CCXCVI. THE THERMOCHEMISTRY OF THE OXYGEN-HAEMOGLOBIN REACTION.

II. COMPARISON OF THE HEAT AS MEASURED DIRECTLY ON PURIFIED HAEMOGLOBIN WITH THAT CALCULATED INDIRECTLY BY THE VAN'T HOFF ISOCHORE.

BY FRANCIS JOHN WORSLEY ROUGHTON,

WITH

G. S. ADAIR, J. BARCROFT, G. GOLDSCHMIDT, W. HERKEL,
R. M. HILL, A. B. KEYS AND G. B. RAY.

From the Physiological Laboratory, Cambridge.

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INTRODUCTION.

IN Part I [Roughton, 1935] data have been given for:

(A) The relation between the heat liberated and the percentage saturation of haemoglobin with oxygen when haemoglobin is equilibrated with insufficient oxygen to saturate it completely.

(B) The effect of laking and of purification on the heat of reaction of oxygen with haemoglobin.

(C) The relation between the heat of the reaction and the *pH* and buffer content of the solution. This question has also been treated theoretically.

(D) Furthermore Bateman & Roughton [1935] have shown that the "slow" heat of reaction between O_2 and Hb (as measured by the classical methods of calorimetry) agrees within experimental error with the "rapid" heat liberated in 0.008–0.02 sec.

These results all conform to simple expectations. The present paper deals with the last item of the programme, sketched in Part I, namely:

(E) The comparison, for purified haemoglobin solutions, of the heat of the reaction, as measured directly, with that calculated indirectly from the effect of temperature on the oxyhaemoglobin dissociation curve by means of the Van't Hoff Isochore, which runs [Adair, quoted by Barcroft, 1928]

$$Q_y' = \frac{RT_1T_2}{T_1 - T_2} \log_e \frac{C_1}{C_2} \quad \dots\dots(1),$$

where Q_y' = heat of reaction at percentage saturation y ; R = the gas constant, i.e. 1.98 calories; C_1, C_2 are the respective concentrations of O_2 in the gas phase required to maintain the haemoglobin at percentage saturation y at absolute temperatures T_1, T_2 respectively.

Such a comparison (in the case of purified Hb) has not been made since the pioneer work of Barcroft & Hill [1909], in which, however, abnormally high values for the heat of reaction were obtained. To secure reliable data of this kind, requires preferably a team of workers, owing to the lability of the haemoglobin. In the present paper experimental data have been obtained under two different sets of conditions:

(I) At *pH* 6.8, *M*/15 phosphate buffer (by Goldschmidt, Ray & Roughton).

In this region it was experimentally possible to get reliable data over a large

range of temperature (0–40°) and thus to test equation (1) very thoroughly. In particular it was desired to check the view of previous workers, that the effect of temperature on the dissociation curve is to alter its scale, but not to alter its shape. It was also desired to see whether the effect of temperature could be expressed by a smooth relationship, or whether there were, at or near certain temperatures, breaks similar to those reported by Crozier [1924] for complex physiological processes.

The results were not, however, expected to be so clear-cut from the theoretical point of view, for within the pH range 6.0–9.0, the heat of reaction is affected by the dissociation and subsequent buffering of the oxy-labile H ions which are liberated when haemoglobin is oxygenated (for definition of this term and discussion, *v. Roughton* [1935]). Under these conditions an elaborate theoretical treatment (*v. infra*) is needed to arrive at even a provisional relationship between the direct and indirect heats. The data were therefore supplemented by a set of observations.

(II) At pH c. 9.5 (by Adair, Barcroft, Herkel, Hill, Keys & Roughton).

At this pH, both reduced and oxyhaemoglobin are almost entirely in the oxy-labile ionized form, and the primary reaction $O_2 + Hb^- \rightarrow O_2Hb^-$, is not complicated by any secondary ionic changes. In this range, haemoglobin is relatively stable, but owing to the greater affinity of Hb for O_2 at alkaline reactions, the concentrations of O_2 in the gas phase could not be estimated with nearly as great percentage accuracy as in (I)—hence the calculated values of the heat are subject to a much larger error. Had it been possible to work at pH 5.6, where reduced and oxyhaemoglobin are both almost entirely in the oxy-labile unionized form, this difficulty would have been obviated; unfortunately haemoglobin changes too readily to methaemoglobin at this acid reaction, for this to be feasible.

(III) The last section of this paper (by Roughton), deals with two theoretical problems, which have arisen from consideration of the data obtained under (I) and (II), viz.:

(a) The question of the interpretation of the Van't Hoff Isochore from the standpoint of Adair's [1925] intermediate compound hypothesis of the reaction between oxygen and haemoglobin, according to which the reaction proceeds in four stages.

(b) The theoretical basis of the relationship between the direct and indirect heats, in the pH range 6.0–9.0 when foreign buffers are present.

I. EXPERIMENTS AT pH 6.8.

By S. GOLDSCHMIDT,¹ G. B. RAY AND F. J. W. ROUGHTON.

EXPERIMENTAL METHODS.

A. The dissociation curves.

(a) *Purification.* The haemoglobin from ox blood was purified by the method of Adair *et al.* [1921]. It was dialysed from 4 to 6 days at 0–1° against a *M*/15 phosphate buffer solution of pH 6.90 at 0°. Latterly the dialysis has been speeded up by rocking the dialysing sacs in a large shaker. The final solutions had an oxygen capacity of 20–26 volumes %: and were stored at 0.5° until required.

(b) Tests were made for the presence and amount of the so-called "inactive haemoglobin". A preliminary comparison of the amount of haemoglobin deter-

¹ From the University of Pennsylvania.

mined by the oxygen capacity with that obtained by the method of Stadie [1920] and the refractometer [Stoddard & Adair, 1923] gave a measure of the amount of haemoglobin which did not combine with oxygen. In none of the solutions used did this value exceed 5%, nor did it increase appreciably during the period of experimentation.

