THE NATURE OF OXYHÆMOGLOBIN, WITH A NOTE ON ITS MOLECULAR WEIGHT.

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IN the present paper we propose (1) to set forth some additional evidence for supposing that the union of oxygen with hæmoglobin is a chemical one, (2) to press this supposition to its logical conclusion and on thermodynamical principles deduce the heat generated when one molecular weight of hæmoglobin unites with oxygen, (3) by actual determinations of the heat produced by the union of one gram of hæmoglobin with oxygen, to calculate the molecular weight of hæmoglobin. The paper will therefore divide itself into three portions corresponding to the subjects set forth above[1].

PART I. THE RELATION OF TEMPERATURE TO THE RATE OF REDUCTION OF HÆMOGLOBIN.

Until recently it was supposed universally that hæmoglobin was chemically combined with oxygen and that the reaction involved was governed by the laws of mass action. Some doubt has been thrown upon this by Wolfgang Ostwald[1] who suggested that Bohr's[2] dissociation curve for hæmoglobin could be explained by the simple hypothesis that the oxygen was held by adsorption to the hæmoglobin.

In a recent paper by Barcroft and Roberts[3] it was shown that Bohr's curve, though true for hæmoglobin crystallised out with ether and redissolved, is modified when the solution so obtained is dialysed. The curve then approximates so closely to a rectangular hyperbola that there is no difficulty in supposing the union of oxygen and

[1] The responsibility for the mathematical portion of the work rests with Hill and for the oxygen estimations with Barcroft.

hæmoglobin in salt free solutions to be represented by the simple chemical formula

$$Hb + O_2 \rightleftarrows HbO_2.$$

But inasmuch as the adsorption formula contains two constants it is capable of great adaptation and it is not impossible to draw an adsorption curve with which the determinations of Barcroft and Roberts would not conflict. Some fresh evidence on the subject is therefore desirable.

If nitrogen be bubbled at a constant rate through hæmoglobin solution the velocity of the reduction of oxyhæmoglobin is very much greater at 38° C. than at 18° C. The experiment was carried out in the following way.

A solution of oxyhæmoglobin was made, dialysed and concentrated with the pump in the manner to be described later by Barcroft and

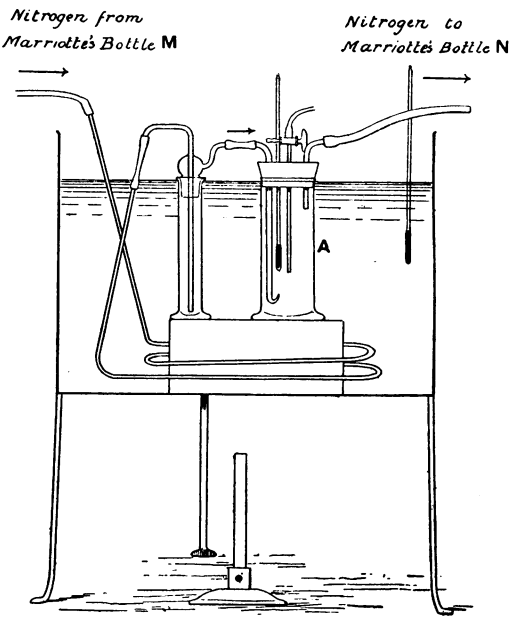

Fig. 1.

Roberts[1]. About 200 c.c. was placed in a cylindrical glass vessel, Fig. 1. This quantity rather more than half filled the vessel and was covered with a layer of oil. The vessel was fitted with a rubber cork through which three tubes led; one, through which the nitrogen entered, went to the bottom of the vessel and then turned upwards and was drawn off

[1] Cp. This *Journal*, *infra*, p. 429.

to a fine point; the second was made of capillary glass tubing and was fitted with a 3-way tap, and this served for the abstraction of samples of hæmoglobin; the third, which only just went through the cork, served as an exit tube for the nitrogen. A thermometer also went through the stopper.

The vessel was placed in a large water bath. In this bath there was also a thermometer and a wash bottle containing pyrogallic acid through which the nitrogen bubbled on its way to the vessel *A*. The nitrogen was delivered from a Marriotte's bottle (*M*) of about 15 litres capacity, and after passing first through a coil of metal tubing in the bath, then through the pyrogallic acid and the hæmoglobin, it was driven into another Marriotte's bottle (*N*) of the same capacity as the first. To set up a flow of nitrogen, water was allowed to enter *M* from a constant high level and to escape from *N* at a constant low level, thus the stream of gas went at a constant rate. The Marriotte's bottles are not shown in the figure. A constant temperature was maintained in the bath to within a fraction of a degree by a Bunsen burner.

The samples of hæmoglobin were withdrawn with a cylindrical 1 c.c. pipette graduated in $\frac{1}{100}$ of a c.c. and analysed in the differential blood gas apparatus of Barcroft and Roberts.

