$\bar{Q}_O$ (direct)	$\bar{Q}_O$ (indirect)	Difference[a]	pH	$\bar{Q}_R$ (direct)	$\bar{Q}_R$ (indirect)	Difference[b]
6.85	7690	7760	+70	6.98	8060	8410	+350
7.07	7830	7610	−220	7.22	8330	8380	+50
7.26	8020	7950	−70	7.44	8590	8940	+350
7.48	8260	8130	−130	7.67	8880	9020	+140
7.73	8470	8190	−280	7.92	9260	8840	−420
8.03	8850	9000	+150				

[a] Mean difference = −80 (regarding signs); = 150 (disregarding signs).

[b] Mean difference = +94 (regarding signs); = 262 (disregarding signs).

where pH_{15} and pH_{28} are the pH values of the solution, with constant base added, at 15° and 28°, respectively.

The agreement between the direct and indirect values of the heats in Table I is well within experimental error. The value of $(\bar{Q}_R - \bar{Q}_O)$ can be estimated from a graphical plot of the data of Table I, and ranges from +300 to +500 cal by the direct method (average, +410 cal over the pH range 7.0 to 8.0), and from 0 to 850 cal by the indirect method (average, +570 cal). The difference between the results by the two methods is not significant; the much greater scatter in the indirect values is due to the fact that only a single pair of temperatures was used for the estimations of $\bar{Q}_O$ and $\bar{Q}_R$. Preferably at least three temperatures should be used (see Fig. 2).

In a similar set of experiments on horse hemoglobin over the same pH range, it was found that $(\bar{Q}_R - \bar{Q}_O)$ by the direct method ranged from +550 to +900 cal (average, +700 cal between pH 7 and 8.4) as compared with +200 to +900 cal (average, +650 cal between pH 7 and 8.4) by the indirect method. The absolute values of $\bar{Q}_R$ and $\bar{Q}_O$ by the direct method were, however, all systematically higher by 1000 to 1200 cal than by the indirect method. The same tendency was noted in much more limited data on human O_2Hb and Hb solutions. The cause of such discrepancies is not clear, although it may be associated with the presence of liquid junction effects in the pH measurements. Similar differences have in fact been found in the case of imidazole, for which the directly measured heat of ionization at 25° = 8800 cal (14) whereas the indirect heat, as calculated from the effect of temperature on pK, is 7700 cal (15). The latter data were based on electromotive force measurements with liquid junctions. In a very recent paper, describing e.m.f. measurements *without* liquid junctions, Datta and Grzybowski (16) reported an indirect value for the heat of ionization of imidazole of 8810 cal at 25°, in excellent agreement with the direct value.

Effect of pH upon $\bar{Q}_O$ and $\bar{Q}_R$

Measurements over the pH range 5.5 to 9.0 and at an ionic strength of 0.2 M were made on three different samples of bovine hemoglobin at temperatures ranging from 12° to 28°, together with measurements on a single sample of horse hemoglobin over the pH range 7.0 to 9.0 at 25°. Experiments were also carried out over two narrower pH ranges of special interest, *viz.* 7.0 to