(c) *Equilibration* of the haemoglobin solutions with varying oxygen tensions was carried out in tonometers, similar in design to those described by Austin *et al.* [1922], with the solutions stored in a small accessory tube prior to equilibration. The gas mixtures were made according to the method of Krogh & Leitch [1919], namely, by washing the tonometer and the attached blood gas reservoir three times with oxygen-free nitrogen and, after a final exhaustion, admitting an amount of CO₂-free air sufficient to give the desired oxygen tension. A measured amount of fully or partially oxygenated haemoglobin was drawn into the reservoir and then nitrogen admitted to a known pressure, usually atmospheric.

(d) *The temperature.* Five different temperatures over the range 0–40° were used, the temperature being regulated to within $\pm 0.1^\circ$. 10 min. were allowed for equilibrium to be attained.

(e) *Analysis of solutions.* The oxygen content of the solutions was, in most cases, determined by the Van Slyke manometric apparatus. All results were obtained in duplicate and checked to within 0.2 ml. O₂ per 100 ml., i.e. to within about 1% oxyhaemoglobin. In one series of experiments the Barcroft differential manometer was used. In this case the oxygen unsaturation (or percentage reduced haemoglobin) was determined by finding out how much O₂ each sample took up on shaking with air. The oxygen capacity of the haemoglobin solution was determined, by the Van Slyke method, upon a mixed solution remaining from the equilibrated samples, this providing a check on any ill-effect resulting from manipulation. In no case was the exposure to low oxygen tensions detrimental to the haemoglobin, except in one experiment the results of which were discarded.

All the solutions were free from CO₂. This was of importance, since CO₂ is now known to have a direct effect on the oxyhaemoglobin dissociation curve apart from that due to its effect on the pH of the solution [Margaria & Green, 1933].

From the difference between the original and final contents of oxygen in the haemoglobin solution, and the data from the preparation of the gas mixtures, the final tension of oxygen at bath temperature was computed by the method suggested by Austin *et al.* [1922]. The final results of the blood analyses were corrected for dissolved oxygen from data given by Ferry & Green [1929] corrected for our experimental temperatures.

In the series of experiments in which the oxygen content of the solution was determined by the Barcroft differential manometer, the final oxygen in the gas phase was also determined by analysis in the Haldane apparatus.

B. *The measurement of the heat of the reaction.*

Most of the details of the experimental technique and procedure will be found in the earlier paper by Roughton [1935]. Here it need only be noted that in the heat measurements:

(a) The haemoglobin solution, which had been prepared for the dissociation curve determinations, was diluted with an equal volume of distilled water, so that the heat measurements should not impose an undue drain upon the supply of purified haemoglobin (of which in each set of experiments about 400 ml. were available). It is unlikely that this degree of dilution would have affected the heat per mol. of O₂ combining with Hb.

(b) In one of the thermos flasks were placed about 200 ml. of the completely

reduced Hb solution, in the other an equal volume of 1% boric acid solution. Since, owing to the shortage of haemoglobin, it was impossible to have the two solutions in the two thermos flasks¹ identical, the temperature drifts at the beginning and end of each experiment were more serious than usual; indeed in one or two cases the correction for drift, as applied in the standard manner, amounted to as much as 30% of the total heat change. Such large drifts are no doubt also in part due to lack of sterility of the Hb solutions, and possibly to other slow secondary changes (such as denaturation) to which purified Hb (especially at a pH relatively so acid as 6.8) may be more prone than is whole blood. In experiments, particularly with haemoglobin several days old, there was reason to suspect such changes. It may be remembered that Brown & Hill [1923] noted that whole blood was by far the most satisfactory material to work on. Had it been possible to have the same Hb solution in each thermos flask, such changes might have cancelled each other out.

It would certainly have been more satisfactory if the drifts had not been so large, but their largeness does not shake our faith in the degree of agreement found below between the heat as obtained (a) directly and (b) indirectly from the dissociation curves, for in correcting for the drifts, the observer has no inkling of what the final result will be until the very end of the calculation.

Fig. 1.

EXPERIMENTAL RESULTS AND CALCULATIONS.

A. The effect of temperature on the dissociation curves.

Experimental results. Fig. 1 demonstrates the effect of temperature on the dissociation curve of purified haemoglobin of the ox in *M*/15 phosphate buffer,

¹ Unfortunately, owing to an accident at a critical moment, it was necessary to carry on with two rather ill-matched thermos flasks.

pH 6.90 at 0°. This family of curves represents the influence of temperature on the shape of the dissociation curve. It is noteworthy that the curves are sigmoidal at all temperatures. A discussion of some previously existing data on this subject may be found in Chapter XVI of Barcroft's book [1928].

The points upon the curves were obtained from two separately prepared solutions of haemoglobin from different ox bloods, yet the curves drawn from one set of determined points serve equally well for the other.

Inspection of the curves confirms the view of previous writers that the effect of temperature is to alter the scale rather than the shape of the dissociation curve. That this is quantitatively true can be shown by multiplying the abscissae of the various smoothed curves by appropriate coefficients, whereupon the curves all reduce to (within experimental errors) a common dissociation curve which is very close to that obtained by Forbes & Roughton [1931] for dilute sheep's blood.

This result is demonstrated best, not by a graph but by Table I.

Table I.