To compare the rates of reduction of oxyhæmoglobin at 18° C. and 38° C. the experiment runs the following course. The bath is started at 18° C.; water, equal in volume to the hæmoglobin solution which we were about to study, was placed in the glass vessel *A* and the bubbling was allowed to commence. When the pressures in various parts of the apparatus had so adjusted themselves that the nitrogen bubbled at a constant rate, the rubber portions of the tubing were clipped, the stopper was taken out of the vessel *A*, the solution of oxyhæmoglobin which had been thoroughly shaken with air was put in instead of the water, some oil was placed on the top of the hæmoglobin and the nitrogen was allowed to bubble through the hæmoglobin. The first sample of hæmoglobin was withdrawn for analysis at the end of 35 minutes. It was analysed by the differential method and found to be 94% saturated. The nitrogen was then stopped. The data furnished by this experiment are recorded in Fig. 2 in which the percentage saturation is plotted vertically and the time from the commencement of the passage of nitrogen through the hæmoglobin solution is plotted horizontally. *AD* on the curve corresponding to Exp. 1 represents the portion of the experiment which we have just described. At this point the temperature of the hæmoglobin was

raised as rapidly as possible to 38° C. The time which this process took is not included in the figure. The nitrogen was once more allowed to pass. By counting the bubbles which entered the Marriotte's bottle *N*, observations were made from time to time to satisfy ourselves that the rate of bubbling was constant. The figure from *D* to *E* represents the subsequent course of the experiment; the actual determinations are shown by encircled points. By a calculation, the basis of which is

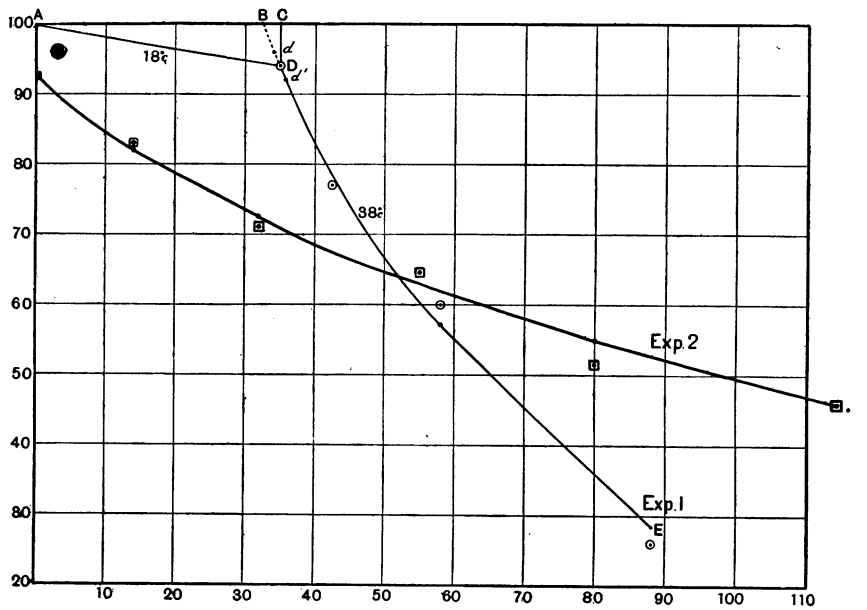

Fig. 2. Curves representing the calculated degree of dissociation at any time in Exps. 1 and 2. Percentage saturation plotted vertically, time in minutes horizontally. The points represent the actual observations.

given below, it is possible to exterpolate the curve *ED* as far as *B* and thus to discover *BC*, the length of time which would have been taken to reduce the hæmoglobin from 100 % to 94 % saturation at 38° C. This is 2·6 minutes. The velocity of the reduction is therefore 35/2·6 = 14 times as great at 38° C. as at 18° C. Over a range of 10° C. the temperature coefficient will be the square-root of what it is over 20° C. or 3·7. The limits of error in the above experiment are necessarily very considerable. The percentage saturation instead of being 94 % might be 92 % or 96 %, in which case *D* would be moved correspondingly down or up *DC* to *d'* or *d* respectively. There would be a corresponding movement of *B*. The experiment shows with

certainty however that the temperature coefficient over a range of 10° C. is between the limits 3·5 and 4·6. This high temperature coefficient is strong evidence in favour of the hypothesis of chemical combination between O_2 and hæmoglobin.

The actual rate of reduction of any hæmoglobin solution will of course depend upon the strength of the solution and the rate of the stream of nitrogen, but if the action is a chemical mass action the curve must be capable of being represented by a formula which is obtained by the following calculation.

If $[O_2]$ be the concentration of oxygen in the solution we have the equation

$$HbO_2 \rightleftarrows Hb + O_2.$$

Now at the surface of any bubbles of gas we have a diffusion of gas into the bubble from the solution and its rate of diffusion is proportional to $[O_2]$.

Hence if the rate of bubbling is always the same the rate of loss of oxygen from the solution is proportional at any moment to $[O_2]$.

Now let $100x/a$ be the percentage saturation, x the number of molecules of HbO_2 present (assuming the Hb to be present as molecules), a the total number of molecules present whether as Hb or HbO_2.

