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THE KINETICS OF BLOOD PIGMENTS: HÆMOCYANIN AND HÆMOGLOBIN.

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Introduction.

Oxygen unites reversibly with the respiratory pigments, hæmocyanin and hæmoglobin. When the equilibrium state has been reached, as many molecules of oxygen are being bound per second by the pigment as are dissociating from it. This paper describes the individual measurement of these two opposing reactions oberved in several kinds of hæmocyanin and hæmoglobin. It follows directly both in method and subject-matter the pioneer researches of Hartridge and Roughton [1923*b*, 1925] on the kinetics of sheep hæmoglobin.

Measurements of the speed of the reactions of oxygen with the respiratory pigments can throw light directly on two groups of questions: (1) Is the speed with which oxygen is taken up or given off by the blood pigments slow enough to be a physiological limiting factor in the metabolism of the animal? (2) What is the mechanism by which the blood pigments bind oxygen? It is also hoped that these studies on the rates of reaction of the small oxygen molecules with large protein molecules may eventually throw some light on the mechanism of enzyme action, and serve as an introduction to a later kinetic study of catalytically active pigments.

I. Method and material.

The experimental method.

The continuous flow method for following rapid reactions taking place in solutions, which was introduced by Hartridge and Roughton [1923*a*], has been used. The two reacting fluids are driven at a high rate of speed into a common chamber where they are thoroughly mixed, and immediately passed down an observation tube, where the colour changes accompanying the reaction can be followed, each point along the

observation tube corresponding to a fixed calculable time after the initial mixing. The apparatus described by Hartridge and Roughton had two principal limitations which have been overcome in the present form. First, it was only adapted to relatively large amounts of material, which were not available when hæmocyanin or hæmoglobin from small animals was being studied. This has been remedied by constructing the device on a micro scale, the observation tube being a little less than 1 mm. in internal diameter. Second, the reversion spectroscope with which Hartridge and Roughton determined the degree of oxygenation of the hæmoglobin is not available for hæmocyanin, while even for hæmoglobin it can only give readings between about 25 and 75 p.c. saturation, thus missing the very important initial and end-points of the curve. Photoelectric cell methods have accordingly been developed, which are not subject to these weaknesses. The increase in total light absorption which accompanies the oxygenation of hæmocyanin when it goes from colourless to blue was used as the criterion for the extent of the reaction when this substance was being studied, while for hæmoglobin the change in relative absorption of different wave-lengths, which is indicated by the purple-to-scarlet shift, was measured. One such method is described in an adjoining paper [Millikan, 1933], and it is hoped in due course to discuss the remainder of the technique elsewhere.

In accordance with the procedure of Hartridge and Roughton the dissociation process has been measured by removing the oxygen liberated by the reaction as fast as it is formed by means of sodium hydrosulphite. This reduces the back reaction to zero. In measuring the rate of combination we have limited ourselves to the first portion of the reaction, where the backward component is negligible or can easily be allowed for. In the reduction process, a solution of oxygenated pigment is driven in one entrance of the mixing chamber and a solution of hydrosulphite in the other, the change of colour in the observation tube being then measured. It was necessary, however, to show that the oxygen absorber actually does act in this capacity, and does not attack directly the molecules of oxygenated pigment. By varying the initial hydrosulphite concentration, Hartridge and Roughton were able to show that in the reduction of hæmoglobin the reagent did act in this desired way, for, after a certain minimal concentration was reached, no further increase produced a change in the velocity of dissociation. I have been able to confirm this result for hæmoglobin, and to extend it to hæmocyanin. Furthermore, I have been able to show that the behaviour is completely different where hydrosulphite acts as a true reducer as in the reduction of methylene blue

to leuco methylene blue or of methæmoglobin to reduced hæmoglobin, and for these two substances the rate of reduction, as measured by the reciprocal of the time required to reach half completion, is directly proportional to the concentration of reducer, as one would expect from the simplest mass action considerations (see Table I, and Fig. 1).

TABLE I. Effect of hydrosulphite concentration on dissociation rate.

Substance and date	Time for half reduction (sec.) 0·2 p.c. $Na_2S_2O_4$	0·4 p.c. $Na_2S_2O_4$
Deoxygenation		
Limulus hæmocyanin, raw, Aug. 1931	0·065 ±0·01	0·065 ±0·01
Limulus hæmocyanin, dialysed, Aug. 1931	0·075 ±0·01	0·075 ±0·01
Limulus hæmocyanin, raw, Apr. 1932	0·092 ±0·01	0·082 ±0·01
Maia hæmocyanin, raw, Feb. 1931	0·013 ±0·005	0·010 ±0·005
Human hæmoglobin, July 1932	0·017 ±0·002	0·014 ±0·002
True reductions		
Methylene blue, Nov. 1930	0·052 ±0·01	0·023 ±0·005
Human methæmoglobin, July 1932	0·053 ±0·012	0·025 ±0·010

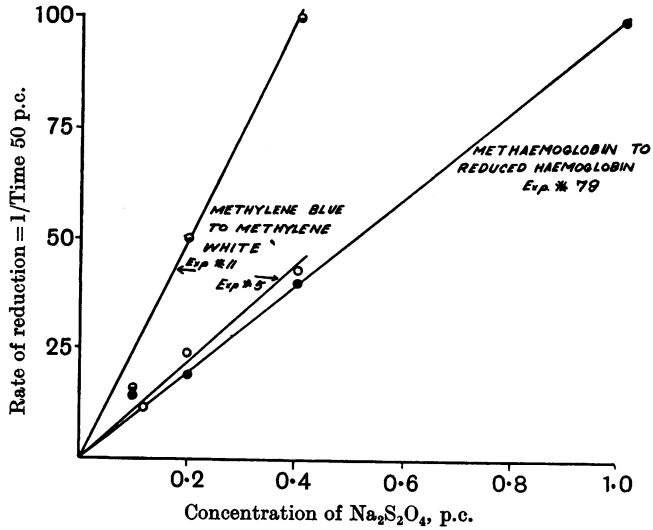

Fig. 1. Effect of reducer concentration in true reductions.

