Hæmoglobin.

A LECTURE DELIVERED BEFORE THE CHEMICAL SOCIETY ON FEBRUARY 11TH, 1926.

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IN making some remarks to-night about one of the subjects dearest to my heart, namely, hæmoglobin, I wish to disabuse you at the outset from any idea that my primary object is to inform you—far otherwise—hæmoglobin is to me a query mark—an unanswered question—a Will-o'-the-wisp—something which one day you think you have grasped only to find the next that its real essence has eluded you. You recollect what Thackeray said about George IV: "To make a portrait of him at first seemed a matter of small difficulty. There is his coat, his star, his wig, his countenance simpering under it: with a slate and a piece of chalk I could at this very desk perform a recognisable likeness of him. And yet after reading of him in scores of volumes, and hunting him through old magazines and newspapers, having him here at a ball, there at a public dinner, there at races and so forth, you find you have nothing—nothing but a coat and wig and a mask smiling below it—nothing but a great simulacrum. . . . I look through all his life and find but a bow and a grin—I try and take him to pieces, and find silk stockings, padding stays, a coat with frogs and a fur collar, a star and a ribbon, a pocket handkerchief prodigiously scented, one of Truefitt's best nutty-brown wigs reeking with oil, a set of teeth and a huge black smock under-waistcoat, more under-waistcoats and under that nothing." And so in my more despondent moments it seems to be with hæmoglobin. Its colour fascinates you, you crystallise it only to find that it crystallises in innumerable forms, you determine its osmotic pressure to find that may be anything, you redissolve the crystals to find that the material has altered as the result of crystallisation. Is the attempt to pursue hæmoglobin worth while? Well, gentlemen, at the end of this lecture you may answer that question as you will. For my part it is just here that the analogy with "The first gentleman in Europe" breaks down, for you must remember that Thackeray went on: "I own I once used to think it would be good sport to pursue him, fasten on him, pull him down. But now I am ashamed to mount and lay good dogs on to summon a full field and then to hunt the poor game." Hæmoglobin never can appear poor game to the biologist, for the life of the warm-blooded animal—yours and mine—depends upon its existence, there is no other substance which is capable of transporting oxygen

in just the same amount and in just the same way. How nature discovered the pieces from which to effect the synthesis must ever be a fascinating subject of inquiry to the biologist—and to find out what those pieces are and in what way the synthesis has been effected he must digress from his own proper sphere into that of chemistry. Yet as ribbons, wigs, and stars go, those of George IV were probably more interesting than most, and if I at once disclaim the objects of telling what hæmoglobin really is, and make it clear that I am merely trying to pass an hour by discussing some of its more superficial properties, it may be that you will find these a little interesting, even if you regard the methods of the biologist as being at one time crude and at another clumsy, and his presentation of a chemical problem naïve and amateurish.

I.

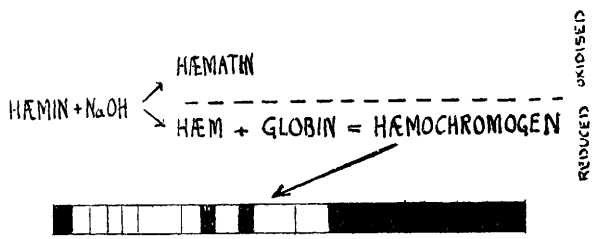
The time-honoured conception of hæmoglobin was that it consisted of two moieties united in some unknown way. These were called hæmatin and globin, the latter being a protein, the former a substance containing iron. It was supposed further that these could be broken the one from the other by acid or alkali. Within the last two years the position as put forward above has been gone into in considerably greater detail. Let us start with hæmoglobin and work backwards. If in the presence of a reducing agent hæmoglobin be made alkaline, a substance, hæmochromogen, is formed with a characteristic spectrum, which possesses a band of great density in the region of λ560. This substance was assumed to be hæmatin, in the reduced form and in alkaline solution. That is to say, it was assumed to be protein-free. This conception appears to be incorrect, for we can obtain hæmochromogen, not analytically, but synthetically, the best starting point being a substance called hæmin.

Hæmin is made by adding glacial acetic acid to dried blood in the presence of sodium chloride and is a well-defined crystalline substance to which the formula $C_{34}H_{35}O_3(?)N_4Fe,HCl$ was given by Hoppe-Seyler. From it, *in the presence of a reducing agent*, the base hæm, $C_{34}H_{35}O_3N_4Fe(?)$, may be liberated by the addition of sodium hydroxide. According to the old conception, this base should have been hæmochromogen, but it is not so, having a quite different and ill-defined spectrum. One further step is necessary to produce hæmochromogen, namely, the addition of globin to the hæm. Therefore we obtain the following scheme (Scheme 1).

The conception put forward above opened up an entirely new field for discovery, in which three principal facts may be emphasised.

First, that globin is only one of a great many substances which

will perform the same function, thus forming a whole group of hæmochromogens. Nicotine, pyridine, hydrazine, ammonia,



SCHEME I.

albumin, etc., all perform the same rôle, that is, when added to hæm, which has a comparatively indistinct spectrum, they yield substances producing spectra with the typical characteristics of that of hæmochromogen, and it must be remembered that the spectrum of hæmochromogen is the most typical and best defined of all blood spectra. We therefore may now extend our scheme as follows:

				Oxidised.
Hæmin + NaOH	Hæmatin			
	Hæm + Ammonia	$\rightleftharpoons$	NH_3 Hæmochromogen	
	+ Glycocoll	$\rightleftharpoons$	G. ,,	
	+ Hydrazine	$\rightleftharpoons$	H. ,,	Reduced.
	+ Pyridine	$\rightleftharpoons$	P. ,,	
	+ Nicotine	$\rightleftharpoons$	N. ,,	
	+ Albumin	$\rightleftharpoons$	A. ,,	
	+ Globin	$\rightleftharpoons$	G. ,,	

SCHEME. II

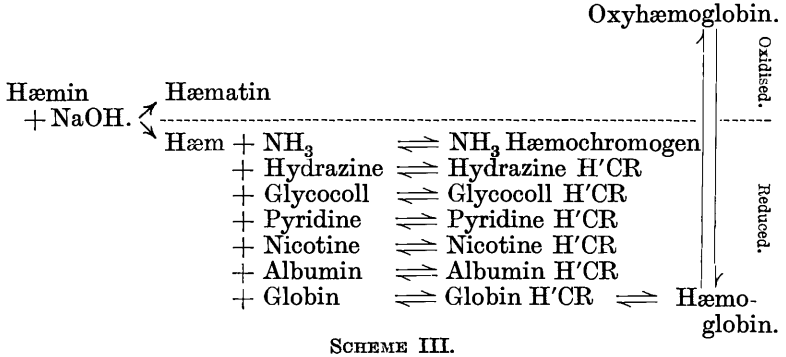
Secondly, whilst all these substances yield hæmochromogens with spectra of the same type, there are appreciable differences between the actual spectra of the different members of the family; in one the bands will be a little towards the red, in another a little towards the blue—but so little that casual observation would not show the difference.

Thirdly, all these hæmochromogens seem to be kept *in statu quo* as the result of balanced action, and here it may be noted that for the production of most of them a great preponderance of the components is necessary. The globin hæmochromogen stands nearly but not quite alone in so far as the action is almost complete.

The globin compound has as well other distinctive properties; it is one of the few hæmochromogens which are soluble in a high degree, and, but chiefly, the globin compound stands alone, in

that it and it only can form a hæmoglobin. Anson and Mirsky, by regulation of the hydrogen-ion concentration, obtained hæmoglobin from globin hæmochromogen. Robin Hill has been at great pains to obtain the analogues from other hæmochromogens, but without success.

