CXV. HAEMOCYANIN.

PART II. THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DISSOCIATION CURVE OF THE OXYHAEMOCYANIN FROM THE BLOOD OF THE COMMON LOBSTER (*HOMARUS VULGARIS*)

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IN Part I of this series the authors [1925] examined the blood of four species of decapod crustacea with the object of ascertaining whether the haemocyanins contained therein were capable of exercising respiratory functions. In so far as their power of combining reversibly with oxygen was concerned, the results left no doubt that the pigments were able so to function. An examination of the dissociation curves revealed two further points of interest. Firstly, they proved to be identical, thus suggesting the identity of the haemocyanins in the four species. Secondly, they departed from the hyperbolic form which would be expected if the combination with oxygen followed the law of mass action as formulated for true solutions, and were, in fact, of an S-shape similar to the characteristic oxygen dissociation curve of haemoglobin. It was obvious that, apart from the isolation and chemical investigation of the pigments, the question of their identity could only be settled with any degree of certainty by an examination of their dissociation curves under strictly comparable conditions of hydrogen ion and salt concentrations. An investigation of the effect of changes in these two variables was, moreover, necessary if attempts were to be made to elucidate the cause of the inapplicability of the mass-law in its simple form. The latter problem is of special interest in view of the analogous problem in the case of haemoglobin. Both pigments, or, to be strictly accurate, both groups of pigments, belong to the class of proteins into the elementary composition of which a metal enters. Further, as implied by their classification as proteins, the physical and physico-chemical properties of their solutions present certain common features. It is therefore probable, especially in view of the similarity between the forms of their oxygen dissociation curves, that the factors controlling or modifying their affinities for oxygen are fundamentally of the same nature. What has hitherto been regarded as the special problem of haemoglobin thus becomes a more general one, and it is doubtful if any solution of it can be considered

satisfactory unless it satisfies the data derived from studies of both series of pigments. On the other hand, the more extensive and varied the material with which a particular phenomenon may be studied the greater are the possibilities for elucidating its cause; it is thus conceivable that advances in our knowledge concerning the factors modifying the affinity of haemocyanin for oxygen may contribute to an explanation of the inflexion in the oxygen dissociation curve of haemoglobin.

These considerations have led the authors to investigate the oxygen dissociation curves of various haemocyanins in dialysed solution and to study the effect thereon of changes in hydrogen ion concentration and of the addition of salts. The present communication deals mainly with haemocyanin from the common lobster, and is confined to the influence of hydrogen ion concentration. A few observations relating to the pigments from other species are also included.

EXPERIMENTAL METHODS.

Preparation of solutions of haemocyanin. The blood, obtained in the manner previously described, was allowed to flow on to solid sodium fluoride (about 1 g. for each 100 cc. of blood) and was stirred vigorously for some minutes to prevent the formation of a gel. After standing for a few hours in the ice-chest, the clot was removed by filtration and the serum concentrated in collodion ultra-filters. The latter process appeared advisable, not only because of the small oxygen capacity of the serum, but in order to compensate for the dilution which occurred in the subsequent process of dialysis. The concentrated serum was then dialysed in collodion membranes against cold water saturated with chloroform until free from salts, filtered from small amounts of protein which had separated, and repeatedly evacuated to remove traces of dissolved chloroform. Adjustments of the hydrogen ion concentration, where necessary, were effected by the addition of a solution of potassium hydroxide.

Determinations of p_H . These were made, unless otherwise stated, electrometrically using a Hildebrand electrode. The measurements were carried out at room temperature, which varied in different experiments from 12° to 20°; no correction was made for deviations of the hydrogen pressure from normal. Before each determination the hydrogen electrode was tested with $M/20$ potassium hydrogen phthalate. The value obtained for the p_H of this solution never deviated by more than 0.02 from the value of 3.97 given by Clark for this buffer solution between the temperatures of 20° and 30°; usually the deviation was not greater than 0.01. As a check against any gross error, the p_H was also, in each case, determined colorimetrically, using standards prepared at intervals of p_H 0.2 as recommended by Clark. For the latter estimations, the haemocyanin solutions were diluted to 1 in 10 with distilled water, and the comparison with the standards carried out in a comparator in the customary manner. Values in satisfactory agreement with those determined electrometrically were obtained over those ranges in which it was possible to use

cresol red or phenol red as indicator. With bromothymol blue and thymol blue, however, the results were less satisfactory. This accords with the experience of other workers, who have also found that the protein error of these indicators is large.