% O ₂ Hb	0° $pO_2 \times 6$	10° $pO_2 \times 3.4$	20° $pO_2 \times 1.9$	30° $pO_2 \times 1.02$	40° $pO_2 \times 0.6$	Forbes- Roughton $pO_2 \times 10.4$
20	—	—	17.8	16.5	17.2	16.2
30	22.0	—	21.5	21.2	21.8	22.5
40	27.0	—	25.9	25.7	26.5	27.0
50	30.0	31.8	29.2	30.1	31.0	31.2
60	33.6	35.0	34.8	35.0	35.8	35.8
70	—	—	43.7	41.7	41.8	41.6
80	—	—	57.0*	52.0	50.6	50.0

The column of figures on the right of Table I are calculated from the curve given in their Fig. 5 by Forbes & Roughton. With the exception of one point asterisked, the effect of temperature at the different parts of the dissociation curve is uniform enough. From the multipliers used in Table I the temperature coefficient per 10° (Q_{10}) of the curves can be obtained. From 0–10° $Q_{10}=1.76$, 10–20° $Q_{10}=1.79$, 20–30° $Q_{10}=1.86$, 30–40° $Q_{10}=1.60$. These figures do not indicate any marked "breaks" in the effect of temperature, although to exclude this possibility beyond doubt, a much greater number of temperatures would be necessary.

Relation between $\log [O_2 \text{ concentration}]$ and $1/T$ at equilibrium. Fig. 2 shows the relation between $\log pO_2 \times 273/T$ at equilibrium and $1/T$ for (1) 30% O₂Hb, (2) 50% O₂Hb and (3) 70% O₂Hb. $pO_2 \cdot 273/T$ is the pressure which the oxygen would have exerted at the standard temperature of 0° in each case, and hence is proportional to the concentration of O₂ in the gas phase whatever the value of T . The curves obtained are practically linear and of very nearly the same slope in each case, thus showing that the effect of temperature on the O₂-Hb equilibrium, as judged by this criterion also, is devoid of the "breaks", referred to as possible in the introduction, and furthermore is roughly the same at different parts of the dissociation curve, as has been found also to be the case by Barcroft [1928] for the rougher data given in Barcroft & King's curves [1909].

Comparison of Q as measured directly with value of Q_v' as calculated indirectly on assumption that the Van't Hoff Isochore can be applied. From Fig. 2 the calculated value of Q_v' using

50% oxyhaemoglobin = 9400 calories

70% oxyhaemoglobin = 9650 calories

30% oxyhaemoglobin = 9300 calories

Mean 9450 calories

The heat so calculated is that of the combination of 1 g. mol. O_2 in the gaseous phase with haemoglobin in the liquid phase. Since the calculated value of the heat appears to be the same at these different degrees of saturation, the direct value should also be constant over this range, and comparison should be justifiable, therefore, with the heat measured calorimetrically when the haemoglobin is brought from 0 to 70 % saturation with oxygen.

Fig. 2.

The directly measured value of Q for the same sample of haemoglobin was found to be $9000 \pm c. 700$ calories over the range 0 to *c.* 70 % oxyhaemoglobin. In a second comparison on haemoglobin, prepared from the blood of another ox:

The value calculated from the effect of five different temperatures (the same as in Fig. 2) on the dissociation curve = 9450 calories.

The directly measured heat at 19° = 9700 calories.

It may therefore be concluded that the value of Q , as measured directly for haemoglobin in *M*/15 phosphate buffer *pH* 6.8, agrees within experimental error with the value of Q_v' , as calculated from the Van't Hoff Isochore in the form of equation (1).

Brown & Hill [1923] found, in the case of borated blood, an appreciable effect of temperature upon the value of Q (direct), viz. about -1200 calories per 10° . This they attribute, on Kirchoff's principle, to a slight difference in the specific heats of oxygenated and reduced haemoglobin solution.

No study has been made yet of the effect of temperature upon Q (direct) for the purified ox haemoglobin, *pH* 6.8, used in these experiments, but as regards

Q_v' (as calculated) the fact that $\log pO_2 \cdot 273/T$ plotted against $1/T$ gives a straight line over the range 0–40° suggests that the indirect value, Q_v' , is not affected by temperature over this range, though owing to the rather considerable effect of experimental error in the dissociation curves on the calculation of Q_v' it would be unwise to exclude the possibility altogether.

II. EXPERIMENTS AT pH 9.5.

By G. S. ADAIR, J. BARCROFT, W. HERKEL, R. M. HILL,
A. B. KEYS AND F. J. W. ROUGHTON.

EXPERIMENTAL METHODS.

(a) *Preparation of the haemoglobin solutions.* The haemoglobin solutions were prepared from ox blood, by a procedure similar to the method (No. 1) for the preparation of carbon monoxide haemoglobin described by Adair & Adair [1934]: instead of adjusting the pH values by dialysis against a standard buffer, the haemoglobin was dialysed against distilled water and the solution was made alkaline with N sodium hydroxide, which was added slowly, with continuous stirring, in order to minimize the risk of denaturation, until the final concentration of sodium hydroxide was $N/16$. The titration curves of Hastings *et al.* [1924] indicate that the pH value of such solutions exceeds 9.5, which is sufficient to ensure that both oxygenated and reduced haemoglobin are present almost entirely in the oxy-labile ionized form.

Two separate preparations were made in this way:

- (i) contained a total haemoglobin content (determined refractometrically) of 0.00996 equivalent/litre.¹ The oxygen capacity determined gasometrically was 0.00899 equivalent/litre, so that 10% of the haemoglobin was "inactive",
- (ii) contained a total haemoglobin content of 0.0102 equivalent/litre, of which 0.00978 equivalent/litre were gasometrically active. The "inactivation" in this case thus only amounted to 2.4%.

