CXII. HAEMOCYANIN.

PART V. THE OXYGEN DISSOCIATION CURVE OF HAEMOCYANIN FROM THE SNAIL (*HELIX POMATIA*) IN DIALYSED SOLUTION.

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THE determination by the authors [1927] of the isoelectric points of the haemocyanins from certain species conclusively demonstrated, in confirmation of the provisional results described in their earlier communications [1926, 1, 2], that the $[H']$ at which the affinity for oxygen of haemocyanin from the crab (*Cancer pagurus*) passed through a minimum did not coincide with the isoelectric point of the protein but was, in fact, considerably on the alkaline side of this point. At the same time, a relation was found to exist between the affinity for oxygen of the pigment from this species and the viscosity of its solutions, the p_H -affinity curve being antibatic to the p_H -viscosity curve, and the p_H corresponding with minimal affinity practically coinciding with that at which the viscosity of the solution passed through a maximum. This relation was established only in the case of haemocyanin from *Cancer*. Nevertheless, an antibasis between viscosity and affinity was also observed with haemocyanin from the lobster (*Homarus vulgaris*); the viscosity appeared, moreover, to be tending towards a maximum, and the affinity towards a minimum, at a p_H which was on the alkaline side of the isoelectric point, but which could not be reached experimentally owing to the slight solubility of the pigment at this particular acidity. It therefore seemed probable that the relation was a general one, and the authors predicted that the affinity of haemocyanin from the snail (*Helix pomatia*), the viscosity of solutions of which was shown to pass through a maximum at about p_H 8.5, would be found to be at a minimum at this acidity. Shortly after the writers had sent their paper to press, a publication by Hogben and Pinhey [1926] came to their notice in which oxygen dissociation curves of the haemocyanin from *Helix* were investigated over a p_H range of 6.8 to 9.05 using the colorimetric method of Pantin and Hogben [1925]. The resulting curves are similar to those obtained with haemocyanin from *Cancer*, and Hogben and Pinhey comment upon them as follows: "Accepting these values (*i.e.* the values of x_{50} , the oxygen partial

pressures corresponding with 50 % saturation), which are consonant with conclusions derived from four different sets of experiments, it would seem that, slight as is the effect compared with the case of crustacean haemocyanin, the haemocyanin of *Helix* exhibits the same fundamental relation to hydrogen ion concentration as the latter, namely, that the effect of increasing hydrogen ion concentration up to a certain point is to diminish the affinity for oxygen, and beyond this point to increase it. The point of minimal value for x_{50} corresponds to a p_H of 8.0 ± 0.1 . That this conclusion is justified in spite of the small order of the effect is, however, quite conclusively reinforced from another consideration. The dissociation curves obtained on the acid side of the critical p_H referred to are not of the same shape as those obtained on the alkaline side. They are flatter at the top, that is to say, they show relatively less affinity for oxygen at high tensions than the alkaline curves passing through the same points at lower tensions." The critical p_H (8.0 ± 0.1) of these authors, although not identical with the p_H (8.5) at maximal viscosity, corresponds with it very closely and hence appears to fulfil the prediction mentioned above. Despite this, however, it has been considered advisable, for a number of reasons, to examine the effect of p_H on the oxygen dissociation curve of haemocyanin from *Helix* by the gasometric method. The results, which are presented below, are not in agreement with those obtained by Hogben and Pinhey, and give no indication of a relationship between affinity and viscosity such as exists in the case of haemocyanin from *Cancer*.

EXPERIMENTAL METHODS.

The serum employed was concentrated and dialysed according to the procedure outlined in earlier communications. Adjustments in p_H were effected by addition of alkali or acid to the dialysed serum, these reagents being employed only on the alkaline or acid sides respectively of the isoelectric point of the haemocyanin in question. Where necessary, the protein was brought from the alkaline to the acid side of the isoelectric point by the method previously described [1926, 2]. All determinations of p_H were made electrometrically under the conditions used throughout this series of investigations.

Determination of the oxygen content of solutions of haemocyanin.