Equilibration of solutions with oxygen at different pressures. More refined methods than those described in Part I have been employed. About 8 cc. of the haemocyanin solution were drawn into a tonometer, which was then swept out and filled under slight pressure with a mixture of nitrogen and oxygen prepared in the proportions necessary to give approximately the desired oxygen pressure. The tonometers were placed in a thermostat and rotated until they attained the temperature of the latter, for which purpose from 15 to 30 minutes were allowed. One stopcock of the tonometer was then caused to project above the surface of the water and opened momentarily in order to equalise the pressure of the contained gases with that of the atmosphere. Rotation in the thermostat was then continued for a further period of from 30 to 45 minutes. Any variations which occurred in the temperature of the thermostat were so small that they were not detectable with a thermometer graduated in tenths of a degree. The exact composition of the gas mixtures in equilibrium with the haemocyanin solutions was determined by analyses of samples of the gases obtained directly from the tonometers; a Haldane portable gas analysis apparatus was employed for this purpose.

Determination of oxygen content of solutions. Two small modifications have been made in the method previously described.

The mixture of hydrosulphite and anthraquinone- β -sulphonate recommended by Fieser [1924] has been used as absorbent for oxygen in place of the hydrosulphite solution described by Van Slyke. Apart from the superior absorbing powers claimed for it by Fieser, the former mixture possesses the advantage that the decomposition of the hydrosulphite, as judged by the formation of sulphuretted hydrogen, proceeds more slowly in the presence of the sulphonic acid than in its absence.

For the extraction of the gases from the equilibrated haemocyanin solutions, 5 cc. samples were introduced into the Van Slyke apparatus in the manner previously described and shaken mechanically for a period of 5 minutes. This extension of the period of extraction, although not necessary with many of the solutions employed, was deemed advisable with certain samples which exhibited a relatively high oxygen capacity at low oxygen pressures, and was accordingly adopted uniformly throughout the experiments.

As evidence for the applicability of the technique employed, it was pointed out in connection with the authors' original experiments that extraction of the serum in the Van Slyke apparatus caused the complete disappearance of the blue colour due to the oxyhaemocyanin. A simple calculation will show that the error due to incomplete extraction is, in most cases, negligible. The volume per cent., v , of oxygen obtained from the sample under examination is, if one omits the factor correcting for the oxygen dissolved at the pressure

existing in the pipette of the apparatus, given by the equation [Van Slyke and Neill, 1924]

$$v = P \frac{0.1316a}{(\text{cc. sample})} \times \frac{1}{1+0.00384t}$$

where P is the partial pressure of the oxygen at the volume a , and t is the temperature. In the experiments recorded in this communication 5 cc. samples of solution were employed; hence the volume of oxygen, measured at N.T.P., extracted from the sample is $v/20$. Since the total volume of liquid introduced into the pipette is 6 cc. (5 cc. solution + 1 cc. water), and the level of the mercury during extraction of the sample is at a point approximately 2 cc. above the 50 cc. mark of the pipette, the pressure of oxygen in the apparatus on the completion of extraction is, neglecting the small temperature correction, $\frac{v}{20} \times \frac{760}{42}$. When $v = 4$, which is about the maximum value obtained in these experiments, the oxygen pressure in the pipette is thus of the order of 3.6 mm.; for most experiments it is considerably less and frequently does not exceed a fraction of a mm. Hence, on withdrawal from the pipette, the samples are in equilibrium with oxygen pressures varying from 0.3 to 3.6 mm.; that the amount of oxygen thereby lost is, in most cases, negligible is evident from an inspection of the dissociation curves. In cases where it would cause an appreciable, though small, error an approximate correction can be obtained graphically from a large scale curve.

