CCCX. THERMOCHEMISTRY OF THE OXYGEN-HAEMOGLOBIN REACTION.

I. DIRECT MEASUREMENTS OF THE HEAT OF REACTION UNDER VARIOUS CONDITIONS.

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BROWN AND HILL [1923] have published the last direct measurements (known to the writer) on the heat of reaction of oxygen with haemoglobin. Since then, the subject has lain practically dormant, nor has much attention been given to the allied problem of calculating the heat of this reaction indirectly from the effect of temperature upon the oxyhaemoglobin dissociation curve. Great advance has, however, been made in the elucidation of other physico-chemical properties of haemoglobin; notably in regard to the molecular weight [Adair, 1925, 1, 2; Svedberg and Fahraeus, 1926], and in regard to the titration curves and buffer power of oxyhaemoglobin and reduced haemoglobin [Van Slyke *et al.*, 1921 and onwards—summarised by Peters and Van Slyke, 1931]. This new knowledge has much bearing upon the thermochemistry of the oxygen-haemoglobin reaction, and indeed points to certain gaps and doubtful assumptions in the previous work.

The objects of the present series of papers were as follows:

(a) To test by direct calorimetry whether the heat, Q , per g. mol. of O_2 (or CO) combining with haemoglobin, when this amount of O_2 (or CO) is employed in saturating the haemoglobin partially, is independent of the percentage saturation attained.

Direct tests of this kind do not seem to have been made and perhaps would hardly be worth making if the reaction between oxygen and haemoglobin were a single, straightforward reaction, *i.e.*

Since, however, the molecular weight of haemoglobin is 68,000, one molecule of reduced haemoglobin combines reversibly with four molecules of oxygen, which, as suggested by Adair [1925, 1, 2], it probably does in four intermediate stages, *viz.*:

Q_1, Q_2, Q_3, Q_4 may not necessarily be the same: if they are different the overall heat, Q , might not be independent of the percentage saturation. If the latter were indeed found experimentally to be the case, it would be concrete evidence in favour of the intermediate compound hypothesis: a converse finding would not, however, disprove the existence of the intermediate compounds. This point will be discussed more fully in a later paper.

(b) To test whether the heat of reaction of oxygen with haemoglobin is affected by (i) laking of the corpuscles, (ii) purification of the haemoglobin.

No definite information on either of these points seems to be available in the previous literature of the subject.

(c) To investigate further the relation between the heat of the reaction, the p_H and the buffer content of the solution.

Brown and Hill [1923] were the first to point out that the heat evolved when oxygen combines with haemoglobin is really the sum of the heats of three separate processes.

(i) The primary reaction between oxygen and haemoglobin—the heat per g. mol. O₂ combining being of the order of 11,000 to 14,000 calories.

(ii) Oxyhaemoglobin being a stronger acid than reduced haemoglobin, oxygenation of the latter leads to the splitting off of H ions, probably from a special NH₃⁺ group in the neighbourhood of the haematin nucleus (which group may, following Van Slyke, be referred to as the oxy-labile group). The heat absorbed per oxy-labile H ion so liberated is considered to be about 10,000 calories.

(iii) The liberated oxy-labile H ions do not remain free in solution but are removed by the buffer substances present therein. The accompanying heat effect obviously depends on the respective concentrations and heats of ionisation of the various buffer systems.

On this basis it is clear that the “overall” heat depends not only on the haemoglobin but also on the nature and concentrations of all other buffers in the system. Under certain conditions indeed (*v. infra*) the overall heat may be expected to differ considerably from the heat of the primary process (i).

Brown and Hill's detailed working out of this conception requires revision in the light of our more recent knowledge: further data over a wider range of p_H are also desirable.

(d) Hartridge and Roughton [1923, 1, 2; 1925], with the aid of their rapid velocity technique, have shown that the combination of oxygen and haemoglobin is, under normal conditions, complete in about 1/100 sec., if the difference in optical properties of oxy- and reduced haemoglobin is used as the criterion of the chemical change. It would be of interest to know whether the large heat effects associated with the reaction also occur at the same speed: a discrepancy therefrom would be of much significance for it would have to be attributed to the occurrence of some other reaction besides the primary combination of oxygen with haemoglobin, which would of course entail a revision of our views as to the mechanism of the whole process.

This question cannot, like the others, be tackled by the ordinary methods of slow calorimetry for in these the temperature changes to be measured are spread out over a period of 10 min. or more, owing to the slowness of diffusion of oxygen in solution and the slowness of heat conduction within the calorimeter: it requires instead the application of the rapid method developed by Roughton [1930, 1, 2].

(e) To compare in the case of purified haemoglobin solutions the heat of reaction of oxygen with haemoglobin, 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. This has not been attempted since the pioneer work of Barcroft and Hill [1909] in which, however, remarkably high values for the heat of reaction were recorded. Both in the latter work and in that of Brown and Hill [1923] on whole blood the oxygen contents of the solutions were estimated by the Barcroft-Haldane blood-gas methods, which are now known to give results which are often, though not invariably, too low [*v. Abeloos et al.*, 1928].

Fairly complete sets of experiments on questions (a) and (b) are given in this paper, together with experiments and detailed theory in regard to question (c). Question (d) is dealt with in the succeeding paper by Bateman and Roughton whilst in a future paper it is hoped to give a comparison of the heat measured directly for purified haemoglobin solutions, both at p_H 6.8 and at *ca.* 9.5, with that calculated indirectly from the effect of temperature on the oxyhaemoglobin dissociation curve together with some theoretical considerations arising therefrom.

The present paper is confined on the experimental side to direct measurements of the heat of reaction by the classical method of slow calorimetry, the technique for which will first be described: then follow the experimental results, together with some theoretical discussion arising out of the effect of p_H on the directly measured heats.

Unless otherwise stated defibrinated ox blood, from the slaughter house, was used for the experiments given in these papers, whether on whole blood or on purified haemoglobin. All experiments were done at 19–20°; oxygen contents were estimated, when required, in the Van Slyke-Neill constant volume apparatus.