The amount of O_2 present $= x +$ the amount in simple solution. The latter term is small compared with the first and may be neglected.

$\therefore \; -\frac{dx}{dt} =$ the rate of loss of oxygen from the solution $= \lambda[O_2]$.

Now the laws of mass action give us

$$-\frac{dx}{dt} \propto k[HbO_2] - k'[Hb][O_2],$$

$$i.e. \quad -\frac{dx}{dt} = k\mu[HbO_2] + \frac{\mu k'}{\lambda}[Hb]\frac{dx}{dt},$$

$$i.e. \quad -\frac{dx}{dt}(\lambda + \mu k'[Hb]) = \mu k \lambda x,$$

$$i.e. \quad -\frac{dx}{dt} = \frac{\mu k \lambda x}{\lambda + \mu k' a - \mu k' x},$$

$$i.e. \quad +\frac{dx\,(\mu k' x - \lambda - \mu k' a)}{\mu k \lambda x} = dt,$$

$$i.e. \quad dx\left\{\frac{k'}{k\lambda} - \frac{\lambda + \mu k' a}{\mu k \lambda}\frac{1}{x}\right\} = dt,$$

and integrating,

$$\left[x\frac{k'}{k\lambda} - \frac{\lambda + \mu k' a}{\mu k \lambda}\log_e x\right] = t,$$

where the square brackets signify "between the limits of integration,"

$$i.e. \quad -(x_0 - x)\frac{k'}{k\lambda} + \frac{\lambda + \mu k' a}{\mu k \lambda}\log_e \frac{x_0}{x} = t.$$

Thus if y_0 be the initial percentage saturation and y the percentage saturation at any time, this may be written

$$\alpha \log_{10}\frac{y_0}{y} - \frac{\beta}{100}(y_0 - y) = \gamma t.$$

The constants α, β, γ involve the rate of bubbling through, the velocity constants, and the temperature.

$$[\alpha \text{ is } (\lambda+\mu k'a)\log_e 10, \quad \beta \text{ is } \mu k', \quad \gamma \text{ is } \mu k\lambda.]$$

Exp. 2 and a part of Exp. 1 show the actual correspondence between the formula and the observed results. In each case the temperature was 38° C. but the bubbling was much more rapid in the case of Exp. 1 than in that of Exp. 2: hence the reduction was more active in the former case. Of the curves shown in Fig. 2 the portion *DE* of Exp. 1 and the whole of Exp. 2 are curves calculated from the above formula. The points which are actually observed lie very close to these curves. Of these two curves, much the most stress is to be laid upon that of Exp. 2 (so far as this aspect of the experiment is concerned). In drawing the curve it is necessary to assume the correctness of the first and two other points in order to decide the constants. Now it might happen that three given points could not be made to lie on any curve which was drawn from the above equation; this however is practically not very likely to be the case, so that the real point at issue is whether the remaining determinations do or do not agree with the curve which at once satisfies the formula and passes through three points. Clearly then the greater the number of determinations the more exacting is the test. In the case of Exp. 1 we only have four determinations on the curve in all. The results so far as they are depicted are reliable: the experiment was carried on longer than is shown and it then appeared that the continued incubation set up an appreciable degree of bacterial action. This was discovered from the fact that after the blood gas determination had been made the fluid in the manometer of the blood gas apparatus gradually crept upwards on the side in which the incubated solution was placed[4].

In Exp. 2 we modified the method of making our hæmoglobin so as to insure greater freedom from bacteria.

The blood was centrifugalised and the corpuscles were washed three times with Ringer's solution. The mass of moist corpuscles was treated with ether to crystallise the hæmoglobin and the thick paste of ethereal crystals was placed in a dialysing tube and dialysed for two days with frequent changing of the distilled water. On account of the ether present, the process of dialysis will have started with the whole contents of the dialyser free from bacteria, and as the ends were closed there was very little opportunity for germs to enter. At the end of the dialysis all trace of ether had gone and the solution was sufficiently

strong to serve for the experiment without concentration. It was centrifugalised to rid it of any solid particles present, either undissolved crystals or traces of precipitated protein from the stroma. At the end of the experiment the samples of solution used in the apparatus were allowed to remain for four hours in the bottles in which they had been analysed: none of them showed any sign of absorbing the oxygen in the bottles.

The results of Exps. 1 and 2 are as follows:

Exp. 1.	Time (minutes) measured from *D*	0		7·5	23		53
	Percentage saturation observed	94		77	60		26
	Percentage saturation calculated	94		79	58		31
Exp. 2.	Time (minutes)	0	14·5	32	55	80	114
	Percentage saturation observed	92·5	83	71	64	55	46
	Percentage saturation calculated	92·5	82	72·5	63	51·5	46

It is clear then that in the case of two solutions, prepared somewhat differently, of different strengths and through which the nitrogen ran at very different velocities the course of the reduction followed a complicated formula based upon a conception of oxyhæmoglobin as a chemical compound.