RESULTS.

Dissociation curves of oxyhaemocyanin from Homarus. The data obtained in five series of experiments, all of which were carried out at a temperature of 23°, are given in Table I.

The first three columns (p_H , O_2 -pressure and O_2 -content) contain the experimentally determined values and require no further comment. In column 4 are shown the corrections which must be added, on account of incomplete extraction by the apparatus, to the figures in column 3 to obtain the true oxygen content of the respective samples. These corrections have been determined graphically in the manner described above and can consequently only be regarded as approximately correct; nevertheless, any error should fall well within the experimental error of the methods employed. In those cases where the corrections would be very small they have been neglected entirely. The values for the physically dissolved oxygen (column 5) have been calculated on the basis that the solubility of oxygen in dialysed haemocyanin solutions is the same as in pure water. For this purpose, it has been assumed, taking the mean of values appearing in tables of physical constants, that water contains 0.62 volumes per cent. of oxygen when in equilibrium with an oxygen pressure of 159 mm. and at a temperature of 23°. The figures in column 6 have been obtained by subtracting the corresponding figure in column 5 from the sum of those in columns 3 and 4. Finally, column 7

Table I

P_{H}	Oxygen pressure (mm.)	Oxygen content (vols. %)	Correction for oxygen retained	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9.65	17.8	0.422	0.010	0.069	0.363	33.0
	27.2	0.544	0.020	0.106	0.458	42.1
	65.1	0.889	0.030	0.253	0.666	60.6
	98.9	1.102	0.040	0.385	0.757	68.8
	152.7	1.378	0.045	0.594	0.829	75.3
	337.8	2.214	0.080	1.320	0.974	88.5
	619.4	3.338	0.100	2.410	1.028	93.5
	650.0	3.477	0.100	2.530	1.047	95.2
					1.100	100.0
9.50	12.9	0.281	—	0.040	0.241	17.2
	26.6	0.512	—	0.085	0.427	34.7
	32.4	0.624	—	0.105	0.519	37.5
	71.8	1.043	0.010	0.240	0.813	58.1
	154.2	1.710	0.020	0.525	1.205	86.1
	190.9	1.806	0.040	0.660	1.185	84.7
	513.2	3.166	0.065	1.800	1.431	102.2
	556.3	3.270	0.070	1.955	1.385	99.0
	654.6	3.608	0.080	2.300	1.388	99.1
8.75	11.6	0.190	—	0.045	0.145	8.5
	20.7	0.313	—	0.085	0.228	13.4
	38.3	1.008	—	0.155	0.853	50.0
	61.0	1.578	—	0.245	1.333	78.2
	151.6	2.165	0.020	0.575	1.610	94.4
	452.3	3.380	0.040	1.720	1.700	99.7
	552.2	3.755	0.045	2.090	1.710	100.3
	655.3	4.135	0.050	2.480	1.705	100.0
					1.705	100.0
8.26	42.8	0.397	—	0.165	0.232	18.6
	58.2	0.571	—	0.225	0.346	27.7
	125.7	1.359	—	0.490	0.869	69.5
	154.9	1.639	—	0.605	1.034	82.8
	258.8	2.262	—	1.020	1.242	99.3
	487.2	3.168	—	1.920	1.248	99.8
	599.7	3.597	—	2.365	1.232	98.6
	696.7	4.036	—	2.750	1.286	102.8
					1.250	100.0
7.76	61.0	0.459	—	0.230	0.229	14.3
	99.9	0.694	—	0.390	0.304	19.0
	153.2	1.054	—	0.605	0.449	28.1
	230.5	1.610	—	0.915	0.695	43.4
	271.7	1.983	—	1.080	0.903	56.4
	542.8	3.630	—	2.160	1.470	91.9
	556.2	3.697	—	2.215	1.482	92.6
	694.1	4.368	—	2.780	1.588	99.2
					1.600	100.0

shows the percentage saturation of the haemocyanin for each oxygen pressure. This has been calculated from the preceding column, using the figures given on the last line of each series of experiments as representing the maximum amount of oxygen with which the haemocyanin is capable of combining. From the data in Table I five dissociation curves have been plotted and are shown in Fig. 1.