The figures given in this paper refer throughout to the heat of the reaction

The heat of solution of O_2 , *viz.* 2800 calories at 17°, must be subtracted from them, if it is desired to obtain the heat of the reaction

EXPERIMENTAL METHODS.

In general outline the method used was as follows.

Reduced blood (or haemoglobin solution) was shaken with oxygen or carbon monoxide in one thermos flask, and the temperature difference between this and a second thermos flask, containing the same amount of fluid but with no chemical reaction occurring therein, was measured thermo-electrically. The technique embodied certain features of the differential method of Hill [1911], and also of the respective methods of Brown and Hill [1923] and of Roughton [1930, 1, 2]. The details of the apparatus were largely governed by what parts were at hand or readily available.

(a) *The calorimeters and their contents* (*v.* Fig. 1). These consisted of two thermos flasks Th , Th' , capacity 400 ml. each, and selected so as to have as nearly the same thermal conductivity as possible. A narrow-mouthed variety of flask (1 in. diameter mouth) was used, so as to cut down heat exchange through the mouth. The flasks were each fitted with rubber corks, through which six holes were drilled. Through two of these holes passed thick-walled glass capillary tubes (internal bore 1 mm.) which were used for filling and emptying the flasks with liquid and/or gas. (These tubes ended flush with the inside surface of the rubber cork.) Two other of the holes were used for the glass U-tubes U , U' , through which could be circulated a stream of constant-temperature water, so that the internal temperatures of the flasks could by this means be readily adjusted so as to be as nearly as possible equal to one another and to that of their outside environment. The fifth hole conveyed the connections of the electric heater, which was used to calibrate the flasks, whilst the sixth hole conveyed a glass tube T , T' reaching to the bottom of the flask and closed at the lower end. These tubes were filled with water, the thermo-junctions were then placed inside

them and the top ends sealed so as to prevent evaporation of the water. Further details as to the electric heating and the thermocouples were as follows:

(b) *The electric heaters.* These were made of fine constantan wire and each had a resistance of about 110 ohms. The heating current was furnished by six 2-volt accumulators in series, and was measured by a milliammeter. The heat supplied = (current)² × (resistance) × (time).

The current was usually 80–100 milliamps, the time 100 sec. and the heat supplied 10–12 calories.

Fig. 1. The calorimeter. Arrangement and connections of twin thermos flasks in box attached to shaker. In order to make the interior construction of the two flasks clear, the spiral tubing which actually encloses the flasks completely is shown as cut away except at the bottom of the flasks.

(c) *The thermocouples.* These were made of copper and constantan and were bent into the shape of a U, one limb of which (consisting of the warm junctions) was pushed into T , whilst the other was pushed into T' . From the centre of the U heavy leads connected to the electric recording system. Sufficient sensitivity was attained with only two or four thermojunctions. The electrical resistance of the thermocouples was from 10 to 20 ohms, and the thermo-electric current was measured by direct deflection of the galvanometer, not potentiometrically, since the E.M.F.'s developed were so small.

(d) *The heat insulation of the thermos flasks and constancy of temperature of external environment.* Each of the thermos flasks was completely encased from top to bottom by a close-fitting spiral of lead tubing, which was connected with a supply of constant-temperature circulating water (constant to $\pm 0.05^\circ$). The two flasks and their encasements were then buried in cotton-wool (1 to 2 in. thick) in a metal box, which was attached in a horizontal position to a board hinged to a large shaking machine. The latter, when set into operation, tilted the flasks to and fro about 100 times per minute. This rate was usually enough to bring the fluids and gases into thermal and chemical equilibrium in about 15 min., without at the same time causing any serious temperature differences between them due to the mechanical heat of fluid friction. In an actual experiment, after the requisite solutions had been placed in the thermos flasks, the shaker was started, and the constant temperature water was circulated round the outside of the flasks and through the inner tubes U , U' .

Usually after about 2 hours' circulation the interior and exterior of the thermos flasks were sufficiently near to each other in temperature for the actual experiment to be performed.

At this stage the inner circulation through U and U' was, by suitable manipulation of clips on the rubber connections, cut off. From this point onwards the circulation only passed through the coils on the outside of the two thermos flasks.

(e) *Preparation of the reduced blood (or haemoglobin) and loading thereof into the thermos flasks.* About 400 ml. of blood or haemoglobin were reduced in a 2-litre bottle by continued boiling and shaking *in vacuo*, according mainly to the directions of Forbes and Roughton [1931], save that small doses of purified nitrogen were often introduced into the bottle after each evacuation so as to make the foam subside more readily. 20 min. were usually enough for complete reduction, but the procedure was always continued 5 to 10 min. more, to make sure that no oxygen was left in the blood. The bottle was then filled with oxygen-free nitrogen (prepared fresh for each experiment by keeping commercial nitrogen in a 10-litre bottle over a solution containing 20% sodium hydro-sulphite and 1% sodium β -anthraquinonesulphonate) and immersed upside down for 1–2 hours in a water thermostat of the same temperature as the circulating water, so as to be at about the right temperature before being put into the thermos flasks.

A known volume, usually 195 ml., of reduced blood (or haemoglobin solution) was then transferred without contact with oxygen into Th and the same volume, though not necessarily of the same fluid, transferred into Th' . Th and Th' were finally left full of oxygen-free nitrogen at atmospheric pressure.

(f) *Further procedure during an actual experiment.* During the temperature equilibration in (e), the shaker was continually in motion. After about 1 hour the "inner" circulation was cut off, and galvanometer readings of the temperature difference between Th and Th' were taken every minute. If the temperature difference was small enough (*i.e.* $< 0.1^\circ$) and if the rate of drift of the temperature difference was also sufficiently slight (*i.e.* of the order of 0.0001° per min. in good experiments) the experiment could be continued. In certain cases the drift was much faster and could not be cut down. In these, and in other cases, where necessary, the drifts were allowed for by means of the usual "cooling curve" procedure.

The first thermos was then calibrated by electric heating for 50 to 100 sec., the shaker working the whole time. Galvanometer readings were taken at minute intervals for 5 min. after the heating. By this time conditions were steady again. The second thermos was then calibrated in the same way.