The control of the *p*H required special experimental precautions, because the sodium hydrosulphite used as an oxygen absorber acts as an acid in solution, and its oxidation products are more acid than it is. The reducing solutions of hydrosulphite were therefore made up in *M*/20 borate or phosphate buffers, with a small measured excess of NaOH added to bring back the reaction to that of the original buffer. Indicators could not be used to determine the *p*H with accuracy because of the bleaching effect of the hydrosulphite, so the reaction was in all cases determined with the glass electrode, and a large number of pre-

liminary tests were made to show that the final equilibrium *p*H was the same as that reached immediately after mixing in the observation tube. Since it was found that the effluent solutions became more acid on standing in the air, it was necessary to make the *p*H determinations during or immediately after a run.

In order to eliminate possible gradual changes in the pigments at different *p*H's, it was the usual procedure to use as stock solutions in the apparatus a single batch of unbuffered pigment on the one hand, and on the other a series of buffered reducer solutions of different reactions.

Substances used.

Three principal respiratory pigments have been used in this study:

(1) The hæmocyanin of the horseshoe crab, *Limulus polyphemus*, dialysed, and also in the native serum.

(2) The serum of the spider crab, *Maia squinado*.

(3) Laked sheep blood in dilute solution.

The first substance, in the purified form, is the best-established example of a "primitive" oxygen carrier, *i.e.* one for which the equilibrium dissociation curve is reproducibly a simple hyperbola. Its behaviour is further simplified by the fact that this curve is relatively insensitive to changes in hydrogen-ion concentration [Redfield, 1930]. *Maia* serum, on the other hand, represents a pigment which has "gone wild." Its equilibrium curve is of variable sigmoid form, and is enormously affected by *p*H. It has a very marked minimum affinity at *p*H 6·2, and large increases on both acid and alkaline sides of this reaction [Hogben, 1926]. The native serum of the horseshoe crab has properties intermediate between these two extreme groups, the equilibrium curve being generally sigmoid in shape, and quite definitely affected in position by change of reaction, though not nearly as much so as that of the Crustacea. There is also a marked minimum affinity at a *p*H of 8·1 [Redfield, 1931].

Laked sheep blood was chosen for comparative study because it represents a hæmoglobin whose equilibrium curve has been very carefully worked out, and because it was the object of the earlier kinetic studies of Hartridge and Roughton with whose results the new data could therefore be compared. Some kinetic experiments have also been made on the hæmoglobins of man, frog, and pig, in order to compare the kinetic behaviour of a number of hæmoglobins with each other. The development of the micro method has for the first time made possible this type of investigation on the smaller or the more valuable animals (viz. frog, man), whose blood is only available in limited quantities.

Limulus serum, treated with toluol to prevent bacterial action, will keep many months without deterioration if stored at 0° C. I am extremely grateful to Dr A. C. Redfield for having generously provided me with a large amount of this material from Wood's Hole, Massachusetts. He drew the blood, filtered it through cheesecloth, added toluol, and shipped

it to me in the ice-boxes of fast liners. Handled in this way, the material remained in apparently good condition. Dialysis was carried out in the usual collodion sacks against distilled water at about 0° C., until the chloride content became less than 1 millimolar as judged by the amount of precipitate formed when an excess of silver nitrate was added to a nitric acid digest of the sample. Control tests were made with known salt solutions.

The formation of melanin proved extremely troublesome in the spider crab serum [Pinhey, 1930], *Limulus* blood being fortunately free of the tyrosinase which produces it. This tendency to blackening, the occasional presence of yellow lipochrome pigments, and the very great variation in the quality and concentration of pigment in spider crab blood made it a very much less satisfactory substance to work with than that of *Limulus* and reduced the accuracy of the results obtained with it. None of the devices used to combat these bad properties of the blood has been completely satisfactory.

Sheep hæmoglobin was prepared as described by Forbes and Roughton [1931] or Hartridge and Roughton [1923 *b*], in order that the results might be comparable with those obtained in earlier work. Human hæmoglobin was obtained a few minutes before each experiment by defibrinating and laking with distilled water a few cubic centimetres of blood drawn from an arm vein. Two or three c.c. of red blood were sufficient for an entire kinetic run with thirty or forty individual points, but the poorer optical properties of hæmocyanin require for it greater concentration, so that 50–75 c.c. of the blue blood were usually necessary.

Calculation of velocity and equilibrium constants.

In calculating the velocity and equilibrium constants for "primitive" pigments, the following expressions were used:

(1) K = equilibrium constant $= \dfrac{1}{p_{O_2} \text{ at 50 p.c. saturation}}$;

Dimensions: $\dfrac{1}{\text{mm. Hg}}$.

(2) $K = \dfrac{k'}{k} = \dfrac{\text{association velocity constant}}{\text{dissociation velocity constant}}$.

(3) $k' = \dfrac{1}{a \times t_{50}}$ = association velocity constant;

a = initial concentration of both oxygen and reduced hæmocyanin in mm. Hg of O_2;

t_{50} = time of half completion of reaction;

Dimensions: $\dfrac{1}{\text{mm. Hg} \times \text{sec.}}$.

(4) $k = \dfrac{0{\cdot}7}{t_{50}}$ = dissociation velocity constant;

Dimensions: $\dfrac{1}{\text{sec.}}$.

These expressions are a simpler and somewhat less accurate version of those given by Hartridge and Roughton [1925]. The following assumptions have been made in deriving them:

(*a*) The dissociation is monomolecular.

(*b*) In measuring the dissociation rate, the hydrosulphite absorbs the oxygen so quickly that there is no back reaction.

For these first two assumptions the evidence, both old and new, is now very strong. They should be valid quite independently of the validity of the remaining three.

(*c*) The association is bimolecular.

As far as the new kinetic studies go, the hyperbolic shape of the equilibrium dissociation curve is our only justification for making this assumption.

(*d*) In measuring the association rate, the back reaction is negligible.

(*e*) In measuring the association rate, the initial concentration of oxygen and reduced pigment are equivalent.