The final scheme (Scheme III) then is :



SCHEME III.

II. *Cytochrome.*

It is not possible to leave the subject of hæmochromogen without making a digression for the purpose of discussing cytochrome. Here, as in the case of hæmochromogen, let the name cytochrome stand for a spectrum, at all events until we can get something more concrete to which we may attach it.

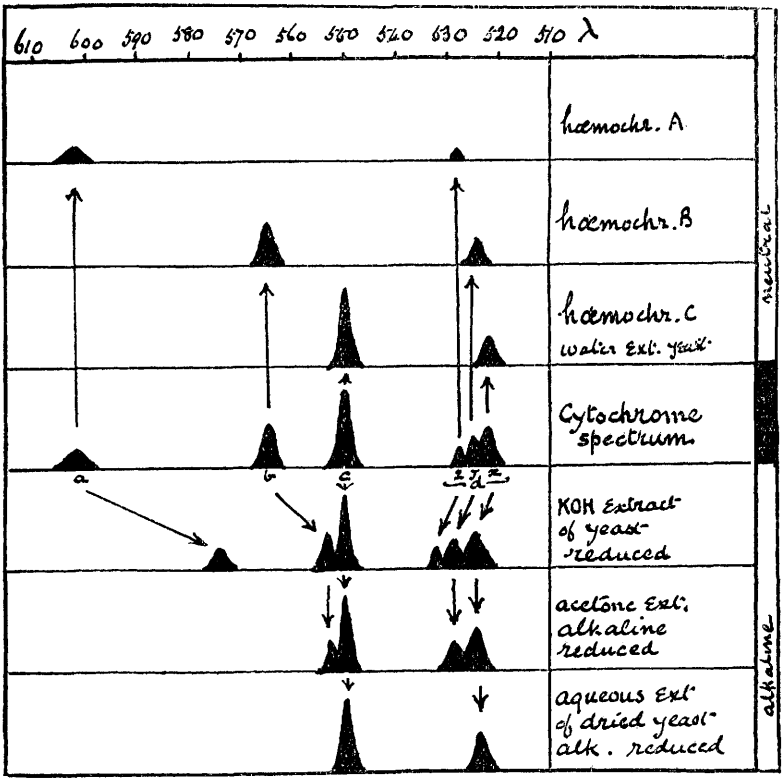
This spectrum was first described by McMunn,[1] but its true significance has only recently been brought to light by the work of Keilin,[2] who has kindly consented to demonstrate it to-night. It may be seen well in the wing-muscle of the bee, in the onion, in yeast, in aërobic bacteria, and, indeed, it may be regarded as of more or less universal occurrence in forms of life which have contracted the oxygen habit. In these there is reason to suppose that it acts as a catalyst, in contrast to hæmoglobin, which acts as a carrier, but pervading both the animal and the vegetable kingdom; it is far more widely distributed than either hæmoglobin or chlorophyll. Not only so, but cytochrome appears in forms of life than which none are more primitive.

This spectrum has never been seen outside a cell; all attempts to extract the cytochrome have ended in changing the spectrum—moreover, it is only seen in the reduced condition—shake yeast with air, the spectrum disappears, to return on permitting the yeast to reduce itself—expose the wing-muscle of the bee to the atmosphere, the spectrum disappears, to return on the exclusion of oxygen by a coverslip.

Superficially this spectrum presents the appearance of possessing four bands, which may be designated as *a*, *b*, *c*, and *d*; of these *c* is the most conspicuous (see Fig. 1).

To say that this spectrum has four bands is only a partial statement of the facts, for the band *d* may be resolved into three overlapping bands which I will call *x*, *y*, and *z*. Thus on any picture

FIG. 1.*



which shows the relative absorption at different parts of the spectrum there are six summits. This is shown crudely in the figure. It must not be supposed that this figure is drawn from accurate spectrophotometric observations; the positions of maximum density only have been measured with care. So far as the relative densities of the various bands are concerned, the figure simply is a rough

* Figs. 4, 7, 8, 9, 10, 11, 12, and 13 are reproduced by permission from the *Proceedings* of the Royal Society, and Figs. 5 and 6 from the *Journal of Physiology*.

representation of the impression one gets from the observation of varying concentrations of the material.

To obtain a better understanding of the cytochrome spectrum, let us turn to alkaline extracts. An extract of yeast in potassium hydroxide gives a spectrum also with six summits, but which has only three bands, because those which correspond to *b* and *c* have become merged into a single band with two summits. The summits in the spectrum of the potassium hydroxide extract I will call a_K, b_K, c_K, z_K, y_K, and x_K, respectively, representing *a*, *b*, *c*, *z*, *y*, and *x*.

If one goes further and makes an acetone extract, which is then made alkaline, only four summits appear; they are b_K, c_K, y_K, and x_K, respectively, a_K and z_K being absent. If one goes still further and dries the yeast, makes an aqueous extract of the dry yeast, and renders that alkaline, two more bands, b_K and y_K, drop out and one is left with c_K and x_K only. And now we have something very familiar; it is none other than a typical hæmochromogen spectrum. The simplest interpretation of the cytochrome spectrum is that it is a mixture of the spectra of three hæmochromogens A, B, and C, of which A is responsible for bands *a* and *z*, B for bands *b* and *y*, and C for bands *c* and *x*, and this interpretation is rendered the more likely by the fact that in different cytochromes the relative intensities of the bands vary. But in this respect the bands are associated in pairs; thus *a* and *z* may be exaggerated, relatively to the rest, or *b* and *y* may be, but not *a* and *y* or say *c* and *z*, or *a* or *b* or *c* alone.

III. *Metallic Porphyrin Compounds.*

Some allusion should here be made to the work of Robin Hill,[4] who has investigated the compounds of various metals with porphyrin. Porphyrin unites with a great number of metals—iron, nickel, cobalt, manganese, zinc, copper, silver, potassium and others. The compounds formed are all on the *hæm* level, if I may put it that way, but of these, only three have the power of being readily oxidisable and reducible. These are the compounds of cobalt, iron, and manganese, *i.e.*, the hæmatins as well as the hæms of these metals can be obtained.

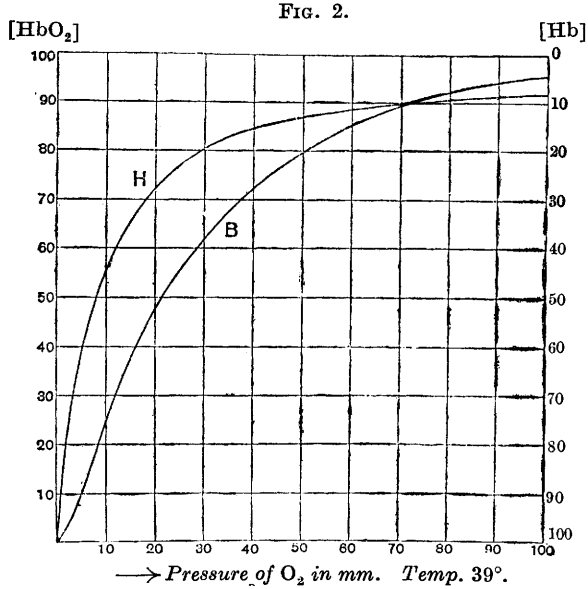
It is remarkable and perhaps significant that, whilst none of these substances has any nitrogenous compound attached, they all, with one exception, have spectra which suggest hæmochromogen rather than hæm. The one exception is the iron compound, and, further, it is the only one to which nitrogenous compounds such as pyridine and globin will attach themselves and thus form true hæmochromogens. Not that the iron–porphyrin compound is precisely the same as hæm, or that the derived hæmochromogens are precisely the same as those formed from blood—the spectral bands are nearer the blue;

presumably the original porphyrins are not the same as that in the hæm obtained from blood.