From 10 to 50 ml. of gas (O_2 or CO) were then measured into a burette with mercury reservoir, and forced by the latter into one of the thermos flasks. The gas was as a rule either dry or saturated with water vapour. Corrections were applied for the heat effects due (a) to the compression of the gas into the flask, (b) to the water vapour in the gas but not for (c) the difference in the temperature between the gas and the inside of the flask, since correction for this was found to be negligible. These corrections, as given by theoretical calculation, were only of the order of 5–10% of the observed heats. In control experiments, in which the thermos flasks held 1% boric acid solution instead of blood, and in which similar volumes of air were forced in as above, the heat effects found agreed closely enough with those calculated on the same basis as the corrections just described. Brown and Hill [1923] avoided these corrections by a special

procedure, and their final accuracy was, on this account, probably better. The present technique, however, has the advantage of enabling the heat liberated when successive amounts of CO (and in some cases of O₂) are introduced into the same flask to be simply measured.

The introduction of the gas took about 2 min., during which time the shaker was still. The shaker was then started up again and galvanometer readings taken every minute for about 20 min. The chemical process, as a rule, was finished in 10 min., and the remaining 10-min. period was used for finding out the rate of drift again.

The second thermos flask was then treated in the same way. If necessary the first thermos was again treated with gas, and then the second and so on—the two thermos flasks always being treated alternately so as to keep the temperature difference between the two flasks small.

At the end of the experiment the flasks were tilted vertically and, if required, blood was run out from each under paraffin oil for analysis of its gas content in the Van Slyke-Neill apparatus. Since the blood was initially reduced, the O₂ or CO content so found = O₂ or CO uptake during the experiments apart from a small amount in physical solution, which could be allowed for.

In some cases the gas uptake by the blood was obtained indirectly from the amount of gas introduced into the thermos flask, the volumes of the gas and liquid phases therein and the oxyhaemoglobin dissociation curve. In a few experiments the final p_{H} was measured by means of the glass electrode.

The two flasks were then thoroughly washed out with water or dilute ammonia, and were finally left completely full with 1% boric acid solution, so as to inhibit bacterial growths. The boric acid was not removed until the beginning of the next experiment.

The thermocouple was connected in series with a Zernicke Zb galvanometer, readings of which were taken on a scale at 4 metres distance. The galvanometer was permanently shunted with a manganin resistance of about 50 ohms, so as to give critical damping of the coil when a high resistance was in series with it. The usual resistance in series was very low, *i.e.* 10–50 ohms, so that the coil was often over-damped, but this did not matter in the present work.

The sensitivity was from 1.5 to 6.0 mm. deflection per 0.001°. A double potentiometer arrangement arranged so as to be as free as possible from parasitic E.M.F.'s, enabled the voltage sensitivity of the galvanometer to be checked with small micro-voltages, and also served partially to compensate the thermoe.m.f.'s if the latter happened to be unduly large in any special case.

(g) *Purity of gases used.* The carbon monoxide was prepared from sodium formate and sulphuric acid and was stored under a slight positive pressure over alkaline sodium hydrosulphite. It was therefore free of O₂ and CO₂, but usually contained 1–4% impurity which was presumably mainly nitrogen.

The oxygen was drawn from an ordinary commercial cylinder and was usually 99% pure.

(h) *Accuracy of the method.* When whole blood, or haemoglobin solution of equivalent oxygen capacity, is saturated with oxygen, there is a rise of temperature of 0.06–0.10°. In good experiments the temperature difference between the flasks remained constant to within 0.001° after each equilibration, or failing that showed only a small and regular drift, the correction for which did not amount to more than 0.003–0.004°; the method was then found to be reliable on the average to $\pm 0.002^\circ$ which would be equivalent to an error of $\pm ca.$ 300 calories in the heat of combination of 1 g. mol. of oxygen with haemoglobin if the concentration of the latter is as in whole blood.

In poor experiments, in which the drifts were greater or more irregular, the error might be 2 or 3 times larger. Amongst the causes responsible for such may be mentioned:

- (i) Unequal dimensions and/or thermal conductivity of the two flasks.
- (ii) Imperfect balancing of the frictional heat due to shaking in the two flasks.
- (iii) The occurrence of slow secondary processes.

The flasks were therefore carefully chosen so as to be equal¹ in size and thermal conductivity (to within 2–3 %), and where possible the same solution was placed in each: in some of the experiments on purified haemoglobin, this, unfortunately could not be done, owing to shortage of material. The experiments on purified haemoglobin were, in fact, less satisfactory than those on whole blood, especially at p_H 6.8: this was in part due to the reason just given, but it may also have been in part due to the readier denaturation of haemoglobin when pure and possibly to other causes as well.

EXPERIMENTAL RESULTS.

(a) *Relation between heat liberated and percentage saturation.*

Three or more instalments of gas (O_2 or CO) were forced into one or both flasks, and the heat developed after equilibration with each in turn was measured. The volume of gas so introduced was such as to saturate the Hb to about 25 %. In this way several points were obtained over the range of 25–90 % saturation.

Unfortunately, however, the heat developed is in each case small, and the experimental error of the method therefore causes a correspondingly great uncertainty in the result. Thus *e.g.* if the temperature rise for 100 % saturation were 0.1° (a normal figure) then the temperature rise for 25 % saturation would be 0.025° , if Q is independent of the % saturation: so an experimental error of 0.001° would cause an uncertainty of 4 % in the answer, whilst an error as large as 0.005° would give a 20 % uncertainty—a figure much too big unless very many experiments were done. Since whole blood generally gave more precise results than purified haemoglobin, the seven experiments on this question were all done with whole blood. Of these two were done with carbon monoxide and five with oxygen.

(1) *Experiments with CO.* The first experiment on this subject with CO gave most satisfactory results. Pig's blood, treated with 1 % boric acid was used in each thermos flask.

In Fig. 2a, the volume of CO added is plotted against the heat change, expressed in cm. of galvanometer deflection. Owing to the very high affinity of the Hb for CO, the amount of residual CO left in the gas phase of the flask after each equilibration was negligible, and the CO uptake is therefore simply equal to the volume of CO introduced into the flask. No corrections were made for the heat effects due to the introduction of CO into the flask, since these being proportional to the volume of CO introduced, would not affect a linear relationship between heat observed and volume of CO introduced. The temperature after each equilibration remained steady to within 0.0002° for 5 min., so no corrections for drift were needed.