(b) *Equilibration and analysis of the Hb solutions.* The solutions were equilibrated in the tonometers described by Barcroft [1934] in a constant temperature bath:

- (i) The oxygen contents of the equilibrated solutions were determined in duplicate by means of the Van Slyke manometric apparatus. The duplicates, on the average, checked to within 0.2 ml. O₂ per 100 ml. solution, i.e. to within about 1% oxyhaemoglobin.

(ii) The oxygen pressure in the gas phase after equilibration was determined, in the usual way, by means of the Haldane gas analysis apparatus. In some cases it was also calculated from a knowledge of (a) the total oxygen introduced into the tonometer at the start (i.e. the sum of the oxygen introduced into the gas phase and the oxygen introduced via the haemoglobin solution), (β) the oxygen content of the haemoglobin solution at the end of equilibration and (γ) the volumes of the gas phase and liquid phase in the tonometer. The oxygen pressure, so calculated, agreed to within 0.1 mm. Hg on the average with the pressure as determined by the direct analysis. The degree of agreement is well within the limits of experimental error of the methods.

(iii) The temperature. Experiments were done over the temperature range 9–39°. No observations were made at the lower temperature of 0°, as in the work at pH 6.8, since in the present case the oxygen pressures at 0° would have only

¹ 1 equivalent of haemoglobin = the weight containing 56 g. iron = 16,800 g.

been of the order of 0.5 mm. Hg, and therefore too small for accurate analysis by the ordinary Haldane apparatus.

(c) *The heat of the reaction.* The details of the measurement were almost the same as at pH 6.8, but since larger volumes of haemoglobin solution were available and much less was required for the dissociation curve determinations, sufficient haemoglobin solution was left over for both the thermos flasks to be filled with the same solution. This, as pointed out in Section I, gives increased accuracy over that obtainable with differing solutions in the two flasks: furthermore, in these experiments the latter were well-matched in size and thermal conductivity. Greater confidence can hence be placed in the direct calorimetric measurements of this Section than in those of Section I.

EXPERIMENTAL RESULTS AND CONCLUSIONS.

Two different preparations of purified ox haemoglobin solution were used.

Fig. 3 shows the dissociation curve points obtained on the first preparation at temperatures 9, 19, 29 and 39°. From these data, the average value¹ of the heat of the reaction $\text{O}_2 \text{ gas (at constant volume) + haemoglobin} \rightleftharpoons \text{oxyhaemoglobin}$ was calculated for the three temperature ranges, viz.

9–19° heat = 12,500 calories,
19–29° heat = 12,400 calories,
29–39° heat = 13,700 calories.

Fig. 3.

The heat, as measured directly at 19°, was 12,400 calories. This is to be compared with the mean of the calculated values for the ranges 9–19° and 19–29°, viz. $12,450 \pm 1200$ calories.

¹ By using, for the calculations, as much of the smoothed dissociation curve as was available at each temperature.

From similar data for the second haemoglobin solution, the calculated values for the heat are as follows:

$$\begin{aligned} 9-19^\circ \text{ heat} &= 15,100 \text{ calories} \\ 19-29^\circ \text{ heat} &= 13,100 \text{ calories} \\ \hline \text{Average} &= 14,100 \text{ calories} \pm c. 1000. \end{aligned}$$

The direct measurement of the heat for this reaction gave 14,250 calories at a temperature of 19°. The indirect measurements may be subject to an error of 1000 calories, since, as already emphasized, they are based on measurements with relatively low gas pressure.

Within the limits of experimental error the calculated values of the heat agree with those determined directly by the calorimetric method at this pH also.

THEORETICAL DISCUSSION.

By F. J. W. ROUGHTON.

THEORETICAL CONSIDERATION OF THE RELATION BETWEEN THE HEAT OF THE REACTION AND THE EFFECT OF TEMPERATURE ON THE DISSOCIATION CURVE.

The simple form of the Van't Hoff Isochore used so far in this work, viz.:

$$Q_v' = R \times \frac{T_1 T_2}{T_1 - T_2} \times \log_e \frac{C_2}{C_1},$$

can be shown to be a necessary consequence both of:

(a) Hufner's theory, according to which the reaction, from the mass action point of view proceeds simply as

(b) Hill's theory, i.e. that the reaction proceeds in the form

Neither of these earlier theories, however, can now be regarded as valid for mammalian haemoglobin: their place has at present been taken by the intermediate compound hypothesis of Adair [1925]. How does the simple form of the isochore fare on this theory?

Writing the reduced haemoglobin molecule as Hb₄ to express the fact that it can combine reversibly with 4 molecules of oxygen, Adair's hypothesis states that the reaction occurs in four stages.

This theory leads to the equilibrium equation

$$y = \frac{\% \text{ oxyhaemoglobin}}{100} = \frac{K_1 C + 2K_1 K_2 C^2 + 3K_1 K_2 K_3 C^3 + 4K_1 K_2 K_3 K_4 C^4}{4(1 + K_1 C + K_1 K_2 C^2 + K_1 K_2 K_3 C^3 + K_1 K_2 K_3 K_4 C^4)},$$

where C = concentration of oxygen in the gas phase.

These equations obviously do not, in general, lead to so simple a form of the Van't Hoff Isochore as that given in equation (1). First then we must ask whether

there are any special conditions in which the simple equation (1) would be theoretically expected to be valid.

One of the basal assumptions made in the thermodynamic deduction of the Van't Hoff Isochore readily gives the clue. For equation (1) to be rigorously valid, the concentration of every reactant, except O_2 , must be the same in the equilibrium at temperature T_1 as at T_2 . This means that

(a) At any given percentage saturation y , the concentration of each intermediate compound must be unaltered by change of temperature. This can only be the case if

(b) The heats of the intermediate reactions, i.e. Q_1, Q_2, Q_3, Q_4 , are all exactly equal.

The following conditions also follow from (a) and (b).