IV. *Equilibrium Constants of Compounds of Hæmoglobin with Oxygen and Carbon Monoxide.*

The curve which represents the equilibrium between oxygen and blood is, as was shown by Christian Bohr,[5], S-shaped in character (Fig. 2, B). This contour adds greatly to the biological value of hæmoglobin and for that reason it was an object of speculation to Bohr himself—as it has been to all his successors—as to what

FIG. 2.



the underlying significance of the inflexion might be. A simple equilibrium of the type

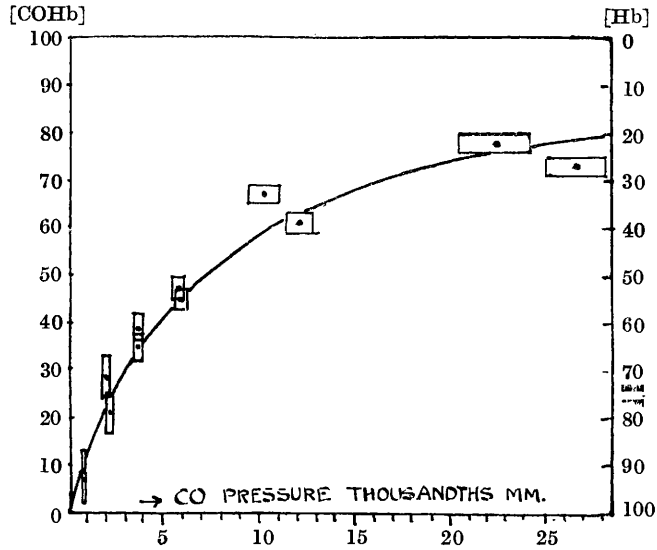
$$[\mathrm{Hb}] \times [\mathrm{O_2}] \rightleftharpoons K\,[\mathrm{HbO_2}]$$

would, of course, be represented by a rectangular hyperbola.

In 1911, Roberts and I [6] found that a dialysed solution of hæmoglobin of considerable purity yielded an equilibrium curve which approximated very much to the hyperbolic type, and we hazarded the view that if complete purity were obtained the curve would actually become a hyperbola (Fig. 2, H). This view met with a general, but as it seems a premature, acceptance; for, on later occasions with I think purer and certainly more concentrated

solutions, we failed to obtain the hyperbolic form, and at Harvard, Adolph and Ferry,[7] with a very perfect technique of its kind, asserted that in no circumstances was the curve anything but S-shaped. I used the phrase "a very perfect technique"—perhaps this phrase was wrong. I should have said a technique which more nearly reached our ideals. Our ideals were : a solution which contained the minimum of impurity and the maximum of hæmoglobin. With regard to the latter point, of course the greater the concentration of hæmoglobin the easier and more trustworthy becomes the gas analysis.

FIG. 3.



(The rectangles represent the calculated experimental error.)

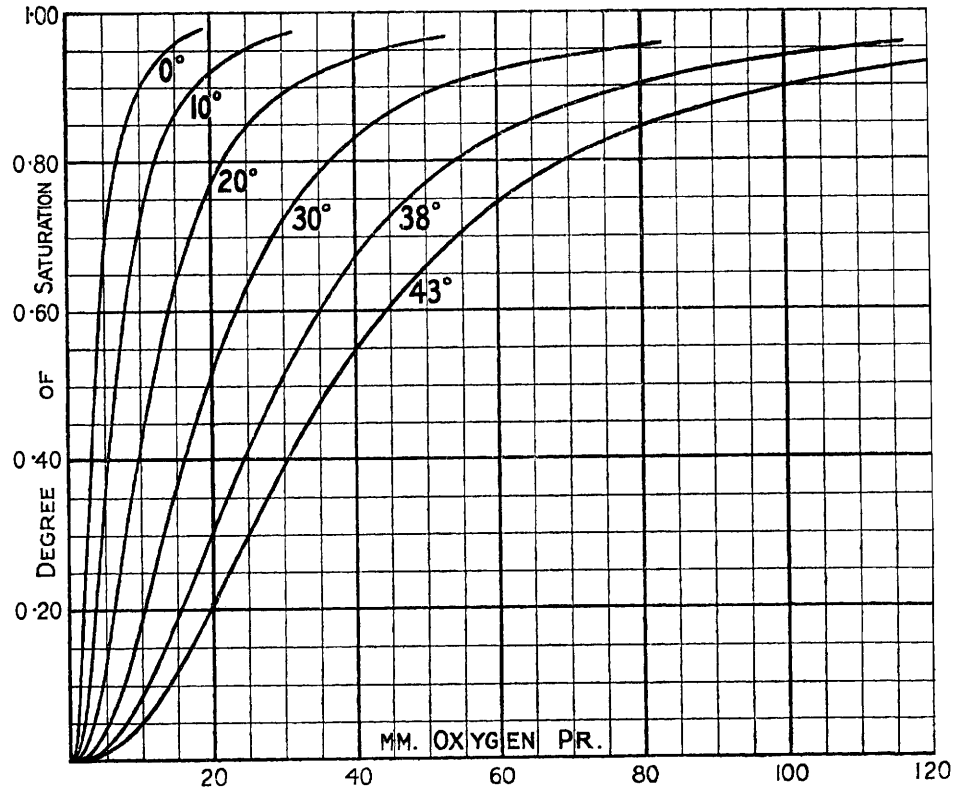
The conceptions put forward by Adair[8] indicated the probability of our ideal being wrong. They suggested that the fundamental equilibrium curve was to be obtained from the investigation of solutions not stronger but more dilute than that used by Roberts and myself. The study of such solutions required an entirely new technique. The relative concentrations of hæmoglobin and oxyhæmoglobin in dilute solution can be determined only by some spectroscopic method, and even that is very difficult to do and can be done only for the middle portion of the curve. The pressures of oxygen involved are so small as to demand very exact and difficult analyses of the atmosphere to which the hæmoglobin is exposed, if the equilibrium be struck at room temperature. On the other hand, if the equilibrium be struck at 35—40°, the gas analysis

becomes easy, but the hæmoglobin becomes unstable. If we review these difficulties in order, we find that the spectroscopic analysis becomes easy when we study the analogous reaction:

$$[\mathrm{Hb}] + [\mathrm{CO}] = K[\mathrm{HbCO}].$$

The difficulties of gas analysis by ordinary methods are, however, seemingly insuperable, for the pressures of carbon monoxide

FIG. 4.



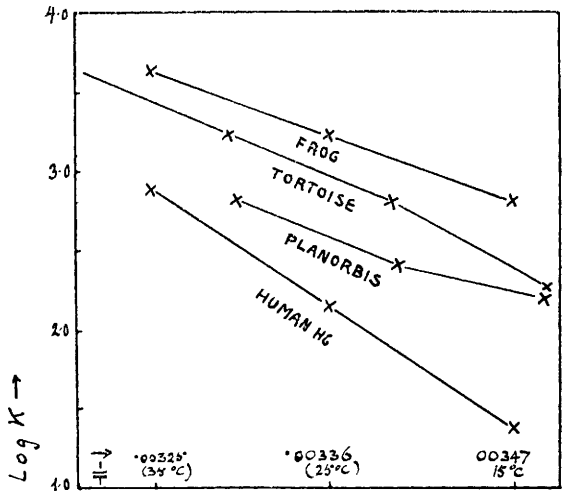
involved are counted in thousandths of a millimetre. Fortunately, it has proved possible at 15° to work out a method—almost audacious in its simplicity—which has enabled us to avoid gas analysis and by a single spectroscopic measurement to estimate the concentrations of all the reacting substances in the above equation. This technique will be demonstrated by my colleague, Dr. Selig Hecht, of Harvard. With its aid we have succeeded in the last fortnight or so in obtaining the curve shown in Fig. 3. Between the limits of 0 and 25

thousandths of a millimetre pressure of carbon monoxide (above which limits the method has not so far proved accurate) the curve is nearly, though not quite, hyperbolic in form.