These last two assumptions can be checked in the following way. The complete differential equation for the association process is

$$\frac{dy}{dt} = k'(a - y)(b - y) - ky,$$

where y = concentration of oxygenated pigment,
a = initial concentration of oxygen,
b = initial concentration of reduced pigment.

This expression was given by Hartridge and Roughton. It can be integrated graphically upon substitution of any arbitrary values for k', k, a, and b. If assumptions (*d*) and (*e*) are made this equation becomes

$$\frac{dy}{dt} = k'(a - y)^2,$$

which can very easily be integrated. Assumption (*d*) was checked by comparing the time of half completion of the reaction as calculated by graphical integration of these two equations, the substituted values being those of actual kinetic runs on *Limulus* hæmocyanin. The difference was just 10 p.c., which is less than the experimental uncertainty in the determination of this quantity. The final assumption (*e*) depends upon the experimental conditions. It was not always possible to make the concentration of the reactants exactly equivalent, but an excess of either one amounting to as much as 40 p.c. can be shown by integration of the corresponding equations to affect the half completion time of the reaction by less than 35 p.c., which is about the experimental uncertainty in this quantity.

The concentration units in which all of the constants are expressed is that in common use for equilibrium curve work, namely, "the equivalent partial pressure in millimetres of mercury." The reduced and oxygenated pigments as well as the dissolved oxygen can be very simply measured in this way, the pigment concentration being expressed in terms of its oxygen capacity. Roughton [1932] has given the thermodynamic

justification for the unit. It is used here because of its convenience, but it can readily be converted into the usual chemical notation by means of the Bunsen solubility coefficient and the gas constant.

It has been pointed out by Adair and by Hartridge and Roughton that the same kinetic and equilibrium equations are obtained if the pigment molecule contains any number of places for oxygen instead of only one, provided that these valencies are all equally strong and are independent of each other. For purposes of calculation one "molecule" of the pigment would, however, be defined as the amount of substance combining with one molecule of oxygen.

II. Experimental results.

Under physiological conditions.

The direct physiological interest in kinetic experiments on the blood pigments lies in the answer to the question: are these reactions slow enough to be limiting factors in the metabolism of the animal? In the vertebrates the question is complicated by the fact that here the pigment is carried in corpuscles, diffusion and chemical reaction velocity becoming joint factors in determining the total rate of gas exchange, as has been shown by Roughton [1932]. Hæmocyanin, however, is dissolved directly in the blood, and this complication does not exist in its case. *In vitro* experiments on hæmocyanin therefore give us a much more direct picture of what goes on in the organism than corresponding ones on hæmoglobin. In two other respects the experimental conditions in the kinetic studies of the blue bloods approach more closely those obtaining in the living animal. Due to optical properties of the pigments, the concentration of hæmoglobin must be reduced to one-hundredth or less of that in the corpuscles in order to make kinetic measurements, and it is conceivable that this great dilution may considerably affect the measured velocity constant of the reaction. Hæmocyanin, however, can be studied at practically blood concentrations. In the second place, normal room temperature, at which these experiments can most easily be carried out, is a normal one for the blue-blooded animals, but is below the body temperature of the mammals.

Table II summarizes the results of kinetic dissociation runs made on the native sera of the spider crab, *Maia*, and the king crab, *Limulus*, at room temperature and at hydrogen-ion concentrations in the neighbourhood of the physiological range [Quagliarello, 1916]. In Fig. 2 are plotted the relevant points of two of the individual experiments. Several

TABLE II. Rate of dissociation of arthropod sera under physiological conditions.

Exp.	Species and date	*p*H	Time, 50 p.c. reduction sec.
50	*Maia*, Nov. 1931	7·4	0·017 ±0·005
51	„ Nov. 1931	7·5 (interpolated)	0·020 ±0·008
52	„ Nov. 1931	7·5 (interpolated)	0·015 ±0·008
71	„ June 1932	7·5	0·020 ±0·004
	Average, *Maia*, *c.* 20° C.		0·017 ±0·006
46	*Limulus*, Aug. 1931	?	0·065 ±0·020
56	„ Feb. 1932	9·0	0·075 ±0·010
57	„ March 1932	8·0	0·075 ±0·010
59	„ April 1932	8·0	0·075 ±0·010
60	„ April 1932	8·0	0·080 ±0·015
	Average, *Limulus*, *c.* 20° C.		0·075 ±0·010

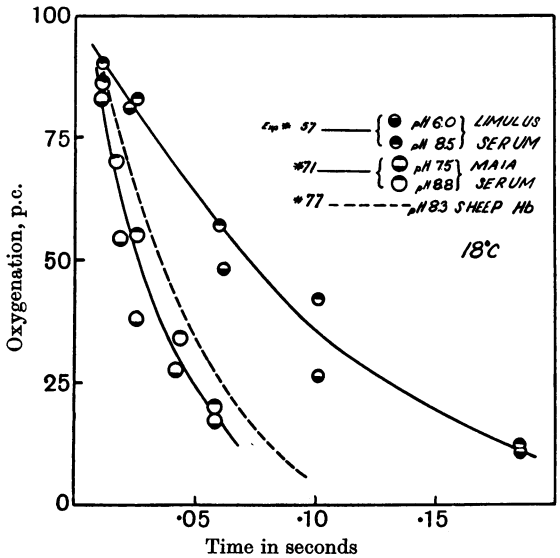

Fig. 2. Rate of dissociation of sera; physiological conditions.

of the points are averages of two or more readings to avoid congestion in plotting. A corresponding curve for sheep hæmoglobin is included for reference. It will be seen that the rate at which oxyhæmocyanin dissociates is of the same general order as that of oxyhæmoglobin, the half reaction taking from a sixtieth to a tenth of a second. There is, however, a very considerable difference between the two hæmocyanin species, the relatively active spider crab possessing blood which yields up its bound oxygen much more rapidly than the more sluggish horseshoe crab. It is improbable that this difference is of any physiological significance, since

even the most slowly dissociating pigment gives up half its oxygen in one-tenth of a second, whereas the time of passage of blood through the tissue capillaries is never less than several seconds.