(c) The dissociation curve at T_2 must be an exact replica of the dissociation curve at T_1 , i.e. it must be possible to make the two curves coincide exactly, just by multiplying the abscissae of one of them by an appropriate constant factor.

(d) The value of Q_y' as calculated from equation (1) must be the same, whatever value of y is chosen.

(e) Q_y' must be equal to Q , the heat as given by direct calorimetry, whatever the range of percentage saturation chosen in the calorimetric measurements.

Conversely if conditions (c), (d), (e) hold good, so then must also conditions (a) and (b).

In point of fact conditions (a) and (b) were implicitly assumed to be true by Adair [1925] in his derivation of the simple form of the isochore (equation (1)) on the intermediate compound hypothesis. These assumptions have not, however, been tested experimentally, there being at present no way of measuring either the concentrations of the individual intermediates or of the heats of their reactions with O_2 , i.e. Q_1, Q_2, Q_3, Q_4 . But the experimental work, both of previous investigators [summarized by Barcroft, 1928] and of these papers, shows that conditions (c), (d), (e) are, within experimental error, realized. Does it follow from this that, if the intermediate compounds exist, their heats of reaction with O_2 must all be equal?

This question has been tackled by numerical trial and error, with negative result, for it has been found that conditions (c), (d) and (e) can hold good (within experimental error) even when values of Q_1, Q_2, Q_3 and Q_4 , which are widely different from one another are chosen. A single example will be sufficient to quote:

Let us, at temperature T_1 , take the first set of values of K_1, K_2, K_3, K_4 put forward by Forbes & Roughton [1931] in Table VI of their paper, viz. $K_1=0.4, K_2=0.15, K_3=0.06, K_4=4.5$. These values fit Forbes & Roughton's dissociation curves within experimental error, and if the scale of O_2 pressures be suitably adjusted, they also fit the curves given in Section I.

Now suppose $Q_1=12,000$ calories, $Q_2=12,000$ calories, $Q_3=24,000$ calories, $Q_4=0$ calories. Choose a temperature T_2 so that $(K_1 \text{ at } T_2)/(K_1 \text{ at } T_1)=2$, therefore $(K_1 \text{ at } T_2)=0.8$.

Then $(K_2 \text{ at } T_1)/(K_2 \text{ at } T_2)=2$, therefore $K_2 \text{ at } T_2=0.075$,

$(K_3 \text{ at } T_1)/(K_3 \text{ at } T_2)=4$, therefore $K_3 \text{ at } T_2=0.015$,

$(K_4 \text{ at } T_1)/(K_4 \text{ at } T_2)=1$, therefore $K_4 \text{ at } T_2=4.5$.

For simplicity we shall assume that the reaction is either so alkaline or so acid that there are no secondary effects due to changes in ionization of the haemoglobin when oxygenated.

Three representative points on the respective dissociation curves at temperatures T_1, T_2 have been calculated from these equilibrium constants and are given in Table II.

Table II.

Temperature T_1		Temperature T_2		Heat of reaction	
pO_2 mm. Hg	% saturation	pO_2 mm. Hg	% saturation	Indirect	Direct
1	10.07	2	9.98	12,090	12,120
2.5	36.30	5	35.95	12,150	12,150
5	77.69	10	77.74	11,990	12,110

It is obvious from the table that the two dissociation curves, if the pressure scale for the T_2 curve is compressed to one half, could not experimentally be separated from one another except by methods which were much more accurate than those at present available, i.e. condition (c) holds within experimental error.

The fifth column gives the heat as calculated from the effect of temperature on the oxygen pressure necessary to give equilibrium at the three different percentage saturations, shown in the second column of Table II, equation (1) being used for the calculation. The values again are too close to be separated by experiment, i.e. condition (d) holds within experimental error.

Finally column 6 gives the values which would be obtained by direct measurement of the heat per g. mol. O₂ combining when the haemoglobin is brought from zero percentage saturation to each of three percentage saturations shown. The mode of calculation of these values may be illustrated by an example:

At temperature T_1 , $pO_2 = 1$ mm., equations (2.1), (2.2), (2.3), (2.4) give:

$$\begin{aligned}
 [Hb_4] &= 67.60\% \text{ of total haemoglobin} \\
 [Hb_4O_2] &= 27.04 \quad " \\
 [Hb_4O_4] &= 4.04 \quad " \\
 [Hb_4O_6] &= 0.24 \quad " \\
 [Hb_4O_8] &= 1.08 \quad "
 \end{aligned}$$

Therefore heat per g. mol. O₂ combining, if directly measured over the range 0–10.07% O₂Hb would be

$$= \frac{\left\{ \begin{aligned} &(27.04 \times 12,000) + 4.04 \times (12,000 + 12,000) \\ &+ 0.24 \times (12,000 + 12,000 + 24,000) \\ &+ 1.08 \times (12,000 + 12,000 + 24,000 + 0) \end{aligned} \right\}}{(27.04) + (2 + 4.04) + (3 \times 0.24) + (4 \times 1.08)} = 12,120 \text{ calories.}$$

Column 6 shows that the directly measured heats would also be practically independent of the percentage saturation, and would be within experimental error, though not mathematically equal to the indirectly calculated heats. So condition (e) would also hold good.