It would seem that at last we are really within sight of the fundamental curve and in a position to push forward and discover the effects on this curve of increasing the concentration of hæmoglobin, of the salts, and of alterations in the concentration of hydrogen and hydroxyl ions in the solution.

I need only remind you that the effect of increasing alteration of hydrogen-ion concentration is to reduce the affinity of oxygen (or carbon monoxide) for hæmoglobin proportionately over the whole curve.

FIG. 5.



The Temperature.—The same may be said of the effect of temperature. The curves in Fig. 4 show the equilibrium of oxygen and hæmoglobin in blood at various temperatures. They are taken from a research by W. E. Brown and A. V. Hill.[9]

Their work has been followed by that of Maçela and Seliskar,[10] who have determined the temperature coefficients of the hæmoglobin, in dilute solution and under constant conditions of hydrogen-ion concentration, of several forms of life. It is remarkable, and again of great biological interest, that the hæmoglobin of man has a temperature coefficient of about 5 for its equilibrium with oxygen (Fig. 5), and that of the frog has a temperature coefficient of only about 2·5 (Fig. 6). Whether human hæmoglobin serves man better by having so high a temperature coefficient is difficult to say, but it would be quite unsuited to the needs of the frog.

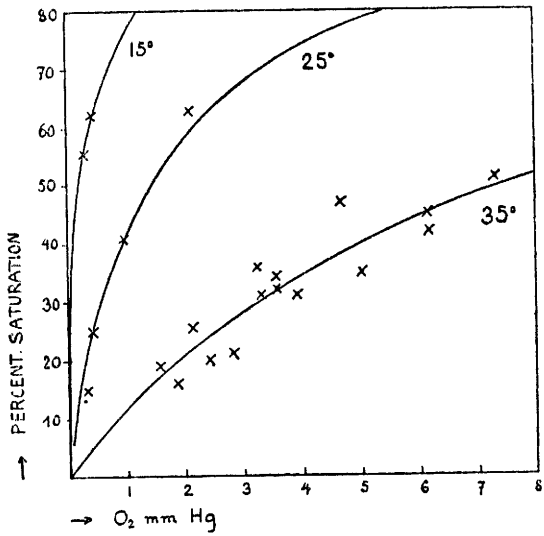
V. *The Velocity Coefficients of the Reactions of Hæmoglobin with Oxygen and Carbon Monoxide.*

The sensitiveness of the equilibrium constant of the reactions

$$Hb + O_2 \rightleftharpoons HbO_2$$

to temperature, hydrogen-ion concentration, etc., naturally leads to the inquiry how are the velocity coefficients of the separate phases of the reaction affected by the conditions under which the reaction takes place. If, for instance, a rise of temperature tends to

FIG. 6.



dissociate oxygen from hæmoglobin, is this because the velocity coefficient, k, of the reaction

$$Hb + O_2 \rightharpoonup HbO_2$$

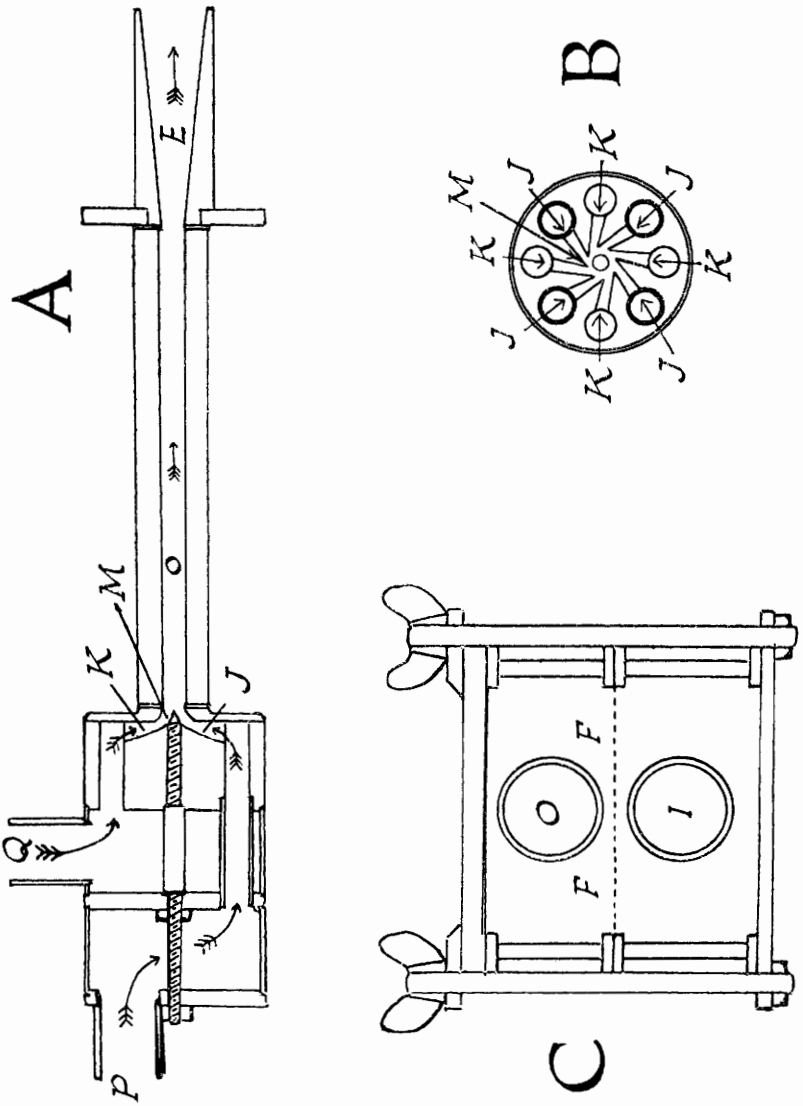
is increased or the velocity coefficient, k', of the reaction

$$Hb + O_2 \leftharpoondown HbO_2$$

is diminished, or are both velocities increased but in different proportions—the state of affairs which would seem most probable *prima facie*—or what? Before the war, A. V. Hill and I made a few experiments on this subject, but it was clear that our experimental methods were quite inadequate—our scheme in general depended on the bubbling of oxygen or nitrogen through hæmoglobin solutions. The actual velocities of the chemical reactions which took place were of so high an order as compared with the rates at which the

gases passed into or out of the solution that the results which we obtained depended merely on the extent to which we were able to

FIG. 7.



"ring the changes" on the physical, as opposed to the chemical, processes involved.

Within the last four years the velocity coefficients of the various

phases of the reactions of hæmoglobin with oxygen and with carbon monoxide have been studied by Hartridge and Roughton [11] with what appears to me to be amazing success. Such a study, of course, demands the most meticulous consideration of detail, and if I treat their method in a very general way it is because the discussion of the details, once embarked upon, would occupy more than the whole time at my disposal.

Let us commence, then, with the consideration of the reaction :

$$Hb + O_2 \leftarrow HbO_2.$$

To carry out our purpose, it is necessary that one or other of the substances produced, the hæmoglobin or the oxygen, should be removed from the sphere of action as fast as it is formed. For this purpose, the procedure is briefly as follows.