We feel justified then in regarding it as such for the purposes of the succeeding experiments.

Part II. Determination of the heat of combination of the gram-molecule of hæmoglobin with oxygen.

On the assumption that the action is of a chemical nature we have used the laws of thermodynamics to give us a value for the heat of combination of the gram-molecule of hæmoglobin with O_2.

Let us assume that the molecule of hæmoglobin contains n atoms of iron, and therefore combines with n molecules of oxygen. The reaction would be:

$$(Hb)_n + nO_2 \rightleftarrows (HbO_2)_n.$$

So that for the equilibrium

$$\frac{[(HbO_2)_n]}{[(Hb)_n][O_2]^n} = K.$$

If $n = 1$ this gives a rectangular hyperbola for the dissociation curve. Barcroft and Roberts[3] obtained six points which within the limits of error lay on such a curve: but this is not conclusive proof of the simple value of n, for it would be possible on the assumption that $n = 2$ to draw

a curve with a different value of K which will satisfy the observations almost as well.

From the laws of thermodynamics we know that K varies with the temperature according to the equation:

$$\frac{1}{K}\cdot\frac{dK}{dT}=\frac{-q}{RT^2} \quad \text{.....(A),}$$

where T is the absolute temperature and q the heat given out by the union of one gram-molecule of hæmoglobin with oxygen[3]. Let us now assume that q remains constant over the range of temperature under consideration, an assumption which is commonly made.

Then integrating the above equation between the limits T and T_0:

$$K=K_0 e^{\frac{-q}{2}\cdot\frac{T-T_0}{TT_0}} \quad \text{.....(B),}$$

where K and K_0 are the equilibrium constants at the absolute temperatures T and T_0. Two points arise out of the equation.

(1) If any two values be taken for T and T_0, are the corresponding values for K always such as to give the same value for q? If so the integration of equation (A) is justifiable.

(2) What is the actual value of q?

We determined to compare the values of K at different temperatures. *If the oxygen tension be kept constant* during the whole experiment K may be taken as proportional to $\frac{\text{Percentage saturation}}{100-\text{percentage saturation}}$.

The problem therefore resolves itself into a determination of the percentage saturation of a given solution of hæmoglobin at different temperatures over as large a range as possible.

Our method of doing this was as follows. A litre flask was used for exposing the hæmoglobin solution to the gas. The strength of the hæmoglobin solution was about 10%: the 10 c.c. used were therefore capable of liberating about 1·3 of O_2 and thus producing a maximum difference of 1 mm. of mercury in the oxygen pressure of the gas in the flask. In reality the difference of oxygen pressure was never so great because (1) the hæmoglobin was never completely reduced and (2) at each increase of temperature a cubic centimetre or more of solution was taken out so that at the end of the experiment there was virtually no hæmoglobin solution left in the apparatus.

The apparatus is shown in Fig. 3. The flask is immersed in a water bath which consists of a saucepan into the lid of which a short tube has been soldered to accommodate the neck of the flask. The bath is

brought to the required temperature and the apparatus vigorously shaken, care being taken to maintain the thermometer reading steady.

In order to take a sample of hæmoglobin for analysis the lid is taken off the bath and rapidly inverted so that the mouth of the flask points downwards and the base upwards. The hæmoglobin solution then falls

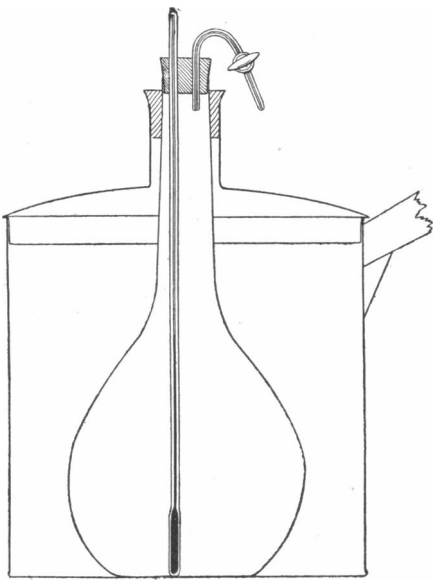

Fig. 3.

into the neck of the flask where only a small surface is exposed to the atmosphere of the flask ; this precaution is necessary in order to prevent the change in temperature caused by taking the flask out of the bath from affecting the saturation of the oxyhæmoglobin. A small piece of rubber tubing of fine bore is attached to the capillary glass tubing which passes through the cork of the flask. As we have worked from lower temperatures to higher ones, mere opening of the tap on this tube will allow a drop of hæmoglobin solution to be driven out, thus clearing the dead space of the tubing. The end of a 1 c.c. pipette is put into the tube and a sample taken out for analysis.

The hæmoglobin was exposed to an atmosphere consisting of oxygen and nitrogen, the former exerting a pressure of 10 mm. of mercury or less. The advantage of a very low oxygen pressure is that it allows of a large range in the percentage saturation at different temperatures.