The rate of oxygen uptake at physiological concentrations is a much quicker affair than the reverse process, reaching half completion in less than 1/300 second for both *Limulus* and *Maia* serum, which is very much too fast to make it a limiting factor in the metabolism of the animal.

The observation tube of the kinetic apparatus does not give a strictly accurate picture of what takes place in the gill capillary, since in the former the oxygen is used up from the supply initially sent down the tube with the reduced pigment, and there is therefore a progressively lower oxygen tension as we proceed along the tube; in the intact animal, on the other hand, oxygen is drawn from outside the capillary, where it exists at a fairly constant concentration. In the kinetic experiments, the initial oxygen tension of about 300 mm. of Hg was chosen so that the average oxygen pressure as the fluid went down the tube would be about that of aerated water, but it is not claimed that the value of 1/300 sec. for half completion tells anything more than the order of magnitude of the rate in the capillary. This happens to be all that is needed to answer the physiological question of limiting factors in oxygen metabolism.

The comparative speeds of dissociation of different blood pigments.

The hæmoglobins of three mammals and one amphibian have been studied under identical (though not physiological) conditions, as well as the hæmocyanins of *Limulus* and *Maia*. The results are summarized in Table III. For these hæmoglobin experiments the blood was hæmolysed

TABLE III. Rates of dissociation compared with oxygen affinity. *p*H 8·6, 22° C. (Kinetic values all obtained in same experimental run.)

Hæmoglobins	Reduction time t_{50}	Reciprocal affinity p_{O_2} – 50 p.c. sat. (mm.)	Authority for affinity values
Frog	0·020	18	Macela and Seliškar [1925]
Sheep	0·028	3	Forbes and Roughton [1931]; Hartridge and Roughton [1923*b*]
Man	0·038	1	Macela and Seliškar [1925]
Pig	0·047	Unknown, but low	
Hæmocyanins			
Spider crab, *Maia*	0·025	16	Hogben [1926]
King crab, *Limulus*:			
Dialysed	0·080	3	Redfield [1930]
Native	0·075	17	Redfield [1930]

and diluted two hundred times in borate buffer. No attempt was made to remove the stromata, salts, or plasma proteins, but their concentration would be low in the final dilute solution. In any case, the effect of salt concentration on the rate of dissociation is probably not large, as its

removal is seen to have little effect on this quantity in *Limulus* hæmocyanin (Table III). The value for *Maia* hæmocyanin given in the table is for the native serum, as no extensive runs were made on this material after dialysis. In Table III are also given the partial pressures of oxygen in equilibrium with half-saturated pigment solutions, as a rough measure of the oxygen affinity. Their significance will be discussed in a later section.

The order of the dissociation reaction.

The kinetic dissociation curve of both hæmoglobin and hæmocyanin has been found to be monomolecular within the limits of error of the

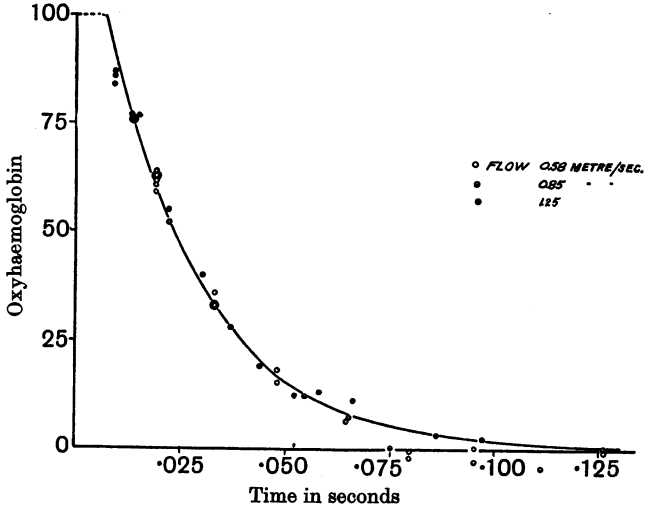

Fig. 3. The dissociation of sheep hæmoglobin. Exp. 77, *p*H 8·3. Curve constructed from equation $y/100 = \rho^{-44.5(t-0.0074)}$; reduction velocity constant $= 44{\cdot}5 \frac{1}{\text{sec.}}$; time of induction $= 0{\cdot}0074$ sec.

experiments, quite independent of the shape of the equilibrium dissociation curve. The data of one experiment on sheep hæmoglobin are plotted in Fig. 3, and replotted logarithmically in Fig. 4. These findings completely confirm the earlier results of Hartridge and Roughton obtained with the reversion spectroscope on the same material, and they furthermore extend the measurements to much higher and lower saturation ranges where the test for monomolecularity becomes more severe. The standard deviation from the logarithmic curve of all points above 3 p.c. saturation is 3·3 p.c. (This is raised to about 5 p.c. if we include the points on the extreme tail of the curve, which were thrown somewhat low by a systematic error of manipulation.) The mutual consistency of points

made with widely differing rates of flow gives satisfactory evidence that these results have a real meaning and are not experimental artefacts.

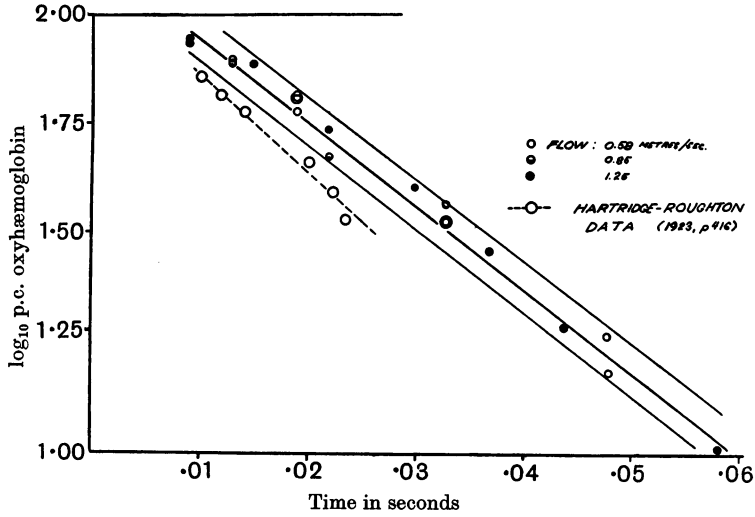

Fig. 4. The order of the dissociation of sheep hæmoglobin. Exp. 77, pH 8·3.