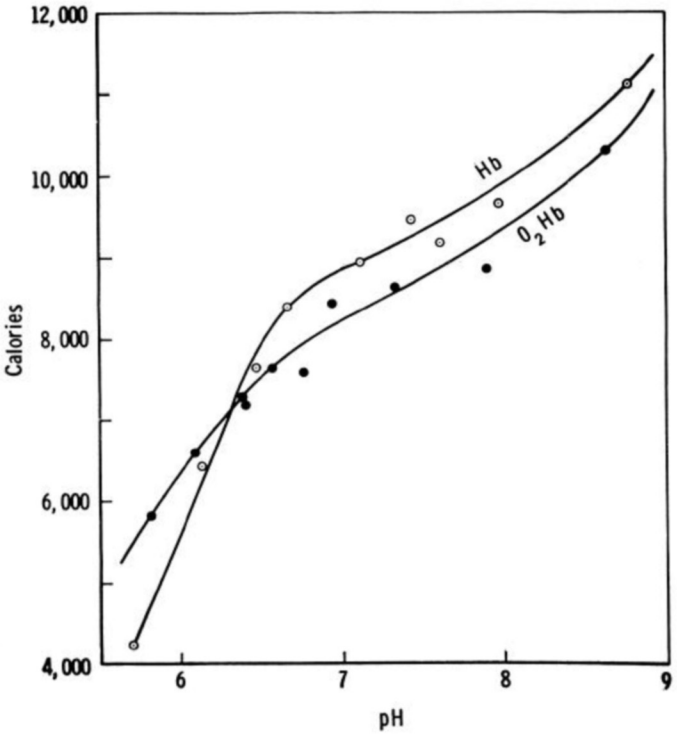

FIG. 1. Thermal titration curves of bovine O_2Hb (●) and Hb (⊙) at 25° and ionic strength of 0.2 M.

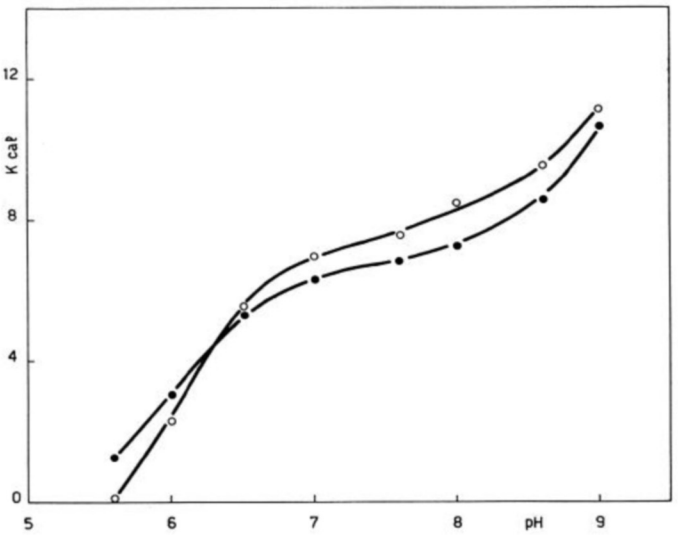

FIG. 2. Indirectly calculated values of $\bar{Q}_O$ and $\bar{Q}_R$ for human Hb (○) and O_2Hb (●), pH 5.5 to 9.0, from data of Antonini *et al.* (13).

7.5 (bovine, horse, and human Hb) and 8.4 to 9.0 (bovine and human Hb).

Fig. 1 gives the results of the most extended set on bovine oxyhemoglobin ($\bar{Q}_O$) and deoxyhemoglobin ($\bar{Q}_R$) prepared from a single animal. The curves show the same features as those in Fig. 2, which gives a plot of the indirect values of $\bar{Q}_O$ and $\bar{Q}_R$ at 30°, as calculated from the titration curve data of Antonini *et al.* (13) at 20°, 30°, and 40° on human hemoglobin. In both cases the two thermal titration curves cross at around pH 6.3 to 6.5, above which the deoxyhemoglobin curve (○) remains systematically above the oxyhemoglobin curve (●) up to (at least) pH 8.6 in Fig. 2, and possibly also in Fig. 1, although in this case there is a clear need of more observations above pH 8.0. The two curves in Fig. 2 obviously close together at and above pH 9.0. More detailed comments on three special regions of the curves follow.