The apparatus (Fig. 7) is schematically in the shape of a **Y** made of tubing—along the limbs of the **Y** enter on one side a dilute solution of the oxide of hæmoglobin, on the other water containing a concentration of sodium hyposulphite to absorb the oxygen, it being ascertained that the sodium hyposulphite does not reduce the oxide by double decomposition. At the junction of the tubes there is a mixing chamber, in which the mixing is so rapid and intimate as to be completed in a time which is negligible compared with the one-hundredth part of a second. The rate at which these fluids are driven through the apparatus is so great that the reaction in the lower limb of the **Y** may be followed with the eye. The fluid, which emerges red from the mixing chamber, is seen to become purple as it passes down the tube. The degree of reduction may be estimated at any point along the tube by the reversion spectroscope, whilst the time taken for the process is calculated from the velocity of flow of the fluid along the tube, the fluid being in turbulent motion.

In the world in which we now are, this reaction is relatively slow; and, as Fig. 8 shows, it obeys the " rules of the game " inasmuch as the curve obtained by plotting the logarithm of the concentration of the oxyhæmoglobin against the time is a straight line.

The reaction :

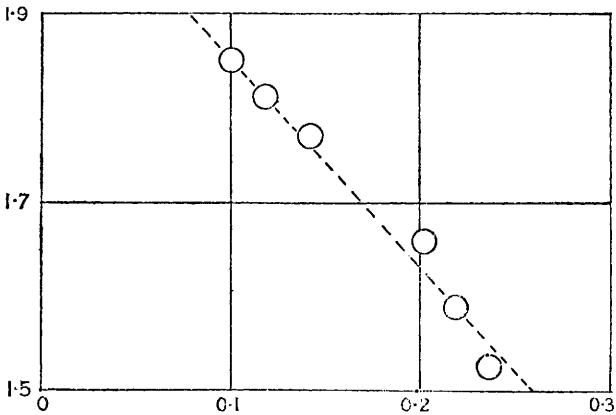
$$O_2 + Hb \rightarrow HbO_2$$

presents much greater difficulties for two reasons. The first is the technical one that it takes place enormously faster, the second is the theoretical one that it has not proved practicable to remove the oxide as it is formed and therefore it is only when the first portions of oxide are forming that the reaction appears with its full velocity. However, just because the disruption of the oxide is slow

as compared with its formation, and because we know the velocity of the disruption, we can correct for it by a process of calculation.

To determine the velocity of oxidation, solutions of hæmoglobin and of oxygen in sufficient concentration to oxidise it are driven at such a velocity that they traverse the lower limbs of the **Y** at the rate of about 600 cm. per second, and over the first few centimetres the chemical change may be seen with the eye, the hæmoglobin turning from violet to red as the oxide forms. The measurements are made with the reversion spectroscope. The time, as before, is measured by the rate at which the fluid travels from point to point, and the degree of oxidation by spectroscopic examination of the fluid from point to point along the tube.

FIG. 8.



Abscissæ = time in seconds.
Ordinates = log of percentage of oxyhæmoglobin.

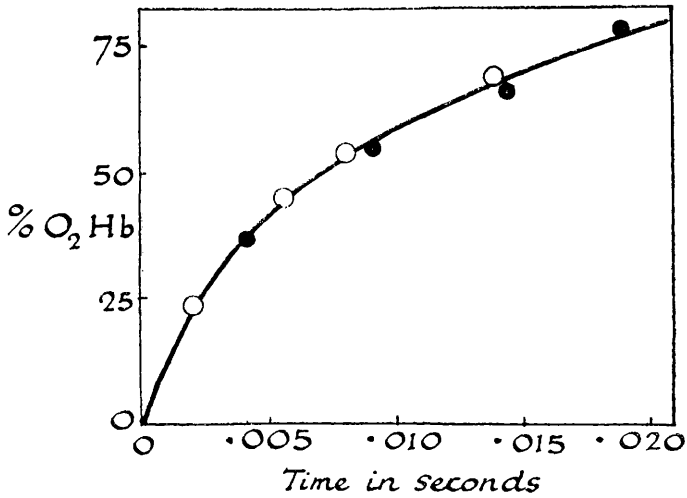
The actual result achieved in an experiment is illustrated by Fig. 9. Here you will see that one-quarter of the whole oxidation takes place in about two-thousandths of a second. To ascertain the figure with an accuracy of but 10% required a certainty of two ten-thousandths of a second in the estimation of the time.

Having said so much about Hartridge and Roughton's method, let me pass to the consideration of their results. Taking first the oxidation, its velocity coefficient possesses properties that are worth noting, the most remarkable of which is that temperature appears to have no effect upon it. Of course I mean temperature within rather narrow limits, for hæmoglobin will not stand heating above about 45°. What the theoretical explanation of this phenomenon may be is a matter which I shall leave to you who know much

more about such things than I do. It would be explained according to the authors on the assumption that all the molecules of oxygen which impinged upon the hæmoglobin molecule—at whatever temperature—adhered to it. The second property, and one which is of great interest to physiologists, is that alteration of the hydrogen-ion concentration of the medium in which the hæmoglobin is dissolved is also without marked effect upon the velocity coefficient of the oxidation phase of the reaction.

It follows, therefore, that the now well-known effects of hydrogen-ion concentration and temperature on the equilibrium constant must

FIG. 9.



be almost entirely the reflexion of the effects of these conditions on the *reduction* phase, and to this we shall now turn.

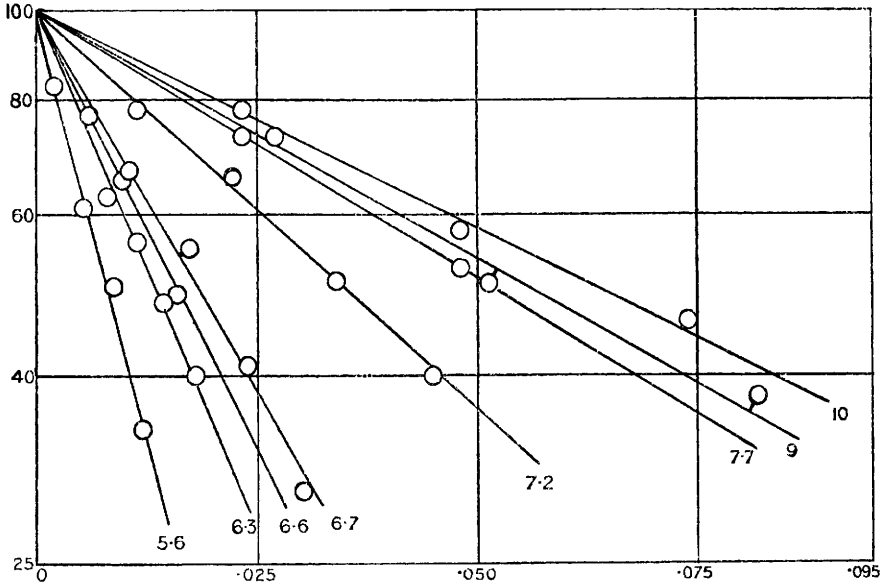
Experiments on sheep's hæmoglobin indicate that the velocity coefficient of the reduction phase has a temperature coefficient of 3·8 for an alteration of 10°, which, as a matter of fact, corresponds to that of the equilibrium constant of the reaction

$$Hb + O_2 \rightleftharpoons HbO_2.$$

The effect of hydrogen-ion concentration is restricted to a small region between $p_H = 7{\cdot}8$ and about $p_H = 5{\cdot}5$, *i.e.*, within 1·2 on each side of the isoelectric point. Within this region the effect is very great and fully accounts for the properties which blood exhibits (Figs. 10 and 11).