In the determinations of the oxygen contents of solutions of haemocyanin from *Cancer* by means of the Van Slyke apparatus [1926, 2], mercuric cyanide was used to facilitate the liberation of oxygen. With haemocyanin from *Helix*, which possesses a much greater affinity for oxygen than that from *Cancer*, especially at low pressures of this gas, the mercuric cyanide reagent by no means completely displaces the oxygen from its combination with the protein, and hence another reagent has been employed. In the choice of this reagent use has been made of the observation of Redfield, Coolidge and Hurd [1926] that potassium cyanide, when used in conjunction with the Van Slyke

apparatus, quantitatively displaces the combined oxygen from *Limulus* blood. There are, however, certain objections to the use of this reagent. Redfield, Coolidge and Montgomery [1928] have recently pointed out that a small quantity of the oxygen combined with the haemocyanin is converted by the potassium cyanide into a form which is no longer liberated on evacuation, although they have succeeded, by suitably modifying their technique, in almost completely suppressing this loss. The strongly alkaline reaction of solutions of potassium cyanide is also a disadvantage, since, under some circumstances, it would cause coagulation of the protein. It seemed probable that these difficulties might to some extent be overcome by using potassium mercuric cyanide in place of potassium cyanide. The former substance has therefore been used to displace oxygen in combination with haemocyanin in all the experiments carried out with the pigment from *Helix*. The technique employed is as follows. 5 cc. of the haemocyanin solution, brought into equilibrium with the gas mixture in the manner previously described, is drawn from an Ostwald pipette into the Van Slyke apparatus and washed in with 2 cc. of water which has been previously evacuated and displaced into the cup of the apparatus. One drop of octyl alcohol is then introduced into the pipette; after which the mercury is lowered and the gases extracted from the solution as completely as possible by shaking for 1 minute. One cc. of a gas-free 1 % solution of potassium mercuric cyanide is now added and the shaking resumed and continued for 3 minutes. At the end of this period, the extracted solution is removed from the apparatus through the trap and the gases analysed in the manner hitherto adopted in this work. The volume (1 cc.) of the 1 % solution of potassium mercuric cyanide used in each estimation has been found sufficient to prevent any recombination of the haemocyanin with oxygen, as shown by the development of blue colour on its coming into contact with the air on removal from the extraction chamber.

That potassium mercuric cyanide resembles potassium cyanide in its action in displacing oxygen from its combination with haemocyanin is doubtless due to the fact that, in its aqueous solutions, it is to some extent dissociated into mercuric and potassium cyanides. Corresponding with this, it is less efficient, in the sense that a greater concentration is required to produce the same effect, than potassium cyanide, and it is consequently uncertain if it possesses any advantages over the latter reagent. It was employed in these experiments, however, because it was thought that its efficiency, although apparently smaller than that of potassium cyanide, would, if referred to the amount of free potassium cyanide which it develops in solution, be actually greater, since the undissociated potassium mercuric cyanide would form a potential supply of potassium cyanide, and hence would resemble the latter in its action on oxyhaemocyanin without possessing the disadvantages, mentioned above, associated with the presence in the solution of moderate concentrations of free potassium cyanide. An experiment was carried out with haemocyanin from *Helix* to test the relative efficiencies of mercuric cyanide, potassium cyanide

and potassium mercuric cyanide. The solution of haemocyanin was brought into equilibrium with air at a temperature of 23° , and estimations were made of the oxygen extracted from a 5 cc. sample by the Van Slyke apparatus (1) in the absence of any cyanide reagent, (2) with the addition of 2 cc. of a saturated solution of mercuric cyanide, (3) with the addition of 0.5 cc. of a 1 % solution of potassium cyanide, and (4) with the addition of 1 cc. of a 1 % solution of potassium mercuric cyanide; it had been previously ascertained that the amounts of cyanide reagent used in the last two estimations were sufficient to decolorise the haemocyanin completely even when in contact with the atmosphere. The manometer readings, which represent the partial pressure of the oxygen in the extracted gases when the latter occupied a volume of 0.494 cc., were 103.5, 107.5, 143.5 and 146.5 mm. respectively. This experiment shows that the effect of the mercuric cyanide is of too small a magnitude for this reagent to be of assistance in connection with estimations carried out with haemocyanin from *Helix*. The values obtained with potassium cyanide and potassium mercuric cyanide agree fairly closely although a slightly greater yield of oxygen has been obtained with the latter reagent. While the difference between the results obtained in these two experiments is greater than the normal experimental error, it is impossible on the basis of this one set of estimations to claim for potassium mercuric cyanide any superiority over potassium cyanide, although such superiority is certainly suggested.