Acid Range (pH 6.7 and Lower)—In the adjoining paper (5) it is shown that in this range the titration curves of O_2Hb and Hb with HCl vary according to whether the titrations are carried out (*a*) by the usual slow procedure, in which several seconds are taken to mix the acid and protein solutions, or (*b*) by mixing the reagents in a Hartridge-Roughton rapid reaction velocity apparatus and recording the pH within a lapsed time of 10 msec after mixture. Above pH 6.7 the discrepancy between the two methods disappears. The meaning of the results at acid pH in this paper is thus obscure, since, on the one hand, the temperature rises are measured within a lapsed time of the order of 10 msec and thus correspond to "rapid" titration conditions, but, on the other hand, the hemoglobin solutions, in the case, say, of a determination at pH 6.0, have first to be acidified to about pH 6.1 and remain there for many minutes before being mixed with further acid in the rapid reaction apparatus to bring them to about pH 5.9. During these "many minutes" the changes in the hemoglobin molecule responsible for the difference between the rapid and slow titration curves will certainly have occurred. Further work is therefore necessary to elucidate the significance of the heat measurements at acid pH.

Middle Range (pH 7.0 to 7.5)—According to the earlier views of Wyman *et al.* (1, 11, 12, 17), $Q_O = Q_R = Q'_O = Q'_R$ = about 6500 cal, corresponding approximately to the heat of ionization of the imidazole group of the histidine residues of the protein molecule. With the values of pK_O, pK_R, pK'_O, and pK'_R originally given by Wyman it could be shown that $(\bar{Q}_R - \bar{Q}_O)$ should have been practically zero in the pH range 7.0 to 7.5. The actual difference of about 600 cal at pH 7.0 in Fig. 1 was, however, confirmed in six other experiments at 25° on bovine hemoglobin, the values of $(\bar{Q}_R - \bar{Q}_O)$ at about pH 7.0 being 360, 430, 600, 620, 890, and 1000 (mean, 650 cal). Fig. 3*A* gives the

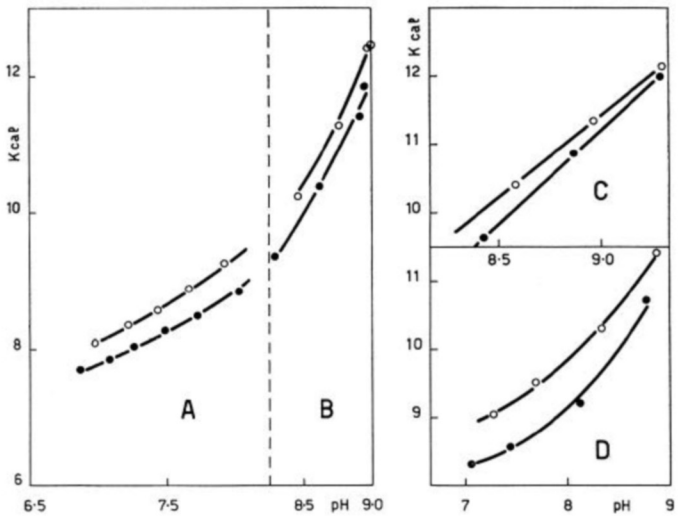

FIG. 3. Directly measured values of $\bar{Q}_O$ and $\bar{Q}_R$ at 25° for bovine hemoglobin, pH 6.8 to 8.1 (*A*), another sample of bovine hemoglobin, pH 8.4 to 9.0 (*B*), human hemoglobin, pH 8.4 to 9.1 (*C*), and horse hemoglobin, pH 7.0 to 9.0 (*D*). ○, Hb; ●, O_2Hb.

results of an experiment on bovine O_2Hb and Hb over the pH range 7.0 to 8.0, the value of $(\bar{Q}_R - \bar{Q}_O)$ in this case (360 cal) being the lowest of the series. For human hemoglobin at about pH 7.0, the mean value of $(\bar{Q}_R - \bar{Q}_O)$ was 600 cal (average of three cases) and, for horse hemoglobin, 300 cal (average of seven cases). These findings led Rossi, Chipperfield, and Roughton (18) to the view that Q_R is greater than Q_O, in the case at any rate of human and bovine hemoglobin—in line, in fact, with a similar and much earlier suggestion by Roughton (4), based on his measurements on the effect of pH upon the heat of oxygenation of bovine Hb. Two years later the experimental results of Rossi *et al.* (18) were generally confirmed by Antonini, Wyman, Brunori, Fronticelli, Bucci and Rossi-Fanelli (13), who, however, put forward an alternative explanation, according to which $Q_O = Q_R = 9000$ cal and $Q'_O = Q'_R = -1500$ cal; their view is discussed later in the present paper and more fully in the adjoining paper (5).