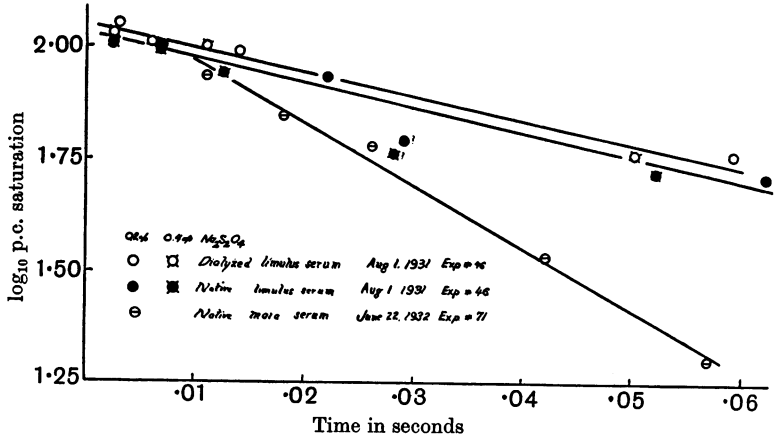

Fig. 5. The order of dissociation of hæmocyanin.

Much less accuracy is obtainable with hæmocyanin, but within the wider limits of experimental error, both *Maia* and *Limulus* sera show the same monomolecular dissociation curves (see Fig. 5), the logarithm of the amount of oxyhæmocyanin decreasing uniformly with increasing time. This is true for *Limulus* serum both before and after dialysis, though the

equilibrium curve is much affected in shape and position by this purification. Neither the shape nor the position of the kinetic dissociation curves is affected by a two- or threefold change in the concentration of hæmocyanin (*Limulus*).

Effect of pH *on dissociation rate.*

The effect of varying the *p*H on the rate of oxygen dissociation differs very considerably from substance to substance. The shifts will be most easily understood in relation to the corresponding changes in equilibrium behaviour, for which the relevant data have been summarized in Fig. 6.

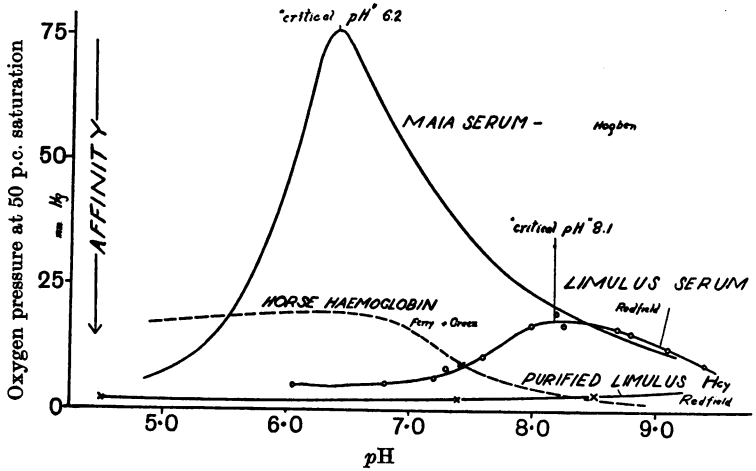

Fig. 6. *p*H effect on equilibrium curves: hæmocyanin and hæmoglobin.

It had been previously found by Hartridge and Roughton that the dissociation rate of dilute sheep hæmoglobin was much higher in acid than in alkaline solution. By repeating their experiments on the new apparatus, I have been able fully to confirm their results, and to show that they can also be carried over to human material (Fig. 7). As was expected from the greater affinity of human hæmoglobin for oxygen [Forbes and Roughton, 1931], the rate of dissociation was in every case considerably lower than that of the sheep, but the character of the change with *p*H was found to be the same. If the velocity constant is plotted against *p*H, the curve is seen to resemble the titration curve of a monovalent acid with a *p*K of about 7, there being a low constant rate at the alkaline end, a high constant rate at the acid end, and an intermediate transition portion.

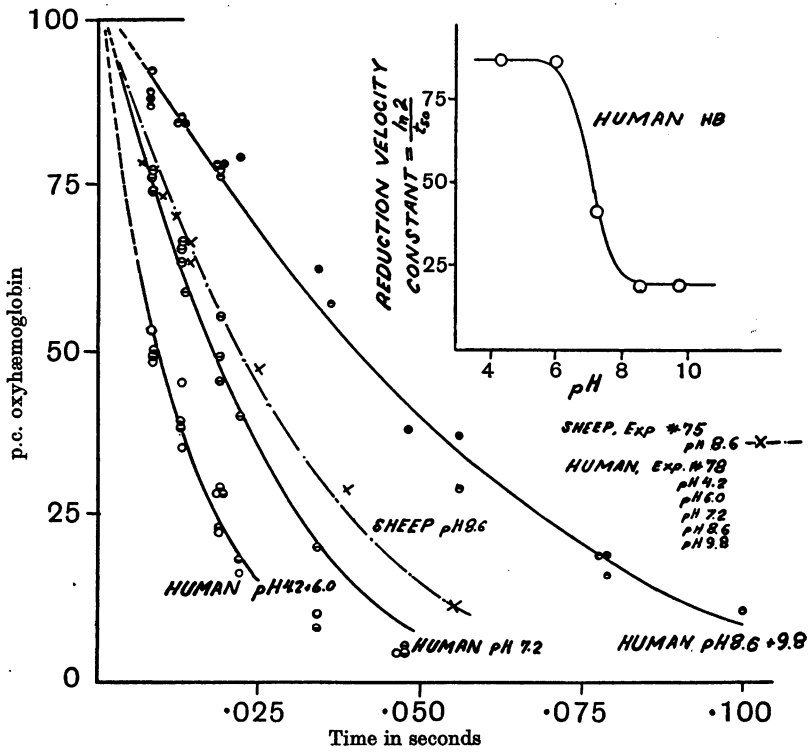

Fig. 7. *p*H effect on rate of dissociation: human hæmoglobin.