The flask was almost filled with distilled water and inverted over water. It contained therefore a little air. The water was then displaced by nitrogen from a cylinder. The cork was put into the neck of the bottle under water. About 10 c.c. of hæmoglobin solution were then forced in through the capillary tube, after which the tap was opened momentarily to equalise the pressure within and without the flask.

The hæmoglobin in the first of the following experiments (Exp. 3) was made in the same way as that used in Exp. 2, whilst that used for Exp. 4 was made as follows. Defibrinated dog's blood was centrifugalised, the serum poured off and the corpuscles given two washings with Ringer's solution. At the end of the last washing the blood was centrifugalised again, the Ringer's solution poured off and the mass of corpuscles dialysed in a tube which was rhythmically raised and lowered in the distilled water throughout the whole period of the dialysis. The distilled water was frequently changed. As the salts passed outwards and the water inwards the corpuscles laked and the hæmoglobin dissolved whilst the stroma remained undissolved. At the end of the dialysis the fluid was centrifugalised: after the sediment had been removed a solution was obtained which contained no solid particles, as shown by microscopic examination.

In the subjoined table (Exp. 3) are placed (*a*) the actual % saturations observed at different temperatures, (*b*) the saturations giving values of K, which inserted with their corresponding temperatures into the above formula (B), satisfy it if q is given the value 27,700.

Exp. 3. Temperature	16°	25°	32°	38° C.
a, % saturation (observed) ...	96	89	77	52
b, % saturation (calculated) ...	97	89	74	54
K, corresponding to *b*, ∞ ...	33	8	2·8	1·17

A similar table is given for Exp. 4. Here the K's are calculated with the value 28,700 for q.

Exp. 4. Temperature	16°	24°	32°	38°	49° C.
a, % saturation (observed) ...	92	71	37	18	6
b, % saturation (calculated) ...	90	71	41	22	6
K, corresponding to *b*, ∞ ...	9	2·4	·69	·28	·06

From the above figures we conclude that:

1. Since the observed saturations are all within the maximum error (5 %) we are justified in regarding q as a constant quantity for any given solution of hæmoglobin at whatever temperature it is observed, and that therefore the equation (A) may be integrated.

2. Since two solutions prepared (*a*) by crystallisation with ether and (*b*) without the use of ether or alcohol, give values for q (27,700 and 28,700) which are so close as to be within the probable limits of error, the value of q may be regarded uniformly as 28,000 calories.

3. It is now possible, with the assumption that $n=1$, when the hæmoglobin molecule is represented by $[\mathrm{Hb}]n$, to draw the dissociation curve for hæmoglobin at any temperature. In making this assumption we are merely anticipating the proof given in the last part of the present paper.

The following are the principles on which such a curve is drawn:

The formula for the dissociation when $n=1$ is:

$$\mathrm{Hb}+\mathrm{O_2} \rightleftarrows \mathrm{HbO_2}.$$

If the total concentration of hæmoglobin be 100 and the percentage present as reduced hæmoglobin be Cr and the oxygen pressure p, the laws of mass action give

$$(Cr)\left(\frac{1}{K}+p\right)=\frac{100}{K}.$$

If then we call (Cr), y; and $\left(\frac{1}{K}+p\right)$, x we arrive at the fact that $xy=$ a constant (at any particular temperature) which involves K. If values of y are plotted vertically downwards and of x horizontally to the right, the curve is a hyperbola in which the distance of any point from the horizontal axis represents the percentage of reduced hæmoglobin present (or $100-{}^0/_0\,\mathrm{HbO_2}$) and any distance measured horizontally from the vertical axis represents, not the pressure, but $\left(\frac{1}{K}+p\right)$.

For 38° C. or 311° absolute:

$$\therefore\ xy=800=\frac{100}{K}, \qquad \therefore\ \frac{1}{K}=8.$$

When the hæmoglobin is entirely reduced $Cr=100$ and p will therefore $=0$ while $x=8$.

If therefore the numerical quantities be represented on the drawing in millimetres, and a vertical line be drawn 8 mm. to the right of the vertical axis, then the pressures are measured from this line, and the hyperbola will pass through it at a point 100 mm. from the top; this point is the origin of the dissociation curve.

For any other temperature T, it is necessary to find a fresh value for K; having found it we get a fresh value for $xy\left(=\frac{100}{K}\right)$ and as before the line representing zero pressure is $\frac{1}{K}$ mm. to the right of the vertical axis.

The fresh value for K comes directly from the formula

$$K=K_{38}\,e^{\frac{-q}{2}\cdot\frac{T-T_{38}}{T.T_{38}}},$$

where $K_{38}=\frac{1}{8}$, $\log e=\cdot 434$, $q=28{,}000$ and $T_{38}=311$.

In the present research the value $xy=800$ for 38° has received some experimental verification. In the table corresponding to Exp. 3, the calculated saturation of oxyhæmoglobin is 54 %, *i.e.* there is 46 % of

reduced hæmoglobin. The tension of the oxygen in the flask should therefore have been $\frac{800}{46} - 8$ mm. $= 9{\cdot}4$ mm. Direct analysis showed it to be $9{\cdot}7 \pm 1{\cdot}0$ mm.