An interesting deduction may be made from the results just described. When the oxygen content of the haemocyanin solution is calculated in the usual manner from the manometer readings obtained in Exps. 1 and 4, the values of 1.266 and 1.794 are obtained respectively. The difference between these values, namely 0.528, clearly represents the vols. % of oxygen which, in Exp. 1, remained in combination with the haemocyanin under the conditions existing in the Van Slyke apparatus immediately prior to the removal of the sample. Now, the total amount of oxygen in combination with the haemocyanin when in equilibrium with air at a temperature of 23° is $(1.794 - 0.590)$ vols. %, 0.590 vols. % being the amount of oxygen physically dissolved in the solution, and it will be shown later that the haemocyanin is 97.5 % saturated under these conditions. It follows that the maximum amount of oxygen with which this particular solution is capable of combining chemically is 1.235 vols. %, and hence in Exp. 1 the haemocyanin was 42.8 % saturated when removed from the Van Slyke apparatus. The oxygen partial pressure with which this solution was in equilibrium in the pipette of the apparatus, when calculated on the lines indicated in Part II [1926, 1], is found to be about 1.5 mm., and the temperature of the apparatus was 18° . Hence, haemocyanin from *Helix* is 42.8 % saturated with oxygen when in equilibrium with a partial pressure of oxygen of 1.5 mm. and at a temperature of 18° . This figure is probably only a rough one, but it serves to illustrate the great affinity for oxygen of haemocyanin from *Helix*. At a temperature of 23° , as will be seen from Fig. 4, the haemocyanin is about 26 % saturated at the same oxygen partial pressure.

RESULTS.

Influence of haemocyanin on the solubility of oxygen in water.

One of the greatest disadvantages attached to the gasometric method for the determination of the oxygen content of solutions of haemocyanin is the uncertainty which exists regarding the influence, if any, which the haemocyanin exerts upon the solubility of oxygen in water. In Part I [1925] of this series, in which the oxygen dissociation curves of the haemocyanin in the sera of four species of decapod crustacea were determined, the amount of oxygen physically dissolved was ascertained by determining, for each specimen of serum examined, the oxygen content of samples equilibrated with increasingly greater oxygen partial pressures until proportionality was obtained between oxygen content and oxygen pressure, and then assuming the applicability of Henry's law. The solubility curves so obtained, while approximating to that for sea-water, indicated, in each case, absorption coefficients which were slightly but definitely smaller than that of sea-water. Assuming that the salt content of the blood was identical with that of sea-water, an assumption for which there is some justification, one could legitimately conclude that haemocyanin depresses the solubility of oxygen in water. When, however, the same method was employed under better conditions and with improved technique in connection with the determination of the oxygen dissociation curves of haemocyanin in salt-free solutions at different $[H']$ [1926, 1, 2], it was found that the results could be best interpreted by consistently assuming an oxygen solubility identical with that of distilled water. The haemocyanin appeared to exert no appreciable influence on the solubility of oxygen. Similar results have been obtained with haemocyanin from *Helix* in the present investigation. On the other hand, Redfield, Coolidge and Hurd [1926], using the same principle, found that the haemocyanin in *Limulus* serum depressed the oxygen solubility, although they admit that their figures "are subject to an error, which amounts to a loss of about 3 to 10 % of the oxygen present in the sample." In a recent paper Redfield, Coolidge and Montgomery [1928] have directly determined the absorption coefficient for oxygen of *Limulus* serum. The value which they obtain is smaller than that for sea-water by about 10 %, and they therefore consider that the haemocyanin in *Limulus* blood depresses the solubility of oxygen by about this amount. In the present investigation the solubility of oxygen in dialysed *Helix* serum has been determined by the method due to the above authors, with the modification that potassium mercuric cyanide has been used in place of the potassium cyanide. The cyanide reagent was evacuated in the Van Slyke apparatus before addition to the serum, and the latter was subsequently evacuated in the high vacuum produced by a rotatory oil pump. It was then equilibrated with air (bar. 761 mm.) at 23° and the oxygen content determined in the usual manner. The values