Thus, in spite of the large differences between the heats of the several intermediate reactions, the haemoglobin would behave over the range 10–80% saturation practically in the same way as it would do if the heats of the intermediate reactions were all the same. The range mentioned represents the limits usually chosen in such studies, since above 80% the dissociation curve becomes too flat for accurate calculations to be made, whereas in the region below 10% the degree of saturation and O₂ pressure are too small to be measured precisely enough. If, however, very accurate measurements could be made at the extreme top of the dissociation curve, i.e. between 99 and 100%, the discrepancies postulated in our example would be brought to light, for in this special range, the curve would depend practically on the value of K_4 alone, and would thus be unaffected by temperature. This region of the dissociation curve, the importance of which has been emphasized elsewhere [Roughton, 1934] has not yet been made accessible to accurate measurement of the type required. These arguments thus lead to the following rather disappointing conclusions:

(i) That constancy, within experimental error, of the heat of reaction of haemoglobin with oxygen, over the whole range of percentage saturation, whether measured directly or calculated indirectly, does not in itself tell us anything about the heats of the intermediate reactions, nor indeed does it either support or disprove their existence.

(ii) That the simple form of the Van't Hoff Isochore (*v.* equation (1)), though a necessary consequence of the earlier theories of Hufner and of Hill, is not a necessary consequence of the intermediate compound theory, though it may, on the latter theory, be approximately or even exactly correct.

THE RELATION BETWEEN THE DIRECTLY MEASURED HEAT AND THE INDIRECTLY CALCULATED HEAT WHEN FOREIGN BUFFERS ARE PRESENT.

The equation $\log_e \frac{C_1}{C_2} = Q_v' (1/T_1 - 1/T_2)$ would not be expected thermodynamically to yield a value of Q_v' agreeing exactly with the directly measured value of Q if the *pH* of the haemoglobin solution is such that there is a change of *pH* on oxygenation. For, in such a case, the concentrations of the oxy-labile ionized and oxy-labile unionized fractions at T_1 and T_2 may not be the same, even if the total concentrations of oxyhaemoglobin and reduced haemoglobin at the two temperatures T_1 and T_2 are the same. The difficulty will only be avoided if the reaction is either so acid that the haemoglobin (oxy- and reduced) is all in the oxy-labile unionized form, or so alkaline that the haemoglobin is all in the oxy-labile ionized form. Of these, only the latter is at present accessible to experimental observation, and it was for the sake of simplifying the issue in this manner that the studies recorded in Section II were made.

We have previously shown [Roughton, 1935] how to calculate, in the intermediate range of *pH*, the relation between the directly measurable heat of reaction and the *pH* and buffer content of the solution; reference should be made to this treatment for a full understanding of the symbols used below. We shall now do the same as regards the indirectly calculable heat. In order to handle the problem, it is necessary to make certain assumptions, which, though not yet experimentally verified, are reasonable ones and, if accepted, lead to the conclusion that Q_v' and Q should, in fact, agree with one another within experimental error even though such an exact equality is not thermodynamically inevitable.

The principal assumption to be made concerns the relation between the oxygen concentration, C , required to produce a given degree of saturation, and the hydrogen ion concentration of the solution. The simplest assumption appears to be of the type

$$C = C_a \frac{H + K_R}{H + K_O} \times \frac{K_O}{K_R} \quad \text{.....(3),}$$

where C_a = concentration required when the reaction is so alkaline that the haemoglobin is completely ionized as regards the oxy-labile portion; K_O , K_R are the oxy-labile ionization constants of oxy- and reduced haemo-globin respectively.

Table III. *Relation between oxygen pressure for 50% saturation and hydrogen ion concentration.*

(Data of Ferry & Green [1929] for horse haemoglobin at 25°.)

$$\text{If } C = C_a \frac{H + K_R}{H + K_O} \times \frac{K_O}{K_R}.$$

<i>pH</i>	6.77	7.0	7.38	7.65	7.95	8.4	8.8
<i>H</i>	1.7×10^{-7}	1.0×10^{-7}	4.17×10^{-8}	2.24×10^{-8}	1.18×10^{-8}	4.0×10^{-9}	1.32×10^{-9}
<i>C*</i> (observed)	9.7	8.0	4.8	3.6	2.0	1.3	1.1
<i>C*</i> calculated (i)	10.0	7.5	4.4	2.9	2.0	1.4	1.1
(ii)	10.5	8.2	5.5	3.5	2.2	1.4	1.1

* C = concentration of oxygen in gas phase, expressed as $\left(\frac{\text{g. mol.}}{\text{litre}} \right) \times 1000$.

(i) $K_O = 2 \times 10^{-7}$, $K_R = 9.4 \times 10^{-9}$.

(ii) $K_O = 2 \times 10^{-7}$, $K_R = 6.8 \times 10^{-9}$.

If $K_O = 2 \times 10^{-7}$, and K_R lies between 7×10^{-9} and 9×10^{-9} , this equation gives a reasonably good fit for the relation between C and H for 50 % saturation of the haemoglobin (horse) in the case of Ferry & Green's data [1929], as shown in Table III.

If equations (1) and (3) are valid at different temperatures

$$\frac{-Q_y'}{RT^2} = + \frac{\partial \log_e C}{\partial T} = \frac{\partial \log_e C_a}{\partial T} + \frac{\partial \log_e (H + K_R)}{\partial T} - \frac{\partial \log_e (H + K_O)}{\partial T} + \frac{\partial \log_e K_O}{\partial T} - \frac{\partial \log_e K_R}{\partial T} \quad \dots\dots(4).$$

If the respective variations of C_a , K_O , K_R and H with temperature are known, then the value of $\frac{\partial \log_e C}{\partial T}$ is calculable, and hence the indirect value for the heat of reaction. Of these

$$(i) \quad \frac{\partial \log_e C_a}{\partial T} = \frac{-Q_{Hb^-}}{RT^2},$$

where Q_{Hb^-} = heat of the reaction $O_2 + Hb^- \rightarrow O_2Hb^- = 13,400$ calories [Roughton, 1935].

$$(ii) \quad \frac{\partial \log_e K_O}{\partial T} = \frac{Q_O}{RT^2},$$

where Q_O = heat of oxy-labile ionization of oxyhaemoglobin which is taken = 9000 calories.

$$(iii) \quad \frac{\partial \log_e K_R}{\partial T} = \frac{-Q_R}{RT^2},$$

where Q_R = heat of oxy-labile ionization of reduced haemoglobin.