Alkaline Range (pH 8.4 to 9.0)—The possibility, hinted at in Fig. 1 and suggested more convincingly in Fig. 2, that $\bar{Q}_R$ may still exceed $\bar{Q}_O$ by a few hundred calories even at approximately pH 8.7, was specially investigated in a further set of experiments, of which the results on bovine O_2Hb and Hb plotted in Fig. 3*B* are an example. The values of $(pH_1 - pH_2)$ and $(pH_3 - pH_4)$ in the latter case were, above pH 8.5, all within the range of 0.08 to 0.23 unit, and the temperature rises in the blank experiments on the Hb and O_2Hb of this sample, at about pH 8.8, agreed with one another to within $\pm 0.0002°$. The difference between $\bar{Q}_R$ and $\bar{Q}_O$ at about pH 8.7 is seen to be about +400 cal; in two other experiments of the same kind, values of 600 and 500 cal were found for $(\bar{Q}_R - \bar{Q}_O)$ at about pH 8.7, making an average for the four experiments of the whole series of +500 cal, which is considered to be significant. For human hemoglobin the mean of experiments on two separate samples at pH 8.7 and 25° gave a difference of +400 cal as compared with a value of approximately +800 from the indirect heat data plotted in Fig. 2. One of these human experiments is shown in Fig. 3*C*. A single direct determination on horse hemoglobin at 25° gave a value of approximately +600 at pH 8.7 (see Fig. 3*D*). That $\bar{Q}_R$ should still be slightly, but we believe significantly, greater than $\bar{Q}_O$ in this pH range, in which $\bar{Q}_O$ is of the order of 11,000 cal, suggests strongly that Q_R itself must, in the case of human and bovine hemoglobin, be of the order of 11,000 cal or more, and must therefore be appreciably higher than has been postulated by Antonini *et al.* (13). It will now be shown how actual values of Q_R can approximately be estimated from the direct calorimetric data on $\bar{Q}_R$ and $\bar{Q}_O$ at about pH 8.7; in the adjoining paper (5) values of Q_R are obtained by a quite independent method. Both procedures unite in indicating values for Q_R definitely above 10,000 cal.

Approximate Calculation of Q_R from Data at pH 8.0 to 8.7 and 25°

In the adjoining paper Rossi-Bernardi and Roughton (5) give the following values for the ionization constants of the Bohr groups of human Hb and O_2Hb at 25°.

$$pK_R = 7.84,\quad pK'_R \doteqdot 5.02,\quad pK_O = 6.84,\quad pK'_O \doteqdot 5.64 \qquad (2)$$

According, however, to Antonini *et al.* (13),

$$pK_R = 7.74,\quad pK'_R \doteqdot 5.48,\quad pK_O = 6.34,\quad pK'_O = 6.28 \qquad (3)$$

There are thus significant differences between the two sets of authors except with respect to pK_R. Even so, it is clear that it is only the alkaline Bohr group of Hb (pK_R) that is appreciably undissociated at approximately pH 8.7. In fact, it turns out (see Reference 5) that pK_R can be estimated approximately (and separately from pK'_R, pK_O, and pK'_O) from the differential titration curves of O_2Hb and Hb within the more alkaline pH range. Similarly it might be possible, from the heat measurements in such a pH range, to obtain an approximate and semi-independent estimate of Q_R. The procedure by which this has been accomplished is as follows. For Hb the following relations hold good.