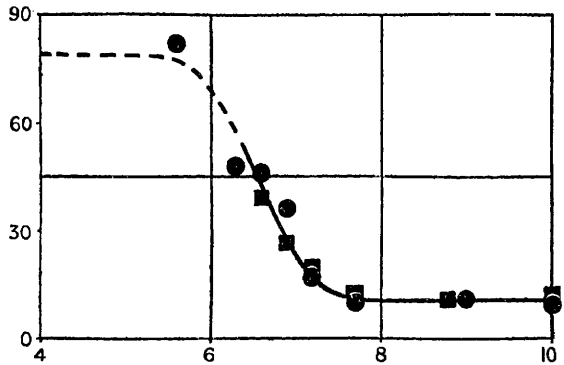
To revert, however, to the original proposition of whether the value of the equilibrium constant as determined does or does not agree

FIG. 10.



Abscissæ = Time in seconds. Ordinates = logarithm of oxyhæmoglobin percentage ; numerals on left of ordinate axis indicate corresponding value of oxyhæmoglobin percentage.

FIG. 11.



Abscissæ = p_H of solution. Ordinates = value of reduction velocity coefficient. Squares represent results obtained in experiment on one sample of blood. Circles represent results obtained in experiment on another sample of blood.

with the quotient of the velocity coefficients, the following table, which summarises the results of four such comparisons, shows, I

think, as good an agreement as can be looked for from experiments of so high a degree of difficulty.

The Comparison of K *with* k′/k.

No. of Expt.	p_H.	Temp.	Mean value of k'.	Mean value of k.	Value of K. Calculated from $K = k'/k$.	Value of K. Observed from dissociation curve.
I.	7·7	17·5°	2875	17·5	164	218
II.	7·2	18·9	2550	17·3	148	112
III.	10·0	15·1	3145	4·5	700	730
IV.	10·0	17·3	3540	8·1	438	336

The values of k', k, and K were subject individually to possible experimental errors of $\pm 20\%$, $\pm 10\%$, and $\pm 15\%$, respectively.

A parallel set of observations has been carried out on the velocity coefficient of the reactions

$$\text{Hb} + \text{CO} \longrightarrow \text{HbCO} \text{ and}$$
$$\text{Hb} + \text{CO} \longleftarrow \text{HbCO}.$$

Of these, the velocity coefficient of the association phase is of the same order as that of the corresponding reaction for hæmoglobin and oxygen; but the velocity coefficient of this dissociation phase is of quite a different order from that of the reduction phase of oxy-hæmoglobin.

Being in possession of the values of k and k' for the reactions

$$\text{Hb} + \text{O}_2 \rightleftharpoons \text{HbO}_2$$
$$\text{Hb} + \text{CO} \rightleftharpoons \text{HbCO},$$

respectively, we should be able to calculate the corresponding co-efficients for the reaction

$$\text{HbO}_2 + \text{CO} \rightleftharpoons \text{HbCO} + \text{O}_2,$$

or, as it is more correctly written,

$$\text{HbO}_2 \rightleftharpoons \text{Hb} + \text{O}_2$$
$$\text{Hb} + \text{CO} \rightleftharpoons \text{HbCO}.$$

But at this point a most interesting discrepancy has appeared. The hæmoglobin appears to be much more reactive towards carbon monoxide if this gas is presented to it within about one-tenth of a second after the removal of the oxygen than it is subsequently. Whether, in that tenth of a second some tautomeric change takes place, or, if not, what the alternative may be, is still under investigation, so there we must leave the matter.

VI. *The Importance of the Protein.*

Within recent years the point of interest with regard to hæmoglobin has been shifting. Formerly, it was entirely centred upon the relation of the oxygen to the hæmatin, and this interest extended to the significance of the pyrrole group and the possible connexion with chlorophyll. Little progress has been made in this direction; the researches of Willstätter more particularly have turned men's minds rather away from the pyrrole group; on the other hand, the protein moiety of hæmoglobin has in one way or another been steadily receiving more recognition. The points of importance with regard to the protein may be considered under two main groups, those which embrace the proportion of hæmoglobin as an amphoteric electrolyte, and those which have to do with its specificity.

(a) *Hæmoglobin as an Amphoteric Electrolyte.*—Hæmoglobin acts as an acid in alkaline solution and as a base in acid solution. The former action has been studied in much greater detail than the latter. In strict parlance one should, I suppose, speak of hæmoglobinic acid and of sodium or potassium hæmoglobinate—the latter being the form in which hæmoglobin exists for the most part in human blood.

This very important fact at once raises a number of points such as:

(α) The number of dissociable sodium atoms for each atom of iron.

(β) The strength of the acid.

(γ) Effect on the strength of the acid of oxidising the hæmoglobin.

(δ) The repercussion of the above considerations on the system in which hæmoglobin is found in the blood, which contains also sodium bicarbonate, sodium chloride, and carbonic acid. This system becomes complicated by the fact that the hæmoglobin is in corpuscles, the envelopes of which appear to be relatively impermeable to kations as compared with anions. This system is of paramount importance to the physiologist, but to the chemist it is so special a case that I shall pass very lightly over it.

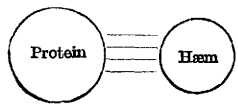
α. Very different views have been held as to the number of gram-atoms of sodium which could dissociate from 16,800 grams of hæmoglobin (the weight which contains 56 grams of iron and unites with 32 grams of dissociable oxygen). The matter recently formed the subject of an exhaustive research by the workers in Dr. Van Slyke's laboratory,[12] who have come to the conclusion that the number was much higher than had formerly been supposed. Their estimate is 12.

β and γ. Hæmoglobin in the reduced form is a very weak acid. In the oxidised form it is, according to A. V. Hill, some seventy

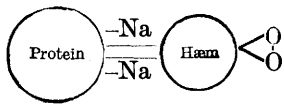
times as strong, and is then of the same order of strength as sodium bicarbonate.

δ. It follows that when hæmoglobin is oxidised in the presence of sodium bicarbonate the sodium becomes redistributed, the hæmoglobin taking more, and therefore the amount of sodium bicarbonate decreases whilst the amount of free carbon dioxide increases. On the other hand, the addition to the system of carbon dioxide or any other acid will attract sodium from the hæmoglobin and tend to release the oxygen, which has less affinity for the free acid than for the sodium hæmoglobinate.

The scheme put forward by Hill [13] to explain these facts is that hæmoglobin may be regarded as



whilst oxyhæmoglobin in the presence of sodium bicarbonate is



(b) *Specificity.*—That the hæmoglobins which are derived from different species of animals, and even from different members of the same species, differ may be shown in a number of ways. That they differ in crystalline form has been known for a long time, and the crystallographic differences have formed the subject of a work, monumental in bulk, by Reichert and Brown.[14] Moreover Landsteiner and Heidelberger,[15] in a very elegant research on the solubility of hæmoglobin, have shown that the difference between one form and another is sufficiently great to render them more or less independently soluble. After a solution has been saturated with the hæmoglobin of one animal, it is still capable of dissolving some at all events of the hæmoglobin of another.