Before discussing these curves, some explanation is necessary respecting, firstly, the selection of the above-mentioned value for the physically dissolved oxygen and, secondly, the determination of the saturation value for the various

haemocyanin solutions. It was anticipated, when the experiments were commenced, that correction for physically dissolved oxygen could be made, as was done in the first paper of this series, from the upper portion of a curve in which oxygen content was plotted against oxygen pressure. Proportionality between these magnitudes did, in fact, occur in the region of high pressures, and the solubility curves deduced therefrom were found to be in fairly satisfactory agreement with the value of 0.62 for 159 mm., thus indicating that the haemocyanin had no influence on the solubility of oxygen. It therefore seemed more accurate to employ the theoretical figure of 0.62 throughout the experiments than to use the actual solubility curves determined in each individual experiment, especially as observed departures from this value were usually in the direction of a slightly greater solubility. Whilst high values could be attributed to experimental error, another explanation is more probable,

Fig. 1.

namely, the incomplete saturation of the haemocyanin even at the high pressures employed. That this is the case in the experiments at p_H 9.65 is evident from an inspection of Fig. 2, in which oxygen content (curve *A*) and combined oxygen (curve *B*) have been plotted against oxygen pressure, curve *B* being obtained from curve *A* on the above assumption that the solution dissolves oxygen to the same extent as pure water. This particular curve has been chosen partly because it represents the extreme case encountered and partly because of its importance. It further illustrates the difficulty of determining the saturation value. Saturation has obviously not been reached at the highest pressure employed and it has therefore been necessary to select a value somewhat arbitrarily. As can be seen from Table I, this has been estimated as 1.1 vol. per cent. In the remaining experiments the corresponding curves indicated that saturation was reached at lower pressures, and hence less difficulty was experienced in this respect.

The general influence of p_H on the dissociation curves can be seen from Fig. 1. Within the limits (p_H 9.65 to 7.76) examined in these experiments an increase in alkalinity produces two effects: it causes the curve to rise more steeply from the origin and to approach saturation less abruptly. As a consequence of these two effects the various curves intersect one another, and it may, in general, be assumed that a given curve will cross, in succession, every curve lying on its acid side. There will, no doubt, be a limit to this process, but its determination must await further experimental work.

Fig. 2.

Since this intersection of the dissociation curves is probably of fundamental importance for any theory of the causes underlying the inflexion of the curves and has not, as far as the authors are aware, been observed in the case of haemoglobin, it appeared desirable to obtain further evidence of the phenomenon. The following experiment was therefore carried out. Equal volumes of a dialysed haemocyanin solution were brought to different H -ion concentrations by the addition of varying amounts of 0.1087 N potassium hydroxide, the final volumes being made up to 20 cc. by the addition of distilled water. The p_H and oxygen content, when equilibrated with air (oxygen pressure 153 mm.), of each solution were then determined. The experimental figures will be found in Table II.

Table II.

Vol. of dialysed haemocyanin sol. (cc.)	15	15	15	15	15	15	15
Vol. of KOH (cc.)	0	0.5	1.5	2.1	2.7	3.3	3.9
Distilled water to (cc.)	20	20	20	20	20	20	20
p_H	7.59	7.84	8.33	8.52	8.79	9.02	9.31
Oxygen content (vols. %)	0.801	0.951	1.806	1.850	1.807	1.589	1.450
Combined oxygen (vols. %)	0.204	0.353	1.209	1.253	1.210	0.992	0.853

The results have been plotted in Fig. 3 which shows the relation between p_H and the amount of oxygen bound by equal quantities of haemocyanin, at the same concentration, when at a temperature of 23° and in equilibrium with an oxygen pressure of 153 mm. The curve shows a maximum at a p_H of about

8.60, which is in good agreement with the positions of the curves in Fig. 1; since there is no doubt that the dissociation curves of the more alkaline haemocyanin solutions are initially steeper than the remainder, this confirms the intersection of the curves. The maximum shown in Fig. 3 naturally holds only for one oxygen pressure, namely, 153 mm. With lower oxygen pressures it would obviously be displaced towards the left, *i.e.* would occur at lower H-ion concentrations, whilst with higher pressures displacement would be to the right.