obtained in three estimations on the same sample were 0.610, 0.599 and 0.593 vols. % respectively. Calculating from the mean of these values the vols. % of oxygen dissolved by the serum at an oxygen partial pressure of 159 mm., a value of 0.615 is obtained, which is in satisfactory agreement with the value of 0.620 for distilled water. Calculations of the volume of oxygen physically dissolved by haemocyanin solutions have accordingly been made on this basis. While it is conceivable that the pigment does actually cause a slight depression in the solubility of oxygen, these results indicate that this depression is of such small magnitude that the error, if any, introduced by neglecting it is not sufficiently great to impair the conclusions finally reached. It should be pointed out that this result does not necessarily conflict with that of Redfield and his co-workers, for the haemocyanins from *Helix* and *Limulus* are different proteins and may behave differently with respect to their influence on oxygen solubility.

Fig. 1.

Influence of p_H on the oxygen dissociation curve of haemocyanin from Helix.

The oxygen dissociation curve of haemocyanin from *Helix* has been examined at a temperature of 23° and over a p_H range of from 4.04 to 9.04. The data are given in Table I. In column 4 the values for the oxygen physically dissolved in the solution have been calculated on the basis outlined above. By subtracting the figures in column 4 from the corresponding figures in column 3, the values for the combined oxygen (column 5) have been obtained. From these the percentage saturation (column 6) of the haemocyanin for each oxygen partial pressure has been calculated, using the figure 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. The significance of column 7 will be pointed out below.

These results have been plotted in Fig. 1, from which it is evident that the points lie about one smooth curve. Haemocyanin from *Helix* thus differs from the pigments from *Cancer* and *Homarus* in that, in the absence of salts, its

Table I.