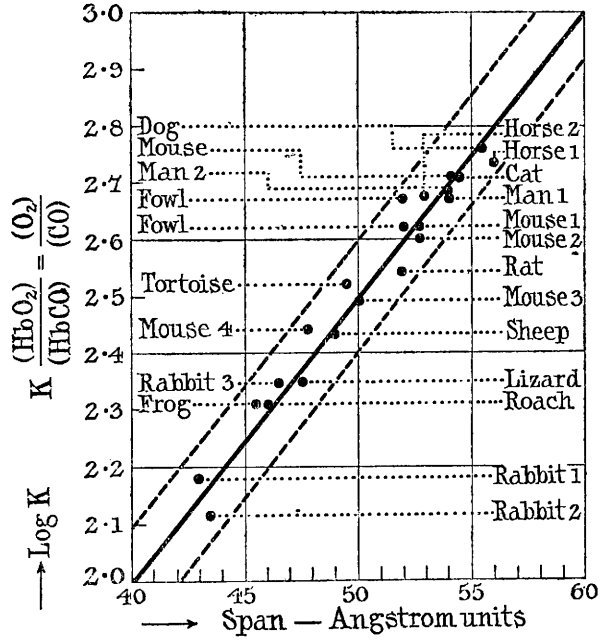
Moreover the spectra are slightly different, the bands being shifted a little towards the red or towards the blue from one hæmoglobin to another. This is true, not only of the bands of oxyhæmoglobin, but also of carboxyhæmoglobin. One band, known as the α-band, has been studied in some detail. In the case of human blood, when carbon monoxide is bubbled through a solution of oxyhæmoglobin this band moves 54 Å.U. towards the blue. In the slang of the laboratory, we say the band has a " span " of 54 Å.U. Most forms of hæmoglobin have a lesser span. Now let me put before you a remarkable and hitherto unexplained relationship, which at present

exists on quite an empirical basis. It merely has been " observed to be so " in the cases which have been tested.

We may express the equilibrium point of the reaction which takes place when carbon monoxide is passed into oxyhæmoglobin as follows :— [16]

$$K[HbO_2] \times [CO] = [HbCO][O_2]$$

FIG. 12.



With different hæmoglobins, K differs. Now let me show you the relation of K to the " span." I do not wish to insist that the relation is in the strict sense linear. We may be on a very flat curve or near the point of inflexion of an S-shaped curve. What I want to emphasise is that *there is a relation.* It would be simple to explain this relation on the hypothesis that there are a limited number of hæmoglobins, say two, which in different animals are mixed together in different proportions. This simple explanation seems to be negatived by the fact that if the hæmoglobin be crystallised and redissolved the solution presents the same properties as the original solution or as the fluid left above the crystals.

On the other hand, no attempt to find any spectroscopic or other differences, either in the hæms or the hæmins derived from these

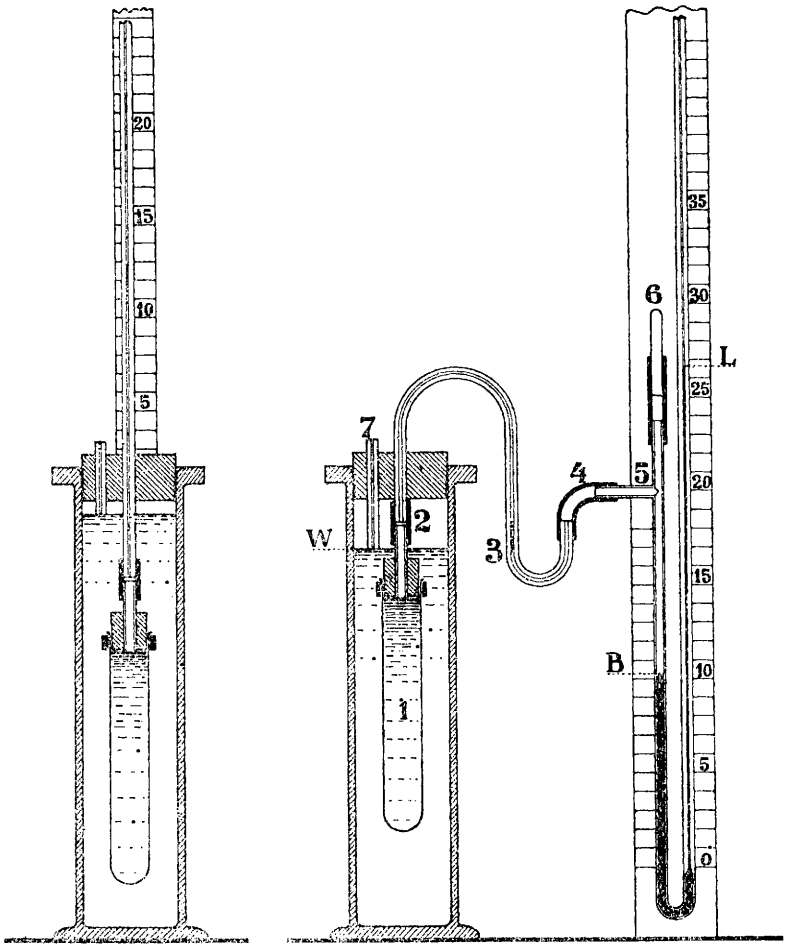
various hæmoglobins, has been successful. In the present state of our knowledge one must attribute this very interesting specific feature to the globins.

VII. *Osmotic Pressure and Molecular Weight.*

And now I come to that part of the subject in which hæmoglobin appears to be in its most illusive mood. The thing which we most want to know about it is perhaps its molecular weight, and in this audience the confession of ignorance on that point is a confession not untinged with shame. We know as a starting point that the least possible molecular weight—that which contains 56 grams of iron—would be somewhere about 16,700. If then we call the molecular weight $(16{,}700)_n$, what is the value of n?

We turn to some indirect method. What is to be said about the osmotic pressure? I may pass rather rapidly over the work of Hüfner and Gansser,[17] who came to the conclusion that the osmotic pressure of a 1% solution of hæmoglobin would be 10 mm. of mercury—a result which would give a value of $n = 1$. By what happy accident Hüfner and Gansser arrived at this result is likely to remain a mystery. Weymouth Reid,[18] in a research which is too little quoted, concluded that 3 was the nearest whole number to his determinations of n. Roaf [19] made the material but rather depressing discovery that n might appear to be almost anything—less than unity, for instance—according to the circumstances in which the measurements were made. It has remained for Adair [20] to reduce to some sort of order the apparently chaotic readings which were found. The possibilities of adsorbed salts, of variable ionisation of the hydrogen or sodium ions, of membrane potentials, of polymerisation of the hæmoglobin, of the onset of putrefaction, of the attainment of a true equilibrium, etc., all had to be taken into account. Take the last two considerations, the attainment of equilibrium and the avoidance of putrefactive changes. In ordinary circumstances it proved quite impossible to complete a measurement in which a satisfactory equilibrium was attained before the solution ceased to be above suspicion. This difficulty was overcome by working at 0·6°, at which temperature a hæmoglobin solution remains good indefinitely. The time factor looms almost as large in Adair's determinations as in those of Hartridge and Roughton. The latter measure events which take place in a fraction of 1/1000 of a second; Adair's osmometric determinations each require a fraction of a year. The work is being carried on in the Low Temperature Station at Cambridge, where Sir Wm. Hardy kindly allows Adair to set up his osmometers (Fig. 13). Such work must be very slow, but already enough information has been

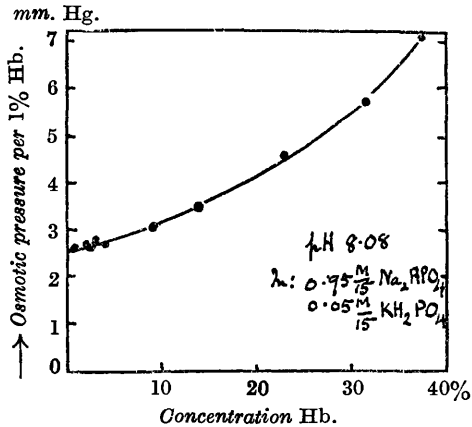
FIG. 13.