Fig. 3.

Fig. 4.

The result of the two changes occurring in the shape of the dissociation curve on increasing the alkalinity of the solution is to cause the S-shape to become less pronounced. At p_H 9.65, as an inspection of Fig. 1 will show, it is no longer apparent; moreover, as far as can be determined by the methods available, the curve approaches saturation asymptotically, as is evident from curve *B* in Fig. 2. At this p_H the curve is, at any rate superficially, of the hyperbolic type which would be expected if the dissociation of oxyhaemocyanin were a unimolecular reaction obeying the law of mass action. If it be assumed that this is in fact the case and that the reaction proceeds according to the equation

then
$$\frac{[\text{HcO}_2]}{[\text{Hc}][\text{O}_2]} = K \quad \dots\dots(2),$$

where the square brackets denote concentrations and K is the equilibrium constant of the reaction. If, further, $[\text{Hc}] = y$, then $[\text{HcO}_2] = 1 - y$; moreover, assuming the validity of Henry's law, $[\text{O}_2] \propto p$, where p is the partial pressure of oxygen in equilibrium with the solution, equation 2 becomes

$$\frac{1-y}{y} = kp.$$

Taking logarithms,
$$\log \frac{1-y}{y} = \log k + \log p.$$

Hence, if $\log(1 - y)/y$ is plotted against $\log p$ a straight line inclined at an angle of 45° to the abscissa should be obtained if equation (1) correctly represents the reaction in question. Under other conditions the slope should be different. Such a curve has been drawn for the results obtained at p_H 9.65 and is reproduced in Fig. 4. The points fall satisfactorily about a straight line inclined to the axis at an angle of 45° .

In view of the relatively high p_H at which this result was obtained the suggestion arose that the disappearance of the point of inflexion and the appearance of a curve, which, if not a hyperbola, at least approximates to one, coincide with the complete conversion of the haemocyanin into its potassium salt. It had been found by the following experiment that the isoelectric point of haemocyanin (*Homarus*) is in the neighbourhood of p_H 4.8. A series of acetate-acetic acid buffers with the same concentration of sodium acetate were prepared and to 9 cc. of each buffer solution 1 cc. of dialysed serum was added. The calculated p_H of the solutions are shown below, while the formation and extent of any turbidity produced on addition of the dialysed serum are denoted by crosses beneath these figures.

p_H	4.2	4.4	4.6	4.8	5.0	5.2	5.4
	+	+	++++	+++++	+++	++	++

The value obtained from this experiment may not accurately represent the isoelectric point, but it is sufficient to show that in the dissociation curves in Fig. 1 the haemocyanin is well on the alkaline side of the isoelectric point and to support the view that at p_H 9.65 the pigment is entirely in the form of its alkali salt. To obtain further evidence on the latter point, 10 cc. of a dialysed haemocyanin solution were titrated with 0.1087 *N* KOH, the p_H of the solution being followed electrometrically during the course of the titration. The results of this electrometric titration are presented graphically in Fig. 5. The

Fig. 5.

curve resembles that of the titration curve of a weak acid and, from its course, it is clear that from about p_H 9.0 onwards practically the whole of the haemocyanin is present in the form of its alkali salt.

Comparison of haemocyanins from different species.

A few preliminary observations are recorded.