In Fig. 4 we show dissociation curves of hæmoglobin drawn on the basis of the values of K in the above tables.

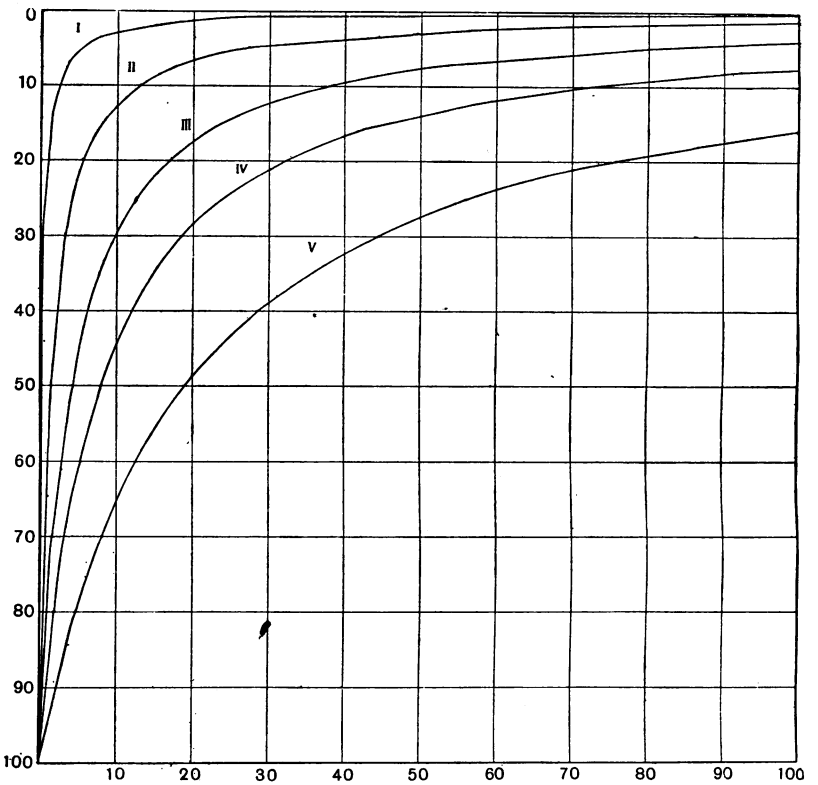

Fig. 4. Dissociation curves I, II, III, IV and V correspond to 16°, 25°, 32°, 38° and 49° C. respectively. Oxygen tension plotted horizontally, percentage saturation of hæmoglobin vertically.

Part III. Determination of molecular weight.

If H be the amount of heat given out when one gram of hæmoglobin unites with oxygen and q the amount when one gram-molecule unites with oxygen, the molecular weight is q/H. A value for q has been obtained above: we propose to describe the method by which we have determined H.

The hæmoglobin solution was made as in Exp. 4. The oxygen was pumped off in the blood gas pump. The receiver of the pump was then filled with nitrogen from a cylinder; the pressure of the gas in the receiver was somewhat in excess of the atmosphere pressure. A 100 c.c. pipette containing a little oil was placed in the position of the burette (see Fig. 2. Barcroft and Roberts. This *Journal*, infra p. 429): on opening the tap the pressure drove the hæmoglobin solution into the pipette, the surface in the pipette being covered with a film of oil. By closing the ends of the pipette the solution of reduced hæmoglobin could be kept unchanged for a short time.

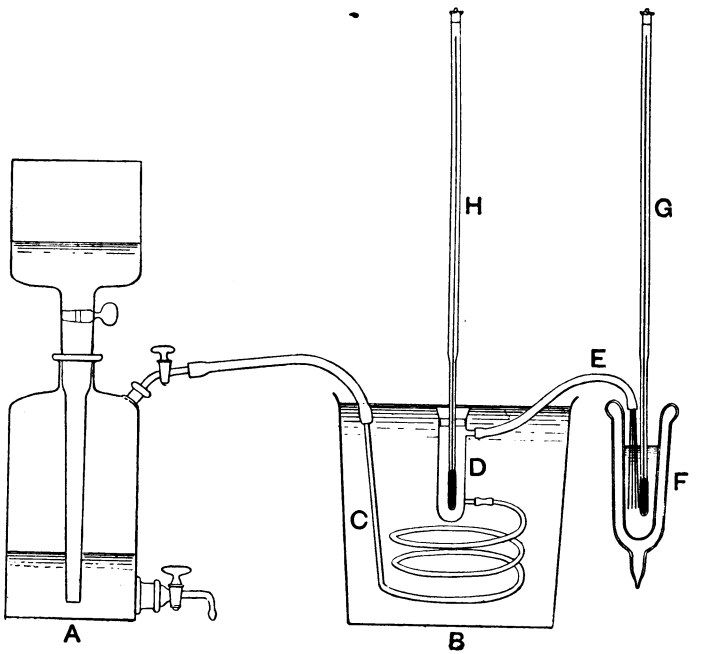

Fig. 5.