Osmometer, Type D (left), with straight column of solution for measuring pressure. Scale in centimetres. Osmometer, Type C (right), for high pressures with mercury manometer: (1) *A rigid collodion tube, 20 c.c. capacity, in jar holding 500 c.c. of outer liquid.* (2) *Rubber connexion. The glass tube from* (2) *to* (3) *is filled with liquid paraffin. Beyond* (3) *there is water.* (4) *Rubber connexion with clip (not shown here).* (5) *Glass* T *tube, with water down to level B.* (6) *Glass rod in rubber tube, for adjusting the pressure.* (7) *Glass tube, open to air, or fitted with a soda-lime tube. W = level of outer liquid; B = level of mercury in manometer limb, 6 mm. in diameter; L = level of mercury in manometer limb, 0·7 mm. in diameter. Above the mercury at L there is a drop of lactic acid.*

obtained to be of considerable interest. Adair will show you a model in which the osmotic pressure in a 1% solution of hæmoglobin is represented vertically whilst the hydrogen-ion concentration and the saline concentration of the solvent are the horizontal co-ordinates. Over a great part of this model the conditions may be varied without any change in the osmotic pressure of the hæmoglobin, which pressure works out pretty regularly at 2·6 mm. of mercury. This figure is given if the solution is not more than 4% hæmoglobin or is not of less saline concentration than 0·01 molar. Where a different and higher osmotic pressure than 2·6 is given per 1% of hæmoglobin, as in cases where the concentration of Hb is more than 4% (Fig. 14)

FIG. 14.



or the pigment is dissolved in salt solution of greater dilution than 0·01*M* (Fig. 15), Adair finds a cause which satisfies him. If we accept his estimate, the value of n would be 4 and the molecular weight of hæmoglobin about 68,000. A substance of this molecular weight, it may be said incidentally, would depress the freezing point of water about 0·00001°.

And now we are face to face with the final puzzle. How are we to reconcile an equation

$$Hb_4 + 4O_2 \rightleftharpoons Hb_4O_8$$

with the form of equilibrium curve as obtained?

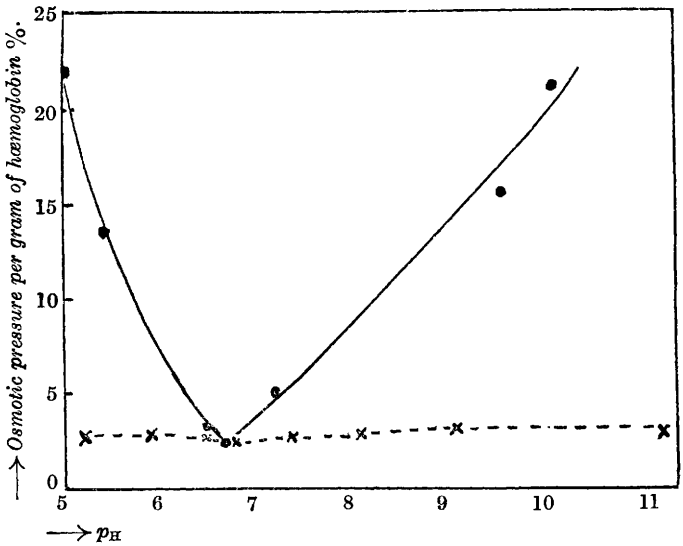
In considering this question I will leave out two factors each of which may produce an appreciable secondary effect on the shape of the curve. These are the alteration in hydrogen-ion concentration produced on oxidation and the effect of the great concentration in

which the hæmoglobin is found, say, in the red corpuscle. These apart, we may consider the reaction as being either

$$\begin{aligned} Hb_4 + O_2 &\rightleftharpoons Hb_4O_2 \\ Hb_4O_2 + O_2 &\rightleftharpoons Hb_4O_4 \\ Hb_4O_4 + O_2 &\rightleftharpoons Hb_4O_6 \\ Hb_4O_6 + O_2 &\rightleftharpoons H_4O_8 \end{aligned}$$

or it may follow what physiologists call the " all or none " principle, there being no possibility of intermediate oxides between Hb_4 and

FIG. 15.



Conditions. Concentration of Hb *less than* 4%.

o Solution in distilled water.
x Solution of saline concentration greater than 0·01*M*.

Hb_4O_8. The latter hypothesis yields a curve of the S-shaped form with which we are familiar in the case of blood.

In the case of successive oxidations, if the equilibrium constants of the four reactions are in the ratios of 4 : 3 : 2 : 1, *i.e.*, if each oxygen may be regarded as of the same value and uninfluenced by the presence or absence of the others, a curve of the hyperbolic form would be produced such as has been obtained in the case of carbon monoxide and a dilute solution of hæmoglobin of relative purity. It may here be remarked that the " all or none " conception may be

regarded as a special case of the conception of successive oxides, the case in which the equilibrium constants of the intermediate oxides have not got finite values, and that being so, it is possible to produce any curve of the general type given by the equation

$$y/100 = Kx^n/(1 + Kx^n),$$

where n is between 1 and 4, by suitably arranging the equilibrium constants of the successive reactions. But we have now passed entirely into the domain of speculation. We know of no reason why the actual factors which do alter the inflexion of the curve should so affect the equilibrium constants of the successive oxidation equilibria, and no one has claimed to have isolated any of the intermediate oxides, or to have obtained anything but the most nebulous evidence of their existence.

Yet so long as the physical basis of the inflexion fails to be understood the dream of the physiologist will be unfulfilled, for the inflexion of the curve is the biologically important fact about it. One day the enigma will be solved, until then we can but strive on in the sure and certain hope that "Nature never has betrayed the heart that loved her."

References.

[1] Anson and Mirsky, *J. Physiol.*, 1925, **9**, 50.
[2] MacMunn, *Phil. Trans.*, 1886, **177**, 206.
[3] Keilin, *Proc. Roy. Soc.*, 1925, *B*, **97**, 312.
[4] R. Hill, *Biochem. J.*, xix.
[5] C. Bohr, *Z. Physiol.*, 1903, **17**, 690.
[6] Barcroft and Roberts, *J. Physiol.*, 1909, **39**, 419.
[7] Adolph and Ferry, *J. Biol. Chem.*, 1921, **47**, 547.
[8] Adair, Fellowship Dissertation presented to King's College, Cambridge.
[9] W. E. Hill and A. V. Hill, *Proc. Roy. Soc.*, 1923, *B*, **94**, 327.
[10] Maçela and Seliskar, *J. Physiol.*, 1925, **60**, 434.
[11] Hartridge and Roughton, *Proc. Roy. Soc.*, 1923, *A*, **104**, 395; 1925, *A*, **107**, 655.
[12] Hastings, Van Slyke, Neill, Heidelberger, and Harrington, *J. Biol. Chem.*, 1924, **60**, 89.
[13] A. V. Hill, *Biochem. J.*, 1922, **51**, 359.
[14] Reichert and Brown, Carnegie Institute Washington Publication, cxvi, 1909.
[15] Landsteiner and Heidelberger, *J. Gen. Physiol.*, 1923, **6**.
[16] Anson, Barcroft, Mirsky, and Oinuma, *Proc. Roy. Soc.*, 1924, *B*, **97**, 61.
[17] Hüfner and Gansser, *Arch. Anat. Physiol.*, 1907, p. 209.
[18] Reid, *J. Physiol.*, 1904, **31**, 438; 1905, **33**, 12.
[19] Roaf, *Proc. Physiol. Soc.*, 1909, p. 1.
[20] Adair, *Proc. Roy. Soc.*, 1925, *A*, **108**, 627.