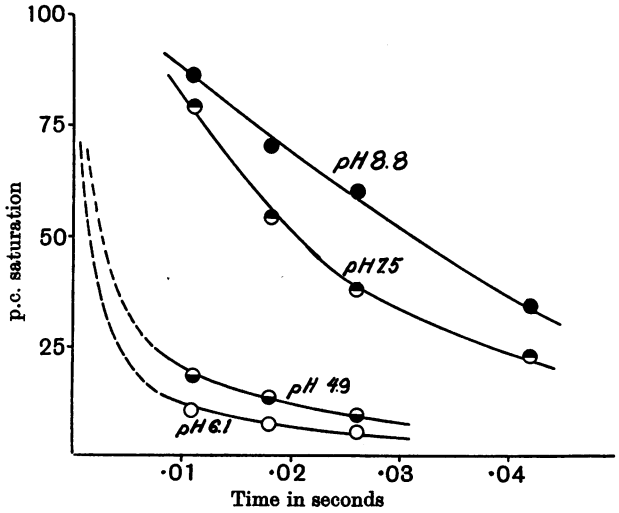

Fig. 8. *p*H effect on rate of dissociation: *Maia* serum. 19° C. Exp. 71. Native serum.

The hæmocyanins of *Limulus* and *Maia* show completely different effects, *Maia* being very sensitive indeed and *Limulus* relatively insensitive (Figs. 8, 9). This reflects the general equilibrium behaviour plotted in Fig. 6. It will be seen that the rate of dissociation of *Maia* serum increases enormously as the solution becomes more acid, but there is no marked slowing up or "come back" in the rate after the critical *p*H (6·2) has been passed, although the oxygen affinity does show such a marked return. *Limulus* serum, on the other hand, shows only a small *p*H effect, the rate increasing slightly with acidification. Here also there

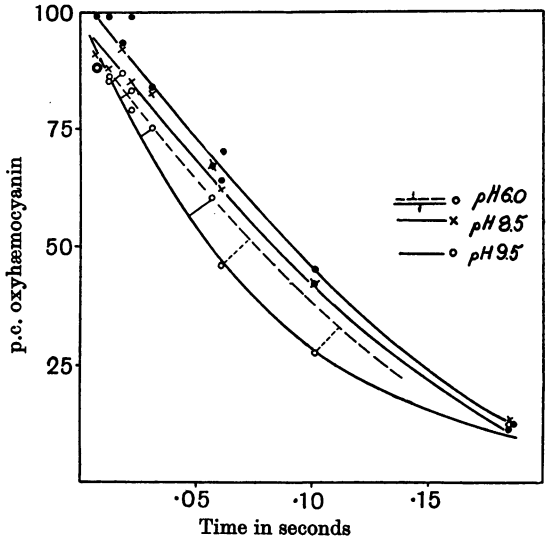

Fig. 9. *p*H effect on rate of dissociation: *Limulus* serum. Exp. 57. Points are individual readings.

are no signs of a return to lower speeds on the acid side of the "critical *p*H" (8·1). The *p*H effect on this species is, however, so small as to be within the rather wide limits of experimental uncertainty. No satisfactory experiments on the *p*H effect of the dialysed *Limulus* material have been made.

Effect of temperature on the dissociation rate.

Increasing the temperature greatly speeds up the dissociation rate of purified *Limulus* hæmocyanin, just as Hartridge and Roughton found it to do for sheep hæmoglobin. The experimental uncertainties in the hæmocyanin temperature work were, however, too great to allow an exact calculation of the temperature coefficient. The value of Q_{10} is probably

about 3·8 (the same as the hæmoglobin value), though one experiment gave as low as 2·2. The shift in the equilibrium constant for this material has been found by Brown [1933] to have a Q_{10} of 2·3.

The rate of combination of oxygen with reduced pigment.

The new experiments have not yielded quite as accurate results for the speed of oxygen association of hæmocyanin as Hartridge and Roughton were able to obtain for sheep hæmoglobin, the values given in Table IV

TABLE IV. Rate of combination of oxygen and reduced pigment.

Exp.	Material	Initial average conc. of reactants mm. Hg of O_2 a	Time of half completion sec. t_{50}	Association velocity constant assuming bimolecularity k'
67	*Limulus* hæmocyanin, dialysed	30	0·012	2·8
68	,, ,, ,,	32	0·008	3·9
65	Sheep hæmoglobin, diluted 1 : 200	25	0·010	4·0
	Sheep hæmoglobin Hartridge and Roughton [1925, p. 674], converted to these units	—	—	4·5–6·3

being possibly in error by as much as 40 p.c. The points of the individual curves are too scattered to give definite information about the order of the reaction. Bimolecularity is assumed in calculating the velocity constant of dialysed *Limulus* serum simply because of the shape of the equilibrium curve. It is also assumed in deriving the same constant for sheep hæmoglobin, in order to compare the results with those of Hartridge and Roughton [1925]. It will be seen that with equivalent initial concentrations of the reactants the two pigments react with oxygen at roughly the same rate of speed.

In order to catch the association reaction at all, a redesigned form of the kinetic apparatus was required, which would allow greater dilutions to be used. This apparatus has an observation tube 2 mm. in diameter (instead of the usual 1 mm. tube), and a 4-jet mixer (instead of the usual 2-jet type). No other changes were made in the experimental arrangements.

III. DISCUSSION OF RESULTS.

The relation of "on," "off," and equilibrium constants.

The purified hæmocyanins of *Limulus*, *Helix*, and *Busycon* are the only respiratory pigments now known which reproducibly give hyperbolic equilibrium curves and for which, therefore, the very simple Hüfner assumptions can reasonably be made. One of these substances is the

logical choice for a comparison of the observed equilibrium affinity with that calculated from the measured rates of the two opposing kinetic reactions. These values for dialysed *Limulus* hæmocyanin are given in Table V.