¹ In the middle of the research one of the flasks was broken and had to be replaced quickly with another flask which was not a good match of the sound one. Later on, this was rectified.

For the three points shown in Fig. 2*a*, the actual values for the heat evolved were in $\frac{\text{cm. deflection}}{\text{ml. CO added}}$:

0.556, 0.525 and 0.558 respectively: Mean = 0.545.

The largest discrepancy from the mean is thus only $100 \times \frac{0.545 - 0.525}{0.545} \% = 3.7 \%$, and is thus well within the experimental error.

Fig. 2*a*.

Fig. 2*b*.

Fig. 2. Relation between volume of carbon monoxide added and heat evolved, when blood and gas phase are equilibrated.

In the second experiment (Fig. 2*b*) calf's blood containing 1% boric acid + phosphate buffer, $M/5$, final p_H about 6.8 was used. The blood in one flask was laked by freezing and thawing and in the other unlaked. This experiment, owing to drifts, was less satisfactory and the results were rather irregular, but in general confirmed the conclusion obtained in the original experiment.

(2) *Experiments with oxygen.* Owing to the smaller affinity of haemoglobin for oxygen (than for carbon monoxide), the oxygen uptake must be somewhat less than the volume of oxygen introduced into the flask. This makes it desirable to withdraw a sample of blood after each equilibration and analyse its oxygen content in the Van Slyke-Neill apparatus. Unfortunately a single experimenter could not do this, as well as the heat experiment, within a period of 1 day—a necessary time-limit if disturbance due to changes in the blood are to be certainly avoided.

The difficulty was got over in the following way. In the two experiments shown in Fig. 3, the analysis of the O₂ content of the blood in the flasks at the end of the experiment showed that the residual O₂ in the gas phase was about 10% of the total volume put in. In these cases the blood was more than 80% saturated, *i.e.* its condition was represented by a point on the flat part of the oxyhaemoglobin dissociation curve at the top. Now on the steep part of the curve below 80% saturation the residual O₂ in the gas phase must be less than 10% of the O₂ taken up, and, furthermore, inspection of the dissociation curve shows that over the range 25–80% O₂Hb the relation between O₂ pressure and % O₂Hb does not differ from a straight line by more than 15%. If the experimental relation between volume of O₂ introduced and heat produced be linear, then it follows that the correction for the residual O₂ in the gas phase would not cause a distortion of more than 15% of 10%, *i.e.* 1.5% from a straight line, if true O₂ uptake were plotted against heat over the range 25–80% O₂Hb. Since

such an error of 1.5% is small compared with that of the method as applied to this problem, *viz.* 5%, it was considered that the plotting of vol. O₂ introduced against heat developed gave a satisfactory test over the range of 25–80% O₂Hb.

The results of two experiments on two different specimens of ox-blood containing 1% boric acid + *M*/10 phosphate buffer p_H *ca.* 7.0 are given in Fig. 3*a* and *b*.

Fig. 3*a*. Fig. 3*b*.

Fig. 3. Relation between volume of oxygen added and heat evolved when blood and gas phase are equilibrated.

In Fig. 3*a*, the first four points lie on a straight line to within 3% whilst the last two points lie above the line owing to the fact that the blood was nearly fully saturated, so that a relatively larger correction for residual O₂ in the gas phase would be necessary. In this case the blood was laked by addition of 1% saponin.

In Fig. 3*b* one thermos flask contained the above blood mixture unlaked whilst the second contained similar blood mixture, but laked by freezing and thawing. These points all fell on a straight line to within 6%.

In a third experiment, not shown, on ox-blood + 1% boric acid p_H 7.5 (about) two points were obtained. For these the divergence from a straight line was 3%. Two other experiments also gave confirmatory results.

The general conclusion is therefore that for oxygen, over the range investigated, *i.e.* 25–80% saturation, and for carbon monoxide over the range investigated, *i.e.* 25–90%, the heat developed is within experimental error proportional to the gas uptake in the case of whole blood, whether laked or unlaked. *Q* appears to be independent of the percentage saturation within this range: these experiments therefore fail to yield any positive evidence for the existence of the intermediate reactions, though they do not, of course, disprove the possibility of their occurrence.

(*b*) *Effect of purification and of laking.*

(1) *Purification.* The heat of combination of oxygen with haemoglobin in laked blood and in purified solution has been compared, both at p_H 6.8 and at p_H *ca.* 9.5. The haemoglobin was purified by Adair's method [Adair *et al.*, 1921] in each case. In the experiments at p_H 6.8 the purified haemoglobin was dissolved in *M*/15 phosphate buffer, whereas the laked blood contained *M*/10 phosphate buffer: had the importance of the concentration of the foreign buffer been appreciated at the time the experiments were done, the same concentration would have been used in the two cases. The discrepancy was, however, allowed for well enough by adding on a figure of 600 calories to the values found in the case of the *M*/10 phosphate solutions: this figure of 600 calories was

arrived at by the method of calculation described in the theoretical section below (*q.v.*). In the comparison at alkaline p_H , both the laked blood and the haemoglobin solution were brought to p_H *ca.* 9.5 by adding an appropriate amount of NaOH: in this p_H range the heat of reaction is theoretically independent both of p_H and of the presence of foreign buffers.

The results of these comparisons are shown in Table I.

Table I. *Comparison of heat of reaction of oxygen with purified haemoglobin and with laked blood.*

p_H	Pure Hb	Laked blood	Unlaked blood
6.8 (phosphate buffer)	9,000 $\pm$ 700	8,700	9300
6.8 " "	9,700	9,400	—
6.8 " "	9,000	8,800	—
<i>ca.</i> 9.5 (NaOH added)	14,250	13,400	—

The heats of the reaction of oxygen with purified haemoglobin are, on the average, 400 calories higher than with laked blood, but the difference can hardly be regarded as outside experimental error.