With the aid of (i), (ii) and (iii) equation (4) thus becomes

$$\frac{-Q_y'}{RT^2} = \frac{-Q_{Hb^-}}{RT^2} - \frac{Q_O}{RT^2} + \frac{Q_R}{RT^2} + \frac{\partial \log_e (H + K_R)}{\partial T} - \frac{\partial \log_e (H + K_O)}{\partial T}.$$

Let Q_{Hb} = the heat of the reaction $O_2 + Hb \rightarrow O_2Hb$. Then by the Conservation of Energy

$$Q_O + Q_{Hb^-} = Q_R + Q_{Hb} \quad \dots\dots(5).$$

Therefore
$$-\frac{Q_y'}{RT^2} = \frac{Q_{Hb}}{RT^2} + \frac{\partial \log_e (H + K_R)}{\partial T} - \frac{\partial \log_e (H + K_O)}{\partial T},$$

or
$$Q_y' = Q_{Hb} - RT^2 \frac{\partial \log_e (H + K_R)}{\partial T} + RT^2 \frac{\partial \log_e (H + K_O)}{\partial T} \quad \dots\dots(6).$$

According to Roughton [1935], $Q_{Hb} = 11,400$ calories, and therefore $Q_{Hb^-} - Q_{Hb} = 2000$ calories, so $Q_R - Q_O$ must, by equation (5), also be taken as 2000 calories, i.e. the heat of combination of oxy-labile H ions with reduced haemoglobin should be appreciably greater than with oxyhaemoglobin. It would be interesting but difficult to test this by direct calorimetric experiments.

According to Roughton [1935], the corresponding value of the directly measurable heat is given by the equation

$$Q = Q_{Hb^-} \frac{K_R}{K_R + H'} + Q_{Hb} \frac{H'}{K_R + H'} + (Q_A - Q_H) \left(\frac{KA}{H' + K} - \frac{KA}{H + K} \right) \quad \dots\dots(7).$$

Where H , H' = the hydrogen ion concentrations of the solution after and before oxygenation respectively; A = molar concentration of the foreign buffer/Hb concentration in equivalents/litre; K = ionization constant of the foreign buffer, Q_A its heat of ionization; Q_H = heat of ionization of haemoglobin.

Equation (6) is of quite different form from equation (7) and hence the value of Q_y' calculated therefrom should not necessarily turn out to be the same as the value of Q calculated from equation (7).

(iv) The variation of $[H]$ with temperature.

If the $[H]$ of a haemoglobin solution at temperature T_1 and a certain percentage saturation is known, the $[H]$ of the same solution (at the same percentage saturation) can be calculated at temperature T_2 by means of an equation based on the following consideration.

When the temperature is changed the titration curve, both of oxyhaemoglobin and reduced haemoglobin, is shifted parallel to itself by 0.24 pH according to Stadie & Martin [1924]. Hydrogen ions are thus released from combination with haemoglobin by rise of temperature and are taken up by the foreign buffer.

If, for example, $T_2 - T_1 = 10^\circ$,

pH_1, pH_2 are the respective pH values at T_1, T_2 ,

H_1, H_2 are the respective hydrogen ion concentrations at T_1, T_2 .

Then $\beta [0.24 - (pH_1 - pH_2)] =$ hydrogen ions ionized from haemoglobin owing to the change of temperature, where β = total buffer power of the haemoglobin solution.

These must be practically all taken up by the foreign buffer. If K_1, K_2 are the respective ionization constants of the foreign buffer acid at temperatures T_1, T_2 , then the amount taken up by the foreign buffer

$$= A \left[\frac{K_1}{K_1 + H_1} - \frac{K_2}{K_2 + H_2} \right].$$

$$\text{Therefore} \quad \beta [0.24 - (pH_1 - pH_2)] = A \left[\frac{K_1}{K_1 + H_1} - \frac{K_2}{K_2 + H_2} \right] \quad \dots\dots(8).$$

The change in H with temperature can thus be calculated, equation (8) being rapidly and simply soluble, by the method of successive numerical approximation. All requirements for the calculation of the "indirect heat" are now to hand. Such calculations have been carried out for haemoglobin of concentration 0.01 equivalent/litre in

(i) $M/15$ phosphate pH 6.695, $\beta = 2.27$;

(ii) $M/15$ phosphate pH 7.042, $\beta = 2.27$.

The percentage saturation chosen for the calculation of Q was 50%, the temperature range $8-18^\circ$, and the following values were assumed for the ionization constants:

pK_O at $18^\circ = 7.00$, at $8^\circ = 7.24$.

pK_R at $18^\circ = 8.50$, at $8^\circ = 8.80$.

pK (phosphate) at $18^\circ = 6.80$, at $8^\circ = 6.83$.

These assumptions conform precisely with those adopted in Table III of the previous paper [Roughton, 1935] for (i) $pH = 6.67$, $\beta_S = 2.0$, (ii) $pH = 7.0$, $\beta_S = 2.01$, for the values of the ionization constants at 18° are the same as those used previously in the direct heat calculations and the values of the constants at 8° have been calculated from the corresponding values at 18° by means of the Van't Hoff Isochore, the values assumed for the various heats of ionization being those which were used in the direct heat calculations, viz. $Q_O = 9000$ calories, $Q_R = 11,000$ calories, Q (phosphate) = 1000 calories.

Table IV shows that the indirect heat and the direct heat, calculated in this manner, agree with one another empirically to within 150 calories—a figure which is well within the limits of error of either of these heats, when determined experimentally.