$$\bar{\beta}_R = \beta_R + \beta'_R + \beta_S \qquad (4)$$

$$\bar{Q}_R\bar{\beta}_R = Q_R\beta_R + Q'_R\beta'_R + Q_S\beta_S \qquad (5)$$

Similarly, for O_2Hb,

$$\bar{\beta}_O = \beta_O + \beta'_O + \beta_S \qquad (6)$$

$$\bar{Q}_O\bar{\beta}_O = Q_O\beta_O + Q'_O\beta'_O + Q_S\beta_S \qquad (7)$$

Elimination of Q_S from Equations 4, 5, 6, and 7 leads to the relation

$$(Q_R - \bar{Q}_R)\beta_R = (\bar{Q}_R - \bar{Q}_O)\beta_S + (\bar{Q}_R - Q'_R)\beta'_R - (\bar{Q}_O - Q_O)\beta_O - (\bar{Q}_O - Q'_O)\beta'_O \qquad (8.1)$$

or

$$Q_R = \bar{Q}_R + D_1 + D_2 - D_3 - D_4 \qquad (8.2)$$

where

$$D_1 = (\bar{Q}_R - \bar{Q}_O)\beta_S/\beta_R \qquad (8.21)$$

$$D_2 = (\bar{Q}_R - Q'_R)\beta'_R/\beta_R \qquad (8.22)$$

$$D_3 = (\bar{Q}_O - Q_O)\beta_O/\beta_R \qquad (8.23)$$

$$D_4 = (\bar{Q}_O - Q'_O)\beta'_O/\beta_R \qquad (8.24)$$

Antonini *et al.* (13) believe, on theoretical grounds, that $Q_R = Q_O$ and $Q'_R = Q'_O$. If these constraints are imposed on Equation 8.1, the values of D_1, D_2, D_3, and D_4 then resolve to

$$D_1 = (\bar{Q}_R - \bar{Q}_O)\beta_S/(\beta_R - \beta_O) \qquad (8.25)$$

$$D_2 = (\bar{Q}_R - Q'_R)\beta'_R/(\beta_R - \beta_O) \qquad (8.26)$$

$$D_3 = \bar{Q}_O\beta_O/(\beta_R - \beta_O) \qquad (8.27)$$

$$D_4 = (\bar{Q}_O - Q'_R)\beta'_O/(\beta_R - \beta_O) \qquad (8.28)$$

At first sight it would appear doubtful whether Equation 8.2 could be used to estimate Q_R independently, since several of the terms on the right-hand side of the equation contain quantities which are not independently measurable or are subject to varying, and possibly wide, degrees of uncertainty. Numerical calculations, however, show that in the alkaline pH range, 8.0 to 8.7, such uncertainties are unlikely to lead to errors of more than about 1000 to 1500 cal in the estimated values of Q_R.

The situation is, in fact, most favorable with regard to the first three quantities on the right-hand side of Equation 8.2. Of these, (*a*) $\bar{Q}_R$ is directly measurable to ±200 cal. (*b*) D_1 is composed of one factor $(\bar{Q}_R - \bar{Q}_O)$ which is directly measurable to ±300 cal (or to within ±200 cal, if the mean of several observations is taken), whereas the other factors, β_R and β_S, are, respectively, calculated from pK_R ($=7.75 \pm \sim 0.1$ for human Hb at 25°) and from $\bar{\beta}_R$, as determined from the experimental titration curve of Hb. Above pH 8.0, β'_R is negligible and, hence, from Equation 4, $\beta_S = \bar{\beta}_R - \beta_R$. Since β_S/β_R is from 3.0 to 4.0 in this pH range, the over-all uncertainty in D_1 is about ±700 cal.