The oxygen for the experiment was taken from a gas holder, Fig. 5 *A*, which had previously been filled from a cylinder over distilled water. The oxygen was therefore saturated with aqueous vapour and changes of temperature in the hæmoglobin solution did not arise from evaporation when the oxygen was bubbled through it, nor was the gas cooled by violent decompression. The gas passed through a long coil of flexible metal tubing immersed in a large water bath. The tube

communicates with a short glass tube, also in the water bath, which contains the bulb of a Beckmann's thermometer. The tube is closed but for the apertures for the entrance and exit of the oxygen. The gas leaves *D* by a piece of rubber pressure tubing; into the end of this are fixed, by means of plasticine, four glass tubular filaments sufficiently long to reach to the bottom of the calorimeter which is a Dewar's flask *F*.

For an hour or so before the commencement of the experiment, the pipette containing the reduced hæmoglobin was immersed in the bath *B*, so that there might be as little difference as possible between the temperature of the hæmoglobin and that of the oxygen entering it, though, so small is the specific heat of oxygen as compared with water, that a very appreciable difference of temperature might exist without vitiating the experiment. (See also controls below.)

Five cubic centimetres of cedar wood oil (which was chosen in preference to olive oil inasmuch as the latter is apt to be acid in reaction, and to clove oil which unites to some extent with water) were placed in the Dewar's flask. The pipette was removed from the water bath and the reduced hæmoglobin allowed to run into the flask, protected from air by the oil.

The temperature changes in the hæmoglobin solution were observed with a Beckmann's thermometer *G*, which could be read with a lens from some little distance. The solution was stirred with a small hollow globe made of very fine glass suspended by a glass filament.

The experiment may be divided into four periods.

(1) That before the oxygen is allowed to flow in which there will be a gradual and regular change of temperature.

(2) That in which the oxygen is flowing: in this there is a rapid rise of temperature.

(3) That following the passage of the gas. The thermometer, getting into equilibrium with the fluid, still rises more rapidly than in period (1),—also possibly the hæmoglobin itself requires an appreciable time to get into equilibrium with the oxygen dissolved in the fluid.

(4) The period subsequent to the chemical action in which the change of temperature should be the same as in period (1).

To calculate the rise of temperature due to the chemical action we subtract from *T*, the rise during periods (1) and (2), *t'*, the change which would have taken place during the same period of time had no oxidation taken place. This is calculated from periods (1) and (4).

The temperature was noted every minute. The results of Exp. 5, which is typical, may be abridged as follows:

Period	Time	Rise of Temperature	Rise of Temp. per min.
I	4 min.	·013° C.	·00325° C.
II	9 min.	·158° C.	·0262° C.
III	1 min.	·012° C.	
IV	4 min.	·013° C.	·00325° C.

$T = ·162°$ C. $t = 10 \times ·00325 = ·032°$ C. $T - t = ·130°$ C.

That bubbling oxygen through distilled water under similar circumstances does not cause a rise of temperature which can be read by the thermometer is shown by the following Exp., in which water was substituted for hæmoglobin solution.

Period	Time	Rise of Temperature	Rise per min.
I	3 min.	− ·001° C.	− ·0003° C.
II	4 min.	− ·001° C.	− ·0002° C.
III	non-existent		
IV	4 min.	− ·004° C.	− ·001° C.

Taking the nett rise of temperature in Exp. 5 as 0·13° C. it is necessary to calculate the actual heat production; several circumstances have here to be considered. (1) The specific heat of the hæmoglobin solution differs from that of water. (2) The oil, the flask and the thermometer are heated as well as the solution. The best way of treating these factors is to find the water equivalent of the flask and its contents. For this purpose a known quantity of water at a known temperature was added and its heating effect observed.

Thus the addition of 5·66 c.c. of distilled water at 33° to the flask which contained 66 c.c. of solution, five of oil, the thermometer and the stirrer raised the temperature 1·110° C. The final temperature was 17°; from which it follows that 90·6 calories raised the equivalent of x grams of water 1·110° C.; therefore $x = 81·6$ c.c. The addition of a second 5·66 c.c. of water (after making the necessary deduction for the increased volume of fluid) gave a value of 84·7: mean value 83·1.

This was slightly too large as the added fluid heated more glass than was occupied by the original 66 c.c.; to correct for this we have assumed that the flask and the thermometer were cylindrical, neglected the base of the flask and the oil and supposed that the amount of glass heated by the 5·66 c.c. of water bore the ratio of $\frac{66 + 5·66}{66}$ to that of the glass heated by the oxygen. This corresponds to a deduction of approximately 1 calorie from the figure given above, leaving 82 c.c. as the water equivalent of the calorimeter and its contents.