(2) *Laking.* That laked blood and unlaked blood have practically the same heat of reaction at p_H 6.6–6.8 (phosphate buffer) is shown by Figs. 2*b*, 3*b* and by the first line of Table I. This p_H range was chosen, because there is practically no difference in the H ion concentration, or according to Maizels and Hampson [1927] in the phosphate concentration, between the interior of the red blood corpuscle and the surrounding fluid. In future experiments it would, however, for reasons given later, be better to compare the heat of reaction of oxygen with corpuscles suspended in isotonic NaCl, with the heat of reaction of the same suspension when laked.

(c) *Effect of p_H and foreign buffer content.*

Table II shows the heat of reaction of purified Hb (prepared from the blood of several different animals) with oxygen at p_H *ca.* 6.8 and p_H *ca.* 9.5. The values, calculated indirectly from the effect of temperature on the dissociation curve

Table II. *Heat of reaction of purified haemoglobin with oxygen at various p_H values.*

p_H and buffer concentration	Directly measured heat	Heat calculated indirectly from effect of temperature on dissociation curve
<i>ca.</i> 6.8, <i>M</i> /15 phosphate	9,000 $\pm$ <i>c.</i> 700	9,450
<i>ca.</i> 6.8, <i>M</i> /15 phosphate	9,700 $\pm$ <i>c.</i> 700	9,450
<i>ca.</i> 6.8, <i>M</i> /15 phosphate	8,800	—
<i>ca.</i> 7.5 distilled water	12,000	—
9.9, <i>M</i> /10 borate	—	15,000
<i>ca.</i> 9.5 (by addition of NaOH to Hb in distilled water)	12,400	12,400
<i>ca.</i> 9.5 " " "	14,250	14,200

of the same haemoglobin solutions (to be described in a later paper) are for convenience also inserted. It will be seen that:

- (i) There is good agreement between the direct and the indirect heats.
- (ii) The variation between different samples at the alkaline reaction is about three times greater than at p_H 6.8.
- (iii) The average heat at the alkaline reaction, *viz.* 13,300, is about 4300 calories greater than the heat at p_H 6.8, *M*/15 phosphate buffer, *viz.* 9300 calories.

A number of experiments have also been done on unlaked blood treated with phosphate and borate buffers of various strengths. The results, which are not included in this paper, did not show as large an influence of the foreign buffer concentration as would be expected on the basis of the theory to be given in the next section. It is known however that the concentration of the foreign buffer in the fluid outside the red blood corpuscles may, at equilibrium, be much higher under certain circumstances than inside the corpuscles, and furthermore that equilibrium may be reached slowly. The foreign buffers may thus play a smaller rôle than if the haemoglobin solution were homogeneous. This makes the interpretation of experiments on unlaked blood obscure, unless there are no foreign buffers present at all in the blood: it is for this reason that any future experiments on the effect of laking are to be performed on corpuscles suspended in unbuffered NaCl solution.

THEORETICAL INTERPRETATION OF THE EFFECT OF p_H AND FOREIGN
BUFFERS ON THE HEAT OF REACTION OF OXYGEN WITH HAEMOGLOBIN.

This problem has already been outlined under heading (c) (p. 2605). In the quantitative treatment now to be given it will be assumed that:

(a) The reduced haemoglobin is treated with enough oxygen to saturate it completely: this avoids complications from compounds intermediate between Hb_4 and Hb_4O_8 .

(b) Of the H ions which combine with haemoglobin on the alkaline side of the isoelectric point there is, per mol. of oxygen-combining capacity, only one (called the "oxy-labile" hydrogen ion) whose ionisation constant is increased by oxygenation and lowered by reduction of 1 g. equivalent of haemoglobin. The experimental results of Hastings *et al.* [1924] are in accord with this view, though they do not prove it conclusively: so also are Hartridge and Roughton's [1923, 2] observations on the rate of dissociation of oxyhaemoglobin at various p_H values. It is a reasonable theory from the chemical point of view and is the simplest way of accounting for several different kinds of experimental result. It has therefore been thought fit to adopt it in this paper.

Symbols and data required.

Let $[Hb^-]$ = concentration in equivalents¹ of reduced haemoglobin, in which the "oxy-labile" hydrogen ion is split off.

$[Hb]$ = concentration of reduced haemoglobin, in which the "oxy-labile" hydrogen ion is still attached.

$[O_2Hb^-]$ = concentration of oxyhaemoglobin, in which the "oxy-labile" hydrogen ion is split off.

$[O_2Hb]$ = concentration of oxyhaemoglobin, in which the "oxy-labile" hydrogen ion is still attached.

A = total concentration of foreign buffer per equivalent of total haemoglobin.

$[H^+]$ = hydrogen ion concentration of the solution in general.

H = hydrogen ion concentration of the solution after oxygenation.

H' = hydrogen ion concentration of the solution before oxygenation.

K_O = oxy-labile ionisation constant of oxyhaemoglobin = $\frac{[O_2Hb^-][H^+]}{[O_2Hb]}$,

whence

$$\frac{[O_2Hb^-]}{\text{Total } Hb} = \frac{K_O}{K_O + H^+}.$$

¹ 1 equivalent of haemoglobin = amount which combines with 1 g. mol. of oxygen, *viz.* 17,000 g.

For horse haemoglobin, in solutions of about the same total electrolyte concentration as in the cases calculated below, K_O is about $10^{-6.57}$ at 38° according to Hastings *et al.* [1924]. The change of temperature from 38 to 18° is allowed for by the Van't Hoff isochore, assuming the heat of ionisation to be 9000 calories; this lowers the value of K_O from $10^{-6.57}$ at 38° to $10^{-7.0}$ at 18° .

In the cases calculated below at 18° K_O is taken $=10^{-7.0}$, and $p_{K_O}=7$.

K_R = oxy-labile ionisation constant of reduced haemoglobin $= \frac{[\text{Hb}^-][\text{H}^+]}{[\text{Hb}]}$,

whence

$$\frac{[\text{Hb}^-]}{\text{Total Hb}} = \frac{K_R}{K_R + \text{H}^+}.$$

On the basis of the same considerations as those just given for K_O , K_R is taken $=10^{-8.5}$ and $p_{K_R}=8.5$ at 18° .