TABLE II

Semi-independent computations of Q_R for human Hb in alkaline pH range

Factor	pH 8.7 (5)	pH 8.7 (13)	pH 8.0 (5)	pH 8.0 (13)
$\bar{Q}_R$	10,800	10,800	9,900	9,900
$(\bar{Q}_R - \bar{Q}_O)$	400	400	600	600
β_S	0.934	0.974	1.545	1.574
β_R	0.246	0.206	0.555	0.526
β'_R	0.0005	0.0014	0.0024	0.0065
β_O	0.031	0.010	0.140	0.049
β'_O	0.002	0.0081	0.010	0.042
D_1	1,520	1,940	1,670	1,800
D_2	30	90	60	160
D_3	550	530	820	950
D_4	80	510	140	950
Q_R	11,700	11,800	10,800	10,000

(*c*) D_2 is composed of one factor $(\bar{Q}_R - Q'_R)$ which may be of the order of 14,000 cal, but of a second factor (β'_R/β_R) which is only of the order of 0.01 at pH 8 and 0.005 or less at pH 8.7. D_2 is thus of the order of 150 cal or less in the pH range 8.0 to 8.7, so that uncertainties in its value are unimportant.

As regards the remaining terms, D_3 and D_4, it is necessary to make approximate assumptions with regard to the values of Q_O and Q'_O as well as of pK_O and pK'_O. Computations have accordingly been made on the basis of (*a*) Reference 5, according to which the values of pK_R, pK'_R, pK_O, and pK'_O are as given in Equation 3.1 above, and $Q'_R \cong -3700$, $Q_O \cong 6000$, and $Q'_O \cong 1500$ cal; and (*b*) Reference 13, according to which the values of pK_R, etc., are as given in Equation 3.2 and $Q_R = Q_O$, $Q'_R = Q'_O \cong -1500$ cal.

The second and third columns of Table II show the results of the computations on these two separate bases at pH 8.7. The assigned values of $\bar{Q}_R$ and $(\bar{Q}_R - \bar{Q}_O)$ are the averages of the direct measurements. The final estimates of Q_R at the foot of the columns agree to within 100 cal, the higher value of D_1 in the second case, *i.e.* 1940 cal (compared to 1520), being compensated by a higher value of D_4, *i.e.* 510 cal (compared to 80).

The fourth and fifth columns of Table II show similar calculations at pH 8.0. These diverge from one another, owing to the increasing size (and uncertainty) of the D_3 and D_4 terms. Such a tendency becomes increasingly pronounced as the pH of calculation is lowered below 8.0. Below pH 7.0, in fact, the divergences are found to rise to several thousands of calories, and the method is obviously out of court. Above pH 9.0 D_2, D_3, and D_4 all settle down to relatively small constant quantities, but D_1 becomes indeterminate, being then the product of a rapidly diminishing quantity $(\bar{Q}_R - \bar{Q}_O)$ and a rapidly increasing quantity (β_S/β_R). Thus the method is in practice limited to a comparatively narrow alkaline pH range around 8.7. The mean value at the latter given in Table II, *viz.* 11,750 cal, is subject to a probable margin of uncertainty of about 1,000 cal; if the computations at pH 8.0 are also included, the mean value for Q_R comes down to 11,100 (±1,000) cal, in reasonable tally with the independent value of Q_R for human Hb given in the adjoining paper (5), *viz.* 10,700 ± 350 cal.

Calculations similar to those of Table II on the mean of the bovine data at approximately pH 8.7 gave Q_R = 11,600 (± ~1,000) cal for bovine hemoglobin at 25°.

An Indication as to Value of $(Q_R - Q_O)$ from Thermal Measurements at pH = $(pK_O + pK_R)/2$

The question also arose as to the possibility of deriving, from the direct thermal measurements, a value for $(Q_R - Q_O)$ to compare with the figure of 5100 cal for human hemoglobin obtained indirectly from the effect of temperature on pK_R and pK_O (5). The following procedure goes some way in this direction.