A similar degree of agreement in the calculations has also been found in the other cases dealt with in Table III of the previous paper [Roughton, 1935], viz. haemoglobin in $M/6$ borate buffer solution pH 7.6, and CO_2 -free haemoglobin,

Table IV. *Comparison of direct and indirect heats for haemoglobin of concentration = 0.01 equivalent/litre in M/15 phosphate.*

pH	Direct heat Q range 0–100 % saturation	Indirect heat Q_{50}'
6.695	9930	10,000
7.042	9120	9,250

without added buffer, pH c. 8.4. Although a generalization would perhaps be unwise there seem fair grounds for concluding that, if the assumptions mentioned above (which, though reasonable, are not as yet experimentally verified) be correct, the heat of combination of oxygen with haemoglobin as measured directly, should agree with the heat, as calculated indirectly by means of the Van't Hoff Isochore, not only in (i) the theoretically simple regions, namely those of very acid or of very alkaline reaction but also in (ii) the relatively complicated situation, which arises when there is present in addition to the haemoglobin a foreign buffer with a heat of ionization very different from that of haemoglobin.

CONCLUSIONS.

The experimental data of this paper have shown that the effect of temperature upon the oxyhaemoglobin dissociation is related to the heat of the reaction by means of the simple form of the Van't Hoff Isochore given by equation (1), within the limits of experimental error. This statement holds good, not only for the theoretically simpler experiments at alkaline pH, but also in the more complex conditions which obtain at pH 6.8, in the presence of foreign buffer (M/15 phosphate buffer).

The significance of this agreement has been reconsidered in the light of the newer knowledge of the mechanism of the oxygen-haemoglobin reaction, which Adair's work [1925] on the molecular weight and Van Slyke's work [summarized by Peters and Van Slyke, 1931] on the titration curves of oxy- and reduced haemoglobin have given us. The theoretical treatment of this section—somewhat cumbersome it must be admitted—shows that the success of the simple form of the Van't Hoff Isochore (1) is compatible, within the limits of experimental error—though not necessarily with mathematical exactitude—with the intermediate compound hypothesis of Adair and with the concept of the oxy-labile ionization of haemoglobin, though it neither strengthens nor weakens the basis on which either of these views at present rests. Although the thermochemical study of the oxygen-haemoglobin reaction has thus brought forth new, and in some cases interesting, data it has not as yet shed any essentially fresh light on the nature of the reaction. This is disappointing in view of the hopes which earlier workers attached to this mode of approach.

The procedure for calculating the relation between the directly measured heat and the indirectly calculated heat when foreign buffers are present is of a general kind and could be applied to other cases, besides the special ones considered in the last subsection. An important case to consider, from the physiological point of view, would be that which exists in blood *in vivo*, wherein the red cell pH is 7.0–7.3 and there is much foreign buffer present, namely in the form of the HCO₃—H₂CO₃—CO₂ system: this has not yet been done, owing to lack of a further essential piece of knowledge, namely the heat of direct combination of CO₂ with haemoglobin in the carbamino-form. Recent work has shown that under physiological conditions the latter reaction is as intimately connected with the oxygenation of haemoglobin, as is the oxy-labile ionization reaction, to which so much attention has hitherto been paid above. The

carbamino-reaction has been left out of account in the present paper, because in all the experiments here quoted, the solutions were freed of CO_2 and hence contained no appreciable amount of carbamino-compounds.

SUMMARY.

1. The effect of temperature on the oxyhaemoglobin dissociation curve has been studied on solutions of purified haemoglobin

- (a) at pH 6.8, in $M/15$ phosphate buffer;
- (b) at pH 9.5.

The results are essentially the same in the two cases, but for reasons given in the text, have been more fully and accurately worked out in (a).

2. Temperature alters the scale, but not the shape, of the dissociation curve: thus the value of the heat, Q_v' , as calculated from the Van't Hoff Isochore

$$Q_v' = \frac{RT_1 T_2}{T_1 - T_2} \log_e C_1/C_2,$$

is independent of y , the percentage saturation chosen for the calculation. The other symbols are defined in the text.

3. The value of Q_v' thus calculated agrees, within the limits of error, with the directly measured heat of the reaction, Q , both at pH 6.8 (average value 9350 calories) and at pH 9.5 (average value 13,300 calories).

4. The theoretical aspects of the comparison mentioned under 3 have not, hitherto, been fully considered in connexion with (i) the possible existence of compounds intermediate in composition between completely reduced haemoglobin, Hb_4 , and fully oxygenated haemoglobin Hb_4O_8 ; (ii) the presence of haemoglobin both in the oxy-labile ionized form Hb^- and the oxy-labile unionized form Hb . One of the objects of this paper was to fill up these gaps.

5. It is pointed out that if intermediate compounds such as Hb_4O_2 , Hb_4O_4 , Hb_4O_6 be present, the overall heat of reaction measured directly need not necessarily agree with the value of Q_v' as obtained by calculation, nor need either heat be independent of the range of percentage saturation chosen, if the heats of the intermediate reactions vary. An example, worked out in the paper, shows, however, that the heats of the intermediate reaction can be quite different from one another without there ensuing any appreciable discrepancy between Q and Q_v' , or any dependence of either upon the percentage saturation y . Experimental agreement between Q and Q_v' and independence of either of y does not therefore prove or disprove the existence of intermediate compounds.

6. The problem of relating the indirectly calculated heat Q_v' to the pH and foreign buffer content of the haemoglobin solution in the pH range 6.0–10.0 is also worked out. Numerical computations for certain typical cases of this kind show that empirically the value of Q_v' should agree closely with the directly measured value of Q even though such agreement is not thermodynamically necessary.

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