p_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation	Percentage saturation (corrected)
4.04	2.89	0.436	0.011	0.425	49.1	47.9
	8.38	0.657	0.033	0.624	72.1	70.3
	11.61	0.687	0.045	0.642	74.2	72.4
	33.38	0.878	0.130	0.748	86.5	84.3
	81.84	1.147	0.319	0.828	95.7	93.3
	128.61	1.363	0.502	0.861	99.5	97.0
	153.62	1.453	0.599	0.854	98.7	96.2
	153.62	1.475	0.599	0.876	101.3	98.7
	—	—	—	0.865	100.0	97.5
4.79	2.74	0.434	0.011	0.423	41.5	40.4
	6.73	0.736	0.026	0.710	69.6	67.9
	29.34	1.032	0.115	0.917	89.9	87.7
	78.92	1.300	0.308	0.992	97.3	94.8
	129.75	1.509	0.506	1.003	98.3	95.9
	154.87	1.613	0.604	1.009	98.9	96.3
	154.87	1.633	0.604	1.029	100.9	98.2
	212.00	1.852	0.827	1.025	100.5	98.0
	—	—	—	1.020	100.0	97.5
6.25	2.85	0.729	0.010	0.719	47.9	46.7
	6.45	1.017	0.025	0.992	66.1	64.5
	11.13	1.132	0.043	1.089	72.6	70.8
	25.24	1.439	0.098	1.341	89.4	87.2
	72.60	1.724	0.283	1.441	96.1	93.7
	114.21	1.936	0.445	1.491	99.4	96.9
	151.06	2.071	0.589	1.482	98.2	96.3
	151.06	2.143	0.589	1.554	103.6	101.1
	—	—	—	1.500	100.0	97.5
6.35	3.79	0.629	0.015	0.614	49.1	47.9
	11.30	0.846	0.044	0.802	64.2	62.6
	27.65	1.161	0.108	1.053	84.2	82.1
	49.25	1.369	0.192	1.177	94.2	91.8
	102.67	1.642	0.400	1.242	99.4	96.9
	123.62	1.708	0.482	1.226	98.1	95.6
	150.48	1.837	0.587	1.250	100.0	97.5
	—	—	—	1.250	100.0	97.5
	—	—	—	1.250	100.0	97.5
7.81	2.83	0.485	0.011	0.474	40.8	39.8
	7.00	0.707	0.027	0.680	58.5	57.1
	7.96	0.780	0.031	0.749	64.5	62.8
	11.10	0.878	0.043	0.835	71.9	70.1
	41.31	1.244	0.161	1.083	93.2	90.9
	53.14	1.318	0.207	1.111	95.6	93.2
	93.51	1.527	0.365	1.162	100.0	97.5
	151.05	1.751	0.589	1.162	100.0	97.5
	—	—	—	1.162	100.0	97.5
8.74	2.90	0.450	0.011	0.439	41.6	40.6
	6.15	0.664	0.024	0.640	60.7	59.1
	10.04	0.751	0.039	0.712	67.5	65.8
	34.45	1.072	0.134	0.938	88.9	86.7
	42.88	1.135	0.167	0.968	91.8	89.5
	81.61	1.322	0.318	1.004	95.2	92.8
	124.24	1.540	0.485	1.055	100.0	97.5
	153.20	1.652	0.597	1.055	100.0	97.5
	—	—	—	1.055	100.0	97.5
9.04	2.50	0.431	0.010	0.421	38.0	37.0
	6.00	0.655	0.023	0.632	56.7	55.6
	7.51	0.735	0.029	0.706	63.7	62.1
	25.17	1.050	0.098	0.952	85.9	83.8
	45.33	1.200	0.177	1.023	92.3	90.0
	84.92	1.431	0.331	1.100	99.3	96.8
	124.12	1.620	0.484	1.136	102.5	100.0
	149.79	1.692	0.584	1.108	100.0	97.5
	—	—	—	1.108	100.0	97.5

oxygen dissociation curve undergoes no detectable change with changes in p_{H} . The curve is, moreover, different in shape from those obtained with crustacean haemocyanins: it rises steeply from the origin and exhibits no point of inflexion. Superficially, it is of the hyperbolic type which would be expected if the dissociation of the haemocyanin were a unimolecular reaction following the mass law in the form in which it is applicable to homogeneous solutions. If such were the case, and the reaction could be represented by the equation

then

$$\frac{[\text{HcyO}_2]}{[\text{Hcy}][\text{O}_2]} = K \quad \text{.....(2)}$$

in which K is the equilibrium constant of the reaction. By putting $[\text{Hcy}] = y$, $[\text{HcyO}_2]$ becomes $1 - y$; since, further, $[\text{O}_2] \propto p$, where p is the partial pressure of oxygen, equation (2) can be written in the form

$$\frac{1-y}{y} = kp \quad \text{.....(3)}$$

or,

$$\log \frac{1-y}{y} = \log k + \log p \quad \text{.....(4)}$$

from which it follows that, provided equation (1) correctly represents the reaction in question, a straight line inclined to the axes at an angle of 45°

Fig. 2.

should be obtained by plotting $\log (1 - y)/y$ against $\log p$. The values of these expressions have been calculated from the figures in Table I and then plotted in Fig. 2. With the exception of those corresponding with high oxygen partial pressures, the points fall satisfactorily about a straight line inclined to the axes at an angle of 45° . It therefore appears that the dissociation of oxyhaemocyanin is correctly represented by equation (1) and it is of interest to construct a curve fulfilling the requirements of this equation and at the same time corresponding as closely as possible with the experimentally determined values. Now, the abscissa of the point at which the straight line in Fig. 2 cuts the axis represents the value of $\log k$ in equation (4); hence, reading this value from Fig. 2, $\log k = -0.61$; consequently $k = 0.245$. Substituting this value