At $pH_M = \frac{1}{2}(pK_O + pK_R)$, $\beta_O = \beta_R$, so that at this pH subtraction of Equation 4 from Equation 6 gives

$$\bar{\beta}_O = \bar{\beta}_R + (\beta'_O - \beta'_R) \tag{9.1}$$

Similarly, subtraction of Equation 7 from Equation 5 gives

$$(\bar{Q}_R\bar{\beta}_R - \bar{Q}_O\bar{\beta}_O) = (Q_R - Q_O)\beta_R + (Q'_R\beta'_R - Q'_O\beta'_O)$$

or

$$(Q_R - Q_O) = \frac{1}{\beta_R}\{(\bar{Q}_R\bar{\beta}_R - \bar{Q}_O\bar{\beta}_O) - (Q'_R\beta'_R - Q'_O\beta'_O)\} \tag{9.2}$$

Substitution of Equation 9.1 into 9.2 after simplification gives

$$\begin{aligned}(Q_R - Q_O) = {} & (\bar{Q}_R - \bar{Q}_O)\bar{\beta}_R/\beta_R + (\bar{Q}_O - Q'_R)\beta'_R/\beta_R \\ & - (\bar{Q}_O - Q'_O)\beta'_O/\beta_R = D'_1 + D'_2 - D'_3\end{aligned} \tag{9.3}$$

For human hemoglobin at 25°, $\bar{Q}_R$ = 8600, $\bar{Q}_O$ = 8000 cal; $\bar{\beta}_R$ = 2.9 (all directly measured). On the basis of Equation 3.1,

$$pK_M = 7.34, \quad \beta_R = 0.42, \quad \beta'_R = 0.012, \quad \beta'_O = 0.044$$

Hence

$$D'_1 = 600 \times 2.9/0.42 = 4200$$

$$D'_2 = (\bar{Q}_O - Q'_R) \times 0.03 \doteqdot 360$$

$$D'_3 = (\bar{Q}_O - Q'_O) \times 0.105 \doteqdot 700$$

Thus $(Q_R - Q_O) \doteqdot 4200 + 360 - 700 \doteqdot 3850$ cal, as compared with the figure in Reference 5 of 5100 cal, a fair tally under all the circumstances. The validity of the present procedure obviously hinges upon D'_1 (a relatively certain quantity) being substantially greater than the less definite terms D'_2 and D'_3. This condition is fairly well met on the basis of the values of pK_R, pK'_R, pK_O, and pK'_O of Reference 5; conversely, the fact that $(Q_R - Q_O)$, as calculated by the present method, comes within about 1200 cal of its value, as calculated from the effects of temperature on pK_R and pK_O (5), does give a measure of independent support to the view that Q_R exceeds Q_O by 4000 to 5000 cal.

Relation between Heats of Ionization of Bohr Groups and Effects of pH on Heat of Oxygenation of Hemoglobin

Interrelated with the effect of oxygenation on the heat of combination of hemoglobin with hydrogen ions is the effect of pH on the heat of oxygenation of hemoglobin. The latter subject was investigated in a preliminary way by Brown and Hill (2) over 40 years ago; a decade later Roughton (4) gave a more comprehensive theoretical treatment in which account was taken of the "alkaline Bohr groups" and the "oxystable groups" of the hemoglobin molecule, but no allowance was made for the "acid Bohr groups," since the latter were not discovered until a few years later by Wyman (1). Roughton (4) applied his theoretical equations particularly to his measurements of the heats of combination of O_2 with bovine Hb in M/15 phosphate buffer at pH 6.8 ($= Q_H$) and at approximately pH 9.5 ($= Q_B$), at which the

alkaline Bohr groups must have been almost completely ionized. The observed difference between Q_B and Q_H, *i.e.* about 4000 cal, could, it seemed, be accounted for only in part by the secondary ionic reactions which occur in hemoglobin over the pH range 6.0 to 9.0; it was suggested that the residue of the effect might be due to Q_R being significantly greater than Q_O.