TABLE V.

Association		Dissociation		Equilibrium	
Exp.	k'	Exp.	k	Calc.	Observed [Redfield]
67	2·8	69	7·4	0·38	0·40
68	3·9	—	—	0·53	—

Exps. 67, 68, and 69 were all made on the same material and on adjacent days. The "observed" value of 0·40 for the equilibrium constant is interpolated from the data of Redfield [1930], and Brown [1933]. I have been able to confirm both the shape of the curve and the value for the equilibrium constant by these workers, on material identical with that used in the kinetic measurements, though the uncertainty of my K value was considerably greater than theirs, being about 0·1. The possible errors for k', k, and K as judged from the experimental uncertainties are 20, 50, and 25 p.c. respectively. The agreement between observed and calculated values of the equilibrium constant is therefore as good as could have been expected, being within 5 p.c. in one case and 35 p.c. in the other.

The situation in regard to hæmoglobin is much less satisfactory. Hartridge and Roughton [1925] obtained about the same agreement between the velocity and equilibrium constants on sheep hæmoglobin as have now been obtained on *Limulus* hæmocyanin. They had determined equilibrium curves on this material, using the reversion spectroscope, and had found them to be hyperbolic in shape. Later investigation, however, especially by Forbes and Roughton [1931], has reproducibly given sigmoid curves for dilute solutions of sheep hæmoglobin. The simple Hüfner theory is no longer able to provide an accurate description of these experimental facts, and we must seek agreement between kinetic and equilibrium data on the basis of other primary assumptions.

Kinetic data can be of help in assigning relative merit to other theories of the nature of the oxygen-respiratory pigment complex, although the quantity and accuracy of the kinetic results so far obtained are not sufficient to give a definitive award to any one. The Hill theory, which has generally been regarded as untenable in its original form since the two independent determinations of Adair and Svedberg, which gave agreeing values for the molecular weight very different from that predicted by this hypothesis, also fails in its prediction of kinetic behaviour of sheep

hæmoglobin in dilute solution. The kinetic equations given under "Method" for a simple pigment in which $n = 1$, can very easily be generalized for any integral values of n, and the velocity constants can be obtained in the same way. The very careful equilibrium curves of Forbes and Roughton, made under conditions which were duplicated as closely as possible in the present kinetic studies, have inflections which give n values between 2 and 3. The equilibrium K calculated from k' and k differs, however, from that obtained directly from the equilibrium curve, being five times too small for $n = 2$ and twenty times too small for $n = 3$. The combined possible errors, though fairly large, are very much less than this discrepancy. Since all values of n greater than 1 give completely unacceptable calculated values for the equilibrium constant, a theory based on mixtures of two kinds of such molecules (in which, for example, one is of the $n = 2$ form, and the other of the $n = 4$ form) such as Redfield has used in interpreting equilibrium curves, would have great difficulty in explaining the results of the kinetic measurements.

There remains the intermediate compound hypothesis suggested by Adair. This theory adequately accounts for the shapes of the carefully made equilibrium curves of Adair [1925], Ferry and Green [1929], and Forbes and Roughton [1931], if the four arbitrary constants predicted by it are suitably chosen. Its principal disadvantage, as a theory, is that it allows too much latitude in the choice of the constants. This lack of specificity is so great that until more evidence is available on the probable values of some of these constants, the attempt to correlate the kinetic and equilibrium data is perhaps futile. At this stage, however, two considerations do deserve emphasis:

(*a*) Analysis shows that it is quite possible for the intermediate compound hypothesis to lead to an accurately monomolecular curve for the dissociation velocity, even if the equilibrium curve is sigmoid (*e.g.* $n = 2$ to 3 on Hill's notation). It is hoped later to develop this matter more fully.

(*b*) Preliminary experiments by Roughton have shown that, under some conditions, there may be a difference between the "slow equilibrium" finally reached when oxygen and hæmoglobin are shaken together in tonometers, and the "quick equilibrium" reached in a fraction of a second when oxygen solutions and hæmoglobin solutions are mixed together in the rapid reaction apparatus. The photoelectric methods are particularly suitable for following up this point further. If it were indeed established it would obviously be of prime importance in the correlation of kinetic and equilibrium data.

The molecular weights of blood pigments.

We have seen how similar in their kinetic properties are hæmoglobin and hæmocyanin, and the equilibrium work of the last few years has but emphasized the essential likeness of these two groups of substances in their behaviour towards oxygen. The differences between the two groups are in general no greater than interspecies differences within each group. It is appropriate, therefore, to ask of what significance is a thirty- to seventyfold difference in their molecular weights, Svedberg's ultracentrifugal determinations having given a value of 5,000,000 for the weight of *Helix* hæmocyanin and 2,000,000 for *Limulus* hæmocyanin as compared with 68,000 for mammalian hæmoglobins. Adair's intermediate compound hypothesis is as easily capable of explaining sigmoid dissociation curves with $n = 30$ or $n = 70$ as with $n = 4$. This theory further suggests a very simple qualitative explanation for the observed change in shape of the curves under different conditions of salt concentration, for if the relative dependence of one oxygen valence on another is determined by the salt concentration, such changes are predicted. For example, in the case of *Limulus* hæmocyanin, the hyperbolic curve obtained for the purified material is predicted by the intermediate compound hypothesis if the tightness with which one oxygen molecule is bound in no way affects the equal tightness with which its neighbour is bound. This will be so no matter what is the molecular weight, and will also explain the indifference of the hyperbolic equilibrium curve of purified *Helix* hæmocyanin to its molecular weight, the Stedmans having shown that the position and shape of the curve are practically independent of the reaction between *p*H 4·0 and 9·0, whereas Svedberg has found the "5,000,000" molecules to be stable only between *p*H 4·5 and 7·5, beyond which they break up into smaller fragments. The addition of salts must now be supposed to introduce restricting influences on the oxygen valencies, so that they become to a certain extent dependent on one another. The altered equilibrium constants when substituted into the intermediate compound equation for oxygen equilibrium will now give it an S-shape, such as that observed experimentally.