K = ionisation constant of the weak acid constituent of the foreign buffer
 $=10^{-6.80}$ at 18° for phosphate buffer ($M/15$)
 $=10^{-9.20}$ at 18° for borate buffer ($M/6$),

Q_{Hb^-} = heat of the reaction $\text{O}_2 + \text{Hb}^- \rightarrow \text{O}_2\text{Hb}^- = 13,400$ cals.

From the numerical values assigned to K_O and K_R it follows that at $p_{\text{H}} > ca. 9.5$, both reduced and oxyhaemoglobin are almost entirely in the oxy-labile ionised form. Q_{Hb^-} is therefore given by the average of the results at p_{H} *ca.* 9.5 in Table II, *viz.* 13,400 cals.

Q_{Hb} = heat of the reaction $\text{O}_2 + \text{Hb} \rightarrow \text{O}_2\text{Hb}$, assumed $= 11,400$ cals. For the reduced and oxyhaemoglobin to be both entirely in the oxy-labile unionised form, it would be necessary to work at $p_{\text{H}} < 5.0$, which is unfortunately too high an acidity for the heat of reaction to be measured directly owing to the difficulty of avoiding methaemoglobin formation under these conditions. The value of 11,400 cals. is one which gives a satisfactory fit between the observed results of Table II and those calculated by the theoretical method which follows.

Q_{H} = heat of combination of 1 g. ion of H with haemoglobin whether in the oxy-labile position or elsewhere $= 9000$ cals. (average of values given later).

Stadie and Martin [1924] have given reasons for believing that the heat of combination of the oxy-labile H ion with haemoglobin is the same as the heat of combination of other H ions.

Q_A = heat of ionisation of foreign buffer
 $= 1000$ calories for phosphate buffer
 $= 4000$ calories for borate buffer.

The buffer power symbols are given later.

Before proceeding to the theoretical calculation it is necessary to refer to the titration curve of haemoglobin, by which is meant the amount of alkali neutralised per equivalent of haemoglobin at varying p_{H} . In Fig. 4 based on Peters and Van Slyke [1931, p. 538] are plotted three titration curves, of which

OO' is for oxyhaemoglobin.

RR' is for reduced haemoglobin.

XX' is the curve calculated for those ionisable groups of the oxyhaemoglobin or the reduced haemoglobin molecule, which are not affected by oxygenation, and which in contradistinction to the oxy-labile part of the molecule may be called the "oxy-stable" residue.

The curve XX' is obtained by subtracting the proportion of reduced haemoglobin which is in the oxy-labile ionised form at each p_H from the ordinate of the point on curve RR' at that p_H , i.e. a quantity $= \frac{K_R}{K_R + H}$, for each equivalent of haemoglobin is subtracted. Exactly the same curve XX' is obtained by subtracting, from OO' , the amount of oxyhaemoglobin which is in the oxy-labile ionised form at each p_H , i.e. $\frac{K_O}{K_O + H}$ per equivalent of haemoglobin.

Fig. 4. Titration curves of oxyhaemoglobin, reduced haemoglobin and "oxy-stable residue" of haemoglobin at temperature 18° (calculated from data of Hastings *et al.* [1924], and Stadie and Martin [1924]).

The curves plotted in Fig. 4 are for a temperature of 18° and so are each shifted $0.5\ p_H$ unit to the alkaline side of the corresponding curves given by Peters and Van Slyke, which are for 38° . This correction for the change of temperature is based on Stadie and Martin's data [1924]. In computing the oxy-stable titration curve, K_O has been taken $= 10^{-7.0}$ and $K_R = 10^{-8.5}$.

Let β_O = buffer power of oxyhaemoglobin at each p_H
 = the tangent of the angle made with the p_H axis by the tangent to the titration curve at each p_H .

β_R = similarly the buffer power of reduced haemoglobin,

β_S = buffer power of the oxy-stable residue of oxygenated or reduced haemoglobin.

Let the point R' in Fig. 4 correspond to the condition of the haemoglobin solution when in the reduced state.

Let the point O in Fig. 4 correspond to the condition of the haemoglobin when oxygenated.

Method of calculation.

We shall now show how to calculate for the general case the heat liberated when one equivalent of haemoglobin is oxygenated. This involves:

(i) Oxygenation of $\frac{K_R}{K_R + H'}$ equivalents of oxy-labile ionised reduced haemoglobin to $\frac{K_R}{K_R + H'}$ equivalents of oxy-labile ionised oxyhaemoglobin.

$$\text{Heat liberated} = Q_{Hb} \frac{K_R}{K_R + H'}.$$

(ii) Oxygenation of $\frac{H'}{K_R + H'}$ equivalents of oxy-labile unionised reduced haemoglobin to $\frac{H'}{K_R + H'}$ equivalents of oxy-labile unionised oxyhaemoglobin.

$$\text{Heat liberated} = Q_{Hb} \frac{H'}{K_R + H'}.$$

(iii) Unionisation of $\left(\frac{H'}{K_R + H'} - \frac{H}{K_O + H}\right)$ equivalents of oxy-labile unionised oxyhaemoglobin.

$$\text{Heat absorbed} = Q_H \left(\frac{H'}{K_R + H'} - \frac{H}{K_O + H}\right).$$

As a result of processes (i), (ii), (iii) the oxyhaemoglobin is now at equilibrium between the oxy-labile ionised and unionised forms at hydrogen ion concentration, H , but there have been liberated $\left(\frac{H'}{K_R + H'} - \frac{H}{K_O + H}\right)$ g. ions of hydrogen. Of these

(iv) $\beta_S (p_H' - p_H)$ combine with the oxy-stable residue of the oxyhaemoglobin molecule.

$$\text{Heat liberated} = Q_H \beta_S (p_H' - p_H).$$

(v) $\left(\frac{KA}{H' + K} - \frac{KA}{H + K}\right)$ are taken up by the foreign buffer.

$$\text{Heat liberated} = Q_A \left(\frac{KA}{H' + K} - \frac{KA}{H + K}\right).$$