for k in equation (3), the value of y , and hence the percentage saturation of the haemocyanin, can be calculated for any value of p . The curve drawn in Fig. 1 is the theoretical curve which has actually been constructed by this method using the above numerical value of k , and the experimentally determined values are, considering the difficulties attached to the estimations, in excellent agreement with it. The greatest consistent divergence occurs at high oxygen pressures. This, however, is not surprising when the method employed for determining the saturation value of the haemocyanin is recalled. For this purpose, it has been assumed that the haemocyanin was saturated with oxygen when the oxygen content was, within the limits of experimental error, proportional to the oxygen partial pressure with which the solution was in equilibrium; the saturation value has then been obtained from the total oxygen content by deducting the amount of oxygen physically dissolved by the solution. Now, if the combination between the haemocyanin and the oxygen follows the law of mass action in the sense indicated above, the haemocyanin must approach saturation asymptotically, as does the theoretical curve drawn in Fig. 1, and it is clear that the increment produced in the amount of oxygen combined with the haemocyanin by increasing the oxygen partial pressure by a relatively large amount will, at the higher pressures employed in these experiments, be so small as to fall within the limits of experimental error. The discrepancy between the upper portion of the curve in Fig. 1 and the experimentally determined points could readily be accounted for in this way, quite apart from the possibility that the solubility of oxygen in the solutions is slightly smaller than has been assumed, a possibility which, if substantiated, would also tend to diminish the discrepancy mentioned above. These considerations indicate that the degree of saturation of the haemocyanin at oxygen partial pressures of from about 70 mm. upwards is more accurately expressed by the theoretical curve than by the actual values determined experimentally, and it would therefore seem that, under the conditions employed in these experiments, haemocyanin from *Helix* is about 97.5 % saturated with oxygen when in equilibrium with an oxygen partial pressure of about 150 mm., *i.e.* when in equilibrium with air. The figures given in column 6 of Table I have accordingly been re-calculated on this basis, the revised values for percentage saturation so obtained being given in the last column of the same table. Fig. 2 has been re-drawn in Fig. 3 from these revised figures, and from this a value of $k = 0.224$ has been obtained. With this new value of k the theoretical dissociation curve shown in Fig. 4 has been constructed; the revised experimental values have also been plotted in this figure.

DISCUSSION.

In view of the history of the investigation of the oxygen dissociation curve of haemoglobin there will necessarily be some hesitation in accepting the implications of the results of the foregoing experiments. Nevertheless, the authors feel that the values obtained for the percentage saturation of haemo-

cyanin from *Helix* at different oxygen partial pressures are, as an inspection of Fig. 4 will show, in such satisfactory agreement with the values calculated from equation (1) on the assumption that the law of mass action is applicable to the system under consideration, that there can be little doubt that the dissociation of the oxyhaemocyanin forms an example of a microheterogeneous

Fig. 3.

Fig. 4.

reaction which takes place according to the laws which have been developed for reactions occurring in homogeneous systems.

The theoretical curve in Fig. 4 has been deduced on the assumption that, in the formation of oxyhaemocyanin, one molecule of haemocyanin reacts with one molecule of oxygen, and, on any other simple assumption, a curve different from that shown would be obtained.

Now, Begemann [1924] has shown that the haemocyanins from *Helix pomatia* and *Carcinus maenas*, when saturated with oxygen, are in combination

with an amount of this element which corresponds to one atom of oxygen for each atom of copper contained in the haemocyanin, while, more recently, Redfield, Coolidge and Montgomery [1928] have examined the haemocyanins from a large number of species and have found that in every case the same relation exists. The work of the latter authors is of such an extensive and convincing nature that it cannot be doubted that the haemocyanins, in general, combine with oxygen in virtue of the copper which they contain and do so, moreover, in the stoichiometrical proportions indicated above. It might be mentioned here that the present authors in some unpublished experiments of a preliminary nature have themselves found that the same ratio between copper content and oxygen capacity exists in the case of haemocyanin from *Cancer pagurus*. These results, taken in conjunction with the fact, established above, that haemocyanin from *Helix* combines with oxygen according to equation (1), indicate that each molecule of the haemocyanin from this particular species contains two atoms of copper. In this connection, it is interesting to note that Redfield, Coolidge and Shotts [1928] have, from the results of their determination of the copper content of haemocyanin from *Limulus* combined with the value of the molecular weight of this protein as determined by Cohn [1925], reached an identical conclusion with respect to the pigment from this species.