The heat given out by the oxidation is therefore $82 \times 0{\cdot}13 = 10{\cdot}66$ calories, *i.e.* $\frac{10{\cdot}66}{66} = {\cdot}162$ calorie per c.c. of the hæmoglobin solution.

The other measurement required was the mass of hæmoglobin oxidised in 1 c.c. of the solution.

At the commencement of the experiment the hæmoglobin may not be entirely reduced, at the end it is not entirely oxidised, so that the total amount of hæmoglobin in the solution bears no relation to the amount which has been oxidised.

Under these circumstances we adopted the method of measuring, with the differential blood gas apparatus, the difference between the amount of oxygen in equal volumes of the hæmoglobin solution as it was before and after the oxidation and deduced the amount of hæmoglobin oxidised. This method has one disadvantage, namely that it assumes a knowledge of the precise ratio between the oxygen and the hæmoglobin —a point about which there is much difference of opinion. For the purposes of the following calculations we have taken Hüfner's figure as being correct—1·34 c.c. of oxygen = 1 gram of hæmoglobin. We put forward no brief for this figure and will not discuss its merit since the use of a slightly different one will not alter the essential argument of our research.

The method which we have adopted has advantages over the alternative one (that of completely oxidising the hæmoglobin and subsequently drying and weighing it) which far outweighed the limited uncertainty respecting the relation of the oxygen to the hæmoglobin.

1. It would be a very difficult and laborious matter to reduce completely—as opposed to approximately—the large mass of hæmoglobin solution necessary for the experiment. Moreover the solution so reduced would be spoiled if it came in contact with even the smallest bubble of air after the reduction.

2. The process of oxidising it completely would be a lengthy one, whilst that of oxidising say two-thirds of the hæmoglobin is a rapid one. The advantage of cutting periods II and III of the experiment as short as possible is very great, since so doing diminishes t in relation to T.

3. If the hæmoglobin is only 60 or 70 % saturated at the end of the experiment the final tension of oxygen in the fluid is but a few mm. and therefore the quantity in solution is practically *nil*. This fact avoids any correction for the heat of solution of the oxygen.

4. If the hæmoglobin were weighed, or even the iron estimated, it

would be necessary to make the very dangerous assumption that there was no methhæmoglobin present.

In the case of Exp. 5, 2·3 c.c. of solution were taken before the commencement of period (1) and the same quantity after the oxidation. The analysis showed that 0·089 gram of hæmoglobin per c.c. of solution had been oxidised. H therefore $= 0{\cdot}162/{\cdot}089 = 1{\cdot}82$ calories. Two similar experiments gave 1·98 and 1·75 calories. The mean value for the heat of combination of one gram of hæmoglobin is therefore 1·85 calories. This value is greater than that given by either Torup[6] or Camis[7].

So far we have assumed in our argument that the molecule of hæmoglobin is $(Hb)_n$, where (Hb) is the least possible molecular weight. This contains one atom of iron, and n may be 1 or any higher whole number. $(Hb)_n$ then has the molecular weight

$$\frac{q}{H} = \frac{28{,}000}{1{\cdot}85} = 15{,}200 : \text{ hence } \mathrm{Hb} = \frac{15{,}200}{n}.$$

But chemical analysis shows the least possible value for the molecular weight of hæmoglobin to be 16,669[8]: to this $\frac{15{,}200}{n}$ must be approximately equal, and n must therefore be 1. Hence the molecular weight of hæmoglobin is the least possible value 16,669. This result agrees with the determinations of Hüfner and Gausser[9], who obtained a similar molecular weight from measurements of osmotic pressure. Waymouth Reid[10] on the other hand found a value 48,000, while Roaf[11] found a value of about 32,000 for hæmoglobin in distilled water, and of 16,000 for hæmoglobin in sodium carbonate solutions. Both used the osmometer method. Roaf has as yet published only a preliminary communication.

It is not at all unlikely that a solution of hæmoglobin prepared by Bohr's method might contain aggregates of molecules, and its dissociation curve can be explained on this theory; possibly all undialysed solutions contain aggregates to a greater or less extent; this however must form the subject of a future paper.

Conclusions.

1. The velocity of dissociation of oxyhæmoglobin obeys an equation derived from the laws of mass action, and has a high temperature coefficient, increasing about 4 times for a rise of 10° C.

2. The variations of the equilibrium constant K, with change of absolute temperature T, follow the equation

$$\frac{1}{K} \cdot \frac{dK}{dT} = \frac{-q}{2T^2},$$

where q is constant and equal to 28,000 calories. From the second law of thermodynamics q is the heat of combination of one gram-molecule of hæmoglobin with oxygen.

3. The amount of heat (H) given out when one gram of hæmoglobin unites with oxygen is 1·85 calories.

4. The molecular weight of hæmoglobin in dialysed solution is the least possible value 16,669.

5. From the high temperature coefficient and from the fact that the reaction obeys several equations based upon a chemical conception of its nature, we conclude that oxygen unites chemically with hæmoglobin according to the formula $Hb + O_2 \rightleftarrows HbO_2$.

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