Limulus. In view of the similarity between the dissociation curves of the haemocyanin in the serum of *Limulus* and of those in other species (see Part I), the former pigment has been examined. The dialysed or undialysed serum differs so markedly in external characteristics from the corresponding sera of *Cancer*, *Maia*, *Palinurus*, or *Homarus*, that further proof that the pigments are different seems almost unnecessary. *Limulus* serum presents a turbid appearance; its colour is pale green by transmitted light and a relatively light blue by reflected light. When dialysed, the turbidity disappears but the colours undergo little change. According to the precipitation method described above in connection with haemocyanin from *Homarus*, the isoelectric point of *Limulus* haemocyanin is not less than 6.3. At a p_H of about 6.8 and in the absence of salts its affinity for oxygen at a temperature of 9° is so great that the methods employed by the authors for the determination of the oxygen capacity of *Homarus* serum are no longer applicable without modification. Under these conditions, the haemocyanin is roughly 80 % saturated at an oxygen pressure of 2 mm. Addition of acid or alkali to the dialysed serum in sufficient amount to bring the p_H to 5.5 or 8.0 respectively does not appear to produce any marked change in the steepness of the dissociation curve. Hence no conclusion can be drawn as to the shape of the initial portions of the curves. For this reason, and because the above p_H values were only determined colorimetrically and are therefore probably not very accurate, the authors are refraining from publishing the actual curves obtained. The above information is sufficient to indicate that *Limulus* haemocyanin differs profoundly from *Homarus* haemocyanin.

Homarus, *Cancer*, *Maia*, and *Palinurus*. The sera from these animals when free from pink pigments, resemble one another closely. As seen by reflected light they appear to be a deep rich blue in colour; by transmitted light they are practically colourless or faintly green. During the process of dialysis the colour by transmitted light is enormously intensified, becoming a deep green or bluish-green; simultaneously, the blue colour by reflected light undergoes considerable diminution. This immediately suggests that the reflected blue colour of the original serum is due to the Tyndall phenomenon, a suggestion which appears to be confirmed by the fact that addition of salt or acid, but not of alkali, to the dialysed serum practically restores its original appearance. As shown above, the isoelectric point of *Homarus* haemocyanin is at about p_H 4.8; using the same method, a similar value was obtained for *Cancer* haemocyanin. The approximate isoelectric points of the pigments from *Maia* and *Palinurus* have not been determined. Despite this resemblance between

the haemocyanins from these four species and the identity of the dissociation curves determined on the undialysed sera, work, which is not yet complete, on the dialysed serum of *Cancer* has given results which conclusively show that *Cancer* haemocyanin is different from *Homarus* haemocyanin. It is therefore probable that the pigments from all four species are different, although they resemble one another in many ways.

SUMMARY.

The work recorded above has shown that, between p_H 7.8 and 9.65, the shape of the dissociation curve of *Homarus* haemocyanin is modified in two directions by changes in H-ion concentration. Increase in alkalinity causes the initial portion of the curve to become steeper while the upper portion simultaneously tends to approach saturation more and more asymptotically. At p_H 9.65 the effect of these two processes is seen at its maximum; as a result, the inflexion of the curve, as far as can be determined by the methods available, disappears while the curve itself is indistinguishable from a rectangular hyperbola. Applying methods similar to those which have been employed in connection with haemoglobin it has been further shown that the behaviour of haemocyanin at p_H 9.65 is consonant with the view that the combination with oxygen follows the law of mass action as formulated for true solutions. It may be concluded from the evidence presented that at this p_H the pigment is present entirely in the form of its alkali salt; hence it would appear that at lower p_H the inflexion in the curve is in some way connected with the fact that the haemocyanin is present, not in one form (*i.e.* as alkali salt or anion), but as a mixture of different forms (*e.g.* anion and undissociated molecule). Further experimental work which, it is hoped, will contribute to the elucidation of this point, is being carried out.

Limulus haemocyanin is different from that of *Cancer*, *Homarus*, *Maia*, and *Palinurus*. The pigments from the last four species resemble one another closely in properties but are probably not identical; in the case of *Cancer* and *Homarus* they are certainly different.

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