The deduction of the curve drawn in Fig. 4 by application of the law of mass action to equation (1) involves the further assumption that the haemocyanin in question is molecularly dispersed in its solutions, although these are of a colloidal nature, and the correspondence between the curve so deduced and the values determined experimentally points to the truth of this assumption. This result, which, it should be emphasised, has been obtained with salt-free solutions and hence does not necessarily hold for solutions containing salts, is of some interest in a general way. Thus, it has been shown above that change in p_H produces no detectable change in the shape of the dissociation curve; it follows that the degree of dispersion of the pigment must remain constant throughout the range of acidity investigated. Nevertheless, the dialysed solutions of haemocyanin from *Helix* employed in these experiments differed markedly in appearance according to the p_H of the solution. In the region of the isoelectric point (p_H 5.3) the solutions exhibited a strong opalescence (Tyndall phenomenon) which was less marked at $[H^+]$ more removed from this point, and, in the more alkaline solutions employed, was scarcely apparent at all. It follows, since the haemocyanin was throughout in a state of molecular dispersion, that these changes were due, not to changes in the degree of aggregation of the micellae, but to changes in their state of hydration.

The most interesting aspect of these experiments is, however, the marked contrast which is shown to exist between the oxygen dissociation curve of haemocyanin from *Helix* and the curves from the crustacean haemocyanins. The behaviour of, for example, the pigment from *Cancer* differs from that of haemocyanin from *Helix* in two important respects: its affinity for oxygen depends upon the reaction of the solution, while its dissociation curve is, under

certain conditions of acidity, of an S-shape. If the shape of the oxygen dissociation curve of haemocyanin from *Helix* may be interpreted, as has been done above, as indicating that the pigment is molecularly dispersed in solution, it would appear probable that the S-shape of the dissociation curves of other haemocyanins is due primarily to aggregation of the molecules. That the molecules are present in the form of aggregates is, moreover, suggested by other considerations. Thus, if it be assumed that the degree of aggregation is n and that the aggregates are not appreciably hydrated and hence occupy a relatively small volume of the solution, then the effective concentration of the reduced haemocyanin, *i.e.* the concentration controlling the rate at which it combines with oxygen when the whole of the pigment is in the reduced form, would be y/n , where y is the actual concentration, and would remain practically constant at this value until $n - 1$ molecules in each aggregate had been converted into the oxidised form. On the other hand, the effective concentration of the oxyhaemocyanin would be identical with the actual concentration: aggregation would not influence the rate at which the oxyhaemocyanin dissociated into oxygen and the reduced pigment. It follows that the percentage saturation of the haemocyanin with oxygen would be practically proportional to the oxygen partial pressure with which the solution was in equilibrium until $n - 1$ molecules in each aggregate had been converted into oxyhaemocyanin. In other words, the initial portion of the oxygen dissociation curve should be a straight line passing through the origin. As pointed out in Part IV [1927], this is actually the case with haemocyanin from *Cancer* at acidities in the neighbourhood of the isoelectric point, and this seems to indicate the correctness of the above reasoning. At acidities more removed from, and on the alkaline side of, the isoelectric point, an S-shaped curve is obtained, which presumably results from the combined effect of aggregation and other factors such as hydration of the micellae. Why the affinity for oxygen of haemocyanin from *Cancer* should be much smaller when it is present as anion than when it is in the undissociated form, whereas that of haemocyanin from *Helix* is uninfluenced by ionisation is not at present clear. This may possibly be due to differences in the constitutions of the two proteins; one would feel inclined, however, to attribute the variation in affinity to the combined effects of aggregation and ionisation, were it not for the fact that Redfield, Coolidge and Hurd [1926] have found that haemocyanin from *Limulus* shows similar variations in affinity, although, as pointed out above, there are