If there is no foreign buffer present, the heat evolved in process (iii) is almost exactly counterbalanced by that evolved in process (iv), and since there is no heat evolved by process (v), the overall heat will be simply the sum of the heats due to processes (i) and (ii), *i.e.*

$$\text{Overall heat} = Q_{Hb} \frac{K_R}{K_R + H'} + Q_{Hb} \frac{H'}{K_R + H'}, \quad \dots\dots(1).$$

But if there is foreign buffer present, the overall heat

$$= Q_{Hb} \frac{K_R}{K_R + H'} + Q_{Hb} \frac{H'}{K_R + H'} - Q_H \left(\frac{H'}{K_R + H'} - \frac{H}{K_O + H}\right) + Q\beta_S (p_H' - p_H) + Q_A \left(\frac{KA}{H' + K} - \frac{KA}{H + K}\right) \quad \dots\dots(2).$$

The relation between the p_H before and after oxygenation is given by the equation

$$\left(\frac{H'}{K_R + H'} - \frac{H}{K_O + H}\right) = \beta_S (p_H' - p_H) + \left(\frac{KA}{H' + K} - \frac{KA}{H + K}\right) \quad \dots\dots(3),$$

so that if the various numerical constants, together with the p_H after oxygenation, are known, the p_H before oxygenation can be calculated. From equations (2) and (3) the total overall heat Q

$$= Q_{Hb} \frac{K_R}{K_R + H'} + Q_{Hb} \frac{H'}{K_R + H'} + (Q_A - Q_H) \left(\frac{KA}{H' + K} - \frac{KA}{H + K}\right) \quad \dots\dots(4).$$

Equations (2), (3) and (4) have been used for numerical calculations of the overall heat of reaction under the various conditions which have so far been studied.

Table III gives the calculated results for haemoglobin of concentration = 0.01 equivalent/litre:

- (i) In $M/15$ phosphate buffer p_H 6.67 and p_H 7.0.
- (ii) In $M/6$ borate buffer p_H 7.6.

According to the writer's measurements with the glass electrode the p_H of blood, treated with 1% boric acid and then rendered CO_2 -free by repeated evacuation and shaking, is on the average 7.6 at 18°. Such boric acid blood was extensively used by Brown and Hill [1923], and so it seemed of interest to apply the calculations to haemoglobin solutions of corresponding condition.

- (iii) With no foreign buffer added, p_H 8.4.

According to the glass electrode measurements this is the average p_H at 18° reached by whole blood when rendered practically CO_2 -free by repeated evacuation and shaking.

It may be remembered that Brown and Hill made some measurements on this kind of blood also.

Table III.

Conc. of Hb in equiva- lents/litre	Nature and conc. of foreign buffer	p_H	p_H'	β_S	Direct heat
0.01	$M/15$ phosphate	6.67	$\left\{ \begin{array}{l} 6.715 \\ 6.728 \end{array} \right.$	$\left\{ \begin{array}{l} 2.0 \\ 1.2 \end{array} \right.$	$\left\{ \begin{array}{l} 9,930 \\ 9,710 \end{array} \right.$
0.01	$M/15$ phosphate	7.0	$\left\{ \begin{array}{l} 7.083 \\ 7.097 \end{array} \right.$	$\left\{ \begin{array}{l} 2.0 \\ 1.2 \end{array} \right.$	$\left\{ \begin{array}{l} 9,120 \\ 8,700 \end{array} \right.$
0.01	$M/6$ borate	7.6	$\left\{ \begin{array}{l} 7.778 \\ 7.825 \end{array} \right.$	$\left\{ \begin{array}{l} 2.0 \\ 1.2 \end{array} \right.$	$\left\{ \begin{array}{l} 10,680 \\ 10,410 \end{array} \right.$
0.01	No foreign buffer added; freed from CO_2 by evacuation	8.4	—	—	12,600
0.01		10.0	—	—	13,400

The figures of Table III thus cover the most important cases dealt with by Brown and Hill [1923] and by Table II of this paper. The values of the heat are calculated both for $\beta_S=2$, this being the value given by Van Slyke's titration curve data for horse haemoglobin, and for $\beta_S=1.2$, there being some experimental evidence that the buffer value of ox haemoglobin may be appreciably less than that of horse haemoglobin, perhaps as much as one-third less. With haemoglobin of a lower buffer power the overall heat of reaction is somewhat less, as would be expected.

The heat of reaction for haemoglobin in $M/15$ phosphate buffer p_H *ca.* 6.8, comes out at about 4000 calories less than the value in the alkaline range, as has indeed been shown experimentally in Table II. If Q_{Hb^-} had been assumed to be the same as Q_{Hb} , *viz.* 13,400 calories instead of being assumed to be 2000 calories less, the calculated difference in the two overall heats would have amounted only to 2000 calories, instead of 4000 calories. Although there is some uncertainty in the calculations owing to lack of knowledge as to the values of K_O , K_R and β_S for the particular ox haemoglobin solutions used, it still seems impossible to account for so large a difference between the value of the heat in alkaline solution and the value of the heat at p_H 6.8 in presence of phosphate buffer, without postulating that the heat of the primary reaction between oxygen and haemoglobin in acid solution, *i.e.* Q_{Hb} , is appreciably less than the heat of the reaction Q_{Hb^-} in alkaline solution. This is a new conclusion, which it is hoped to test out further by other methods.

Brown and Hill [1923] noted that the heat of reaction of oxygen with 1% boric acid blood was about 3000 calories less than the heat of reaction with

CO₂-free blood containing no added foreign buffer. As regards haemoglobin solution, the calculated difference according to Table II is about 2000 calories. Brown and Hill's heat data may, however, be too high (*v. infra*): furthermore the ratio of haemoglobin concentration to boric acid concentration in their 1% boric acid blood may have been less than in the case quoted in Table III—this also would have tended to make their observed difference greater than the calculated one.