Although Adair's theory was put forward on the basis of definite molecular weight data, it is now seen that its usefulness persists even in the absence of the support furnished by the molecular weight, and molecular size becomes of relatively little importance in fitting the oxygen data, so long as one molecule has at least several places for oxygen. It is quite possible that the giant hæmocyanin molecule is made up of

hæmoglobin-like "sub-molecules," in each of which $n = 4$ or some other small number, but so far we have no experimental evidence either for or against this hypothesis.

The equilibrium condition.

When a solution of hæmocyanin or hæmoglobin is in equilibrium with a given partial pressure of oxygen, just as many molecules of oxygen are being bound by the pigment every second as are dissociating from it. If any change in conditions produces an increase in the equilibrium affinity, this can only mean that there has been an increase in the rate at which oxygen molecules have been joining on or a decrease in the rate with which they have been coming off, or both. This is true quite independently of any theory of the mechanism by which they are bound. Hartridge and Roughton found that for sheep hæmoglobin it was the dissociation reaction which bore the brunt of such a change, the joining on process being little if at all affected by changes in *p*H, salts, or temperature. The experiments on hæmoglobins of several animals described in Section II show that qualitatively at least this principle may be extended to interspecies differences, the higher the affinity, the slower being the dissociation (Table III). There are difficulties, however, when we attempt to apply the same principle to the two hæmocyanins which have been investigated. The native hæmocyanins of *Maia* and *Limulus* at *p*H 8·6 have approximately equivalent affinities, but their dissociation rates differ by a factor of three. Furthermore, the large increase in the affinity of *Limulus* serum for oxygen brought about by dialysis fails to be reflected in any considerable change in the rate of dissociation. Another exception is found in the *p*H behaviour of both types of blue blood, for on the acid side of the "critical *p*H" acidification brings increased affinity for oxygen, but fails to bring marked slowing down of the dissociation process.

Two possible explanations for these exceptions suggest themselves:

(1) there is some compensating change in the rate of the combining reaction, or

(2) the published equilibrium curves fail to portray the equilibrium conditions at the moment of the kinetic measurements.

More extensive kinetic association data or more information about "fast" and "slow" equilibrium curves will be needed for a decision between the two.

Comparative properties of hæmoglobin and hæmocyanin.

A number of outstanding properties of hæmoglobin and hæmocyanin have been listed in Table VI. A glance at this table will show how our newer kinetic evidence has reinforced the results of equilibrium work in bringing out the essential similarity in the oxygen-binding behaviour of

TABLE VI. Comparative properties of hæmoglobin and hæmocyanin.

Properties of equilibrium curve	Hæmoglobin	Hæmocyanin
1. Shape, native state	Always sigmoid	Always sigmoid
2. Shape, purified	Probably always sigmoid	Sometimes hyperbolic: often sigmoid
3. *p*H effect, native state	None (*Urechis*) Moderate (mammals) Large (fish)	None (pure *Helix*) Moderate (*Limulus*) Large (*Maia*)
Kinetic properties		
4. Approximate time of dissociation, t_{50}, 20° C., *p*H *circa* 8·6	1/40–1/20 sec.	1/40–1/10 sec.
5. Character of dissociation	Accurately monomolecular	Monomolecular within experimental limits
6. Effect of decreasing *p*H on rate of dissociation	Increase	Increase or small change
7. Effect of increasing temperature on rate of dissociation	Great increase $Q_{10}=3{\cdot}8$	Probably great increase $Q_{10}=2{\cdot}2?-3{\cdot}8$
8. Approximate rate of combination, k' (assuming bimolecularity)	4·0 (new)–6·3 (H.R. 1925)	2·8–3·9
Other properties		
9. Metal: Element	Iron	Copper
Pyrrol prosthetic group	Yes	No
10. Ratio, metal: oxygen molecules	1 : 1	2 : 1
11. "Met" oxidation product	Yes	Yes (Conant)
12. Addition products beside oxygen: CO	Yes	?
KCN	Probably no	Yes
13. Molecular weight	68,000 (mammals) millions (*Arenicola*)	2,000,000–5,000,000 for 2 species (no Crustacea)

these pigments, and we have seen above that the molecular weight differences need not affect this similarity. Of what significance are the other differences, such as the metallic element, the ratio of metal to oxygen bound, the existence of other loose complexes? The answer to this question is probably closely bound up with the catalytic rôle of iron and copper compounds in biological processes, and it is from the further study of oxygenation and oxidation, of oxygen transport and oxygen catalysis—perhaps by kinetic methods—that we may hope for further light on the mechanism of oxygen binding by the respiratory pigments.

SUMMARY.

1. The reactions of oxygen with hæmocyanin take place with the same order of speed as those with hæmoglobin.

2. Under physiological conditions, the oxygen dissociation of *Limulus* serum takes about 1/10 of a second to reach half completion; that of *Maia* takes about 1/25 of a second. The association process is half completed in less than 1/300 of a second for both these pigments. These reactions are too fast to be limiting factors in metabolism.

3. The oxygen dissociation of the blood pigments has been found to be monomolecular, independently of the shape of the equilibrium dissociation curve. This is true, in particular, for sheep hæmoglobin, where the reaction has been followed throughout its entire course with a probable error of about 3 p.c.

4. Increasing the acidity of the blood pigments either greatly increases the rate of oxygen dissociation (*Maia* serum, sheep hæmoglobin, human hæmoglobin), or produces little effect on it (*Limulus* serum). It never much reduces the rate, even on the acid side of the "critical *p*H."

5. Increasing the temperature greatly accelerates the dissociation of *Limulus* hæmocyanin, as it does for sheep hæmoglobin.

6. Under identical conditions, the hæmoglobins of pig, man, sheep, and frog, have quite different rates of oxygen dissociation, as do the hæmocyanins of *Maia* and *Limulus*.

7. The equilibrium constant of dialysed *Limulus* serum as calculated from the rate of dissociation and the rate of combination with oxygen agrees with that obtained by direct measurement.

8. The bearing of these experimental results on the problem of the nature of the oxygen-binding mechanism of the blood pigments has been discussed.

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