As an offshoot of the present work we have made a start, in collaboration with Dr. J. C. Kernohan, on a reinvestigation of the effect of pH on the heat of oxygenation of hemoglobin. The earlier theoretical formulas have been modernized and extended to include the acid Bohr effects and other more recent concepts and results, and further experimental measurements have lately been made, by the present method, on the heat of combination of oxygen with bovine and human hemoglobin over the pH range 7.0 to 10.0. As in the earlier work (4), external buffers, *e.g.* phosphate or borate, have in some cases been added to the hemoglobin solutions, but special attention has also been paid to the heat of oxygenation of Hb in 0.2 M KCl, without added external buffer. Two of the present workers, together with their colleague, Dr. Kernohan, have recently taken up positions in other laboratories, and it is in consequence uncertain when the joint work on this aspect can be rounded off. In these circumstances it may be useful to place briefly on record such indications as already appear definite.

For bovine Hb in 0.2 M KCl at 25°, without external buffer, Q_H was found to increase from about 8,000 to 9,500 cal as the pH was raised from 7.0 to 8.0, as compared with the Q_B value of about 11,200 cal at approximately pH 9.5. The excess of Q_B over Q_H agreed roughly, *i.e.* to within ±600 cal, with that calculated from the modernized theoretical formulas if the values of Q_R, Q'_R, Q_O, Q'_O, pK_R, pK'_R, pK_O, and pK'_O postulated by Rossi-Bernardi and Roughton (5) are used.

In two experiments at 25° on human Hb in 0.2 M KCl, prepared from the blood of two separate donors, it was found that $(Q_B - Q_H)$, at pH 7.0 with no external buffer, was at least 6000 cal, *i.e.* some 2000 cal greater than that calculated on the basis of the values of Q_R, etc., given in Reference 5, and 3400 cal greater than that calculated on the basis of Reference 13. Although there is thus a discrepancy of at least 2000 cal still to be explained, it appears that the results of these experiments are more nearly in line with the views of Rossi-Bernardi and Roughton (5) than with those of Antonini *et al.* (13). Much further work, both experimental and theoretical, is clearly needed in this field.

Note on Relations among Q_R, Q_O, Q'_R, and Q'_O

Consider the over-all process $O_2 + Hb + 2H^+ = H'\cdot O_2Hb\cdot H$. This process can occur by two separate paths.

$$\begin{aligned} O_2 + Hb &= O_2Hb && \text{Heat} = Q_B \\ H^+ + O_2Hb &= O_2Hb\cdot H && \text{Heat} = Q_O \\ H^+ + O_2Hb\cdot H &= H'\cdot O_2Hb\cdot H && \text{Heat} = Q'_O \end{aligned} \qquad (a)$$

$$\text{Over-all heat} = Q_B + Q_O + Q'_O$$

$$\begin{aligned} H^+ + Hb &= Hb\cdot H && \text{Heat} = Q_R \\ Hb\cdot H + H^+ &= H'\cdot Hb\cdot H && \text{Heat} = Q'_R \\ O_2 + H'\cdot Hb\cdot H &= H'\cdot O_2Hb\cdot H && \text{Heat} = Q_A \text{ (say)} \end{aligned} \qquad (b)$$

$$\text{Over-all heat} = Q_A + Q_R + Q'_R$$

Since the total heat of the over-all process must be the same whatever the route, it follows that

$$Q_A + Q_R + Q'_R = Q_B + Q_O + Q'_O$$

whence

$$Q_B - Q_A = (Q_R - Q_O) + (Q'_R - Q'_O) \qquad (10)$$

Antonini *et al.* (13), from their data on the effect of temperature on $p_{0.5}$ (the oxygen pressure for half-saturation of human Hb) at pH 5.6 and 9.6, calculate that Q_A is approximately equal to Q_B. Hence, from Equation 10,

$$Q_R - Q_O \doteqdot Q'_O - Q'_R$$

but neither $(Q_R - Q_O)$ nor $(Q'_O - Q'_R)$ is necessarily zero. The adjoining paper (5) does indeed indicate that $(Q_R - Q_O)$ and $(Q'_O - Q'_R)$ may both be of the order of 5000 cal, although in the case of $(Q'_O - Q'_R)$ there is a wide margin of uncertainty, owing to the difficulty of estimating Q'_O and Q'_R.

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