According to the theory put forward above, the minimum value for the overall heat of the reaction should occur when the p_H is midway between p_{K_0} and p_{K_R} and there is present a high concentration (relative to the haemoglobin) of a foreign buffer with a low heat of ionisation, *e.g.* phosphate buffer. In such a case the overall heat of reaction might be expected to be nearly 7000 calories less than the value in alkaline solution. Unfortunately it is not easy to obtain these conditions without having the absolute concentration of haemoglobin too low for accurate measurements of the heat of reaction. This is the reason why the test has not yet been made, though it is hoped to carry it out with an improved apparatus in the near future.

The heat of combination of H ions with haemoglobin. This is an important figure in the above theory. Adair *et al.* [1929] have reviewed and discussed the data obtained up till that date and have, moreover, suggested that the heat may decrease as the p_H is lowered towards the isoelectric point of haemoglobin. Two preliminary experiments were therefore performed to test this point as regards purified ox haemoglobin:

By a modification of the technique described previously, small portions of acetic acid or acetic acid-sodium acetate buffer were mixed with haemoglobin solution, the heat recorded and the p_H measured before and afterwards by means of the glass electrode. Allowance was made for the slight heat of ionisation of acetic acid. The results obtained are shown in Table IV. The values of the

Table IV

p_H range	Heat in calories per g. ion H combining
1st sample of { 8.75-8.0 haemoglobin { 8.0-7.1 { 7.1-6.45	9700 9500 9800
2nd sample of { 8.4-7.35 haemoglobin { 7.35-6.25	8500 8200

heat given in Table IV do not show any marked decline with increased acidity, but the measurements were not very accurate, and further work is needed. The average of the figures reviewed by Adair *et al.* [1929] and obtained since then by Roughton [1930, 1, 2 and Table III of the present paper] is about 9000 calories. The latter value has therefore been used for the calculations given in this paper.

DISCUSSION.

Comparison with the results of previous workers. Adolph and Henderson's [1922] figures show such a wide scatter that it is hardly possible to compare our results with theirs. Only a few of our experiments were done under nearly the same conditions of p_H and buffer content as were those of Brown and Hill [1923] but the general tendency of our figures is to be somewhat lower, *i.e.* 1000-2000 calories, than theirs. No systematic comparison was, however, projected, since

Brown and Hill measured the oxygen taken up by the blood with the aid of the Barcroft-Haldane methods of blood gas analysis. As already mentioned these are known now to give results which are often too low, which would mean that the values for the heat of combination of 1 g. mol. O_2 with haemoglobin given by Brown and Hill would be too high, and to an unknown extent.

None of the results given in this paper or found at any stage in our work approached the high value of 28,000 calories recorded by Barcroft and Hill [1909]. Figures of the same order were, however, found by Macela and Seliškar [1925], using the indirect method (*i.e.* effect of temperature on the dissociation curve): the ratio of the temperature coefficient of the rate of dissociation of oxyhaemoglobin to that of the rate of combination of oxygen with haemoglobin, as reported by Hartridge and Roughton [1923, 2], also suggest a heat value of this order. No explanation has as yet been given of these anomalous results: it is possible that the tendency towards a high heat value may be associated with the same obscure factor, which spasmodically causes the dissociation curve of mammalian oxyhaemoglobin to be hyperbolic instead of sigmoid in shape.

Theoretical conclusions. The data obtained in this paper show that the thermochemistry of the reaction between oxygen and haemoglobin is straightforward in certain points, which had not previously been tested, namely in that the heat per g. mol. O_2 combining with haemoglobin is independent of the percentage saturation, the purity and the habitat of the haemoglobin (*i.e.* whether it is present in solution or in the red blood corpuscle). The thermochemical measurements thus give no crucial evidence either for or against the intermediate compound hypothesis of Adair, nor do they give any indication that the condition of haemoglobin in the corpuscle is different from its condition in solution.

As regards the effect of p_H and foreign buffer content a more comprehensive theory has been worked out than that of Brown and Hill [1923], who did not, through lack of knowledge of the titration curves of oxygenated and reduced haemoglobin (which at that time had not yet been worked out), realise that oxy-labile H ions liberated from one part of the haemoglobin molecule would be buffered, not only by the foreign buffers in the solution (*i.e.* substances other than haemoglobin), but also by other parts of the haemoglobin molecule itself. The present theory needs more extensive test than it has yet received, especially as regards the interesting point that the heat of reaction of oxygen with haemoglobin in the unionised condition is appreciably less than in the ionised condition.

In broad outline, the thermochemistry of the oxygen-haemoglobin reaction, has probably been now almost fully worked out, without having thrown the new light on the mechanism of the O_2 -haemoglobin reaction, which the earlier workers in this field had hoped to obtain thereby. The knowledge gained, however, is of value, not only for its own sake, but also as a basis for studying the thermochemistry of the closely connected reactions of CO_2 in blood. This subject, hitherto but little explored, we hope shortly to take up in detail in the hope of elucidating further some of the more newly recognised processes in CO_2 transport in the blood, especially those in which the enzyme, carbonic anhydrase, on the one hand and carbamino-compounds on the other are concerned.

SUMMARY.

1. A differential calorimeter is described for the measurement of the heat evolved when blood or haemoglobin solution is shaken with various amounts of gaseous oxygen or carbon monoxide. The technique has been applied to certain outstanding points in the thermochemistry of the haemoglobin reactions.

2. The heat of combination of haemoglobin, per g. mol. of oxygen, as measured directly is:

(a) independent of the percentage saturation over the range investigated, viz. 25–80 %; this also holds good for the heat of combination of haemoglobin with carbon monoxide;

(b) practically the same in whole blood, laked blood and purified haemoglobin;

(c) much greater at p_{H} ca. 9.5 (i.e. $Q=13,400$ calories about) than at p_{H} 6.8, $M/15$ phosphate buffer (i.e. $Q=9400$ calories about). The difference is in part due to the heat of combination of oxygen with oxy-labile ionised haemoglobin being greater than the heat of combination with "oxy-labile" unionised haemoglobin, and partly due to the heat effects associated with the secondary ionic reactions, which occur over the p_{H} range 6.0–9.0. The magnitude of these effects depends not only on the p_{H} but also on the nature and concentration of the foreign buffer (phosphate) relative to that of the haemoglobin. Detailed theory and calculations concerning this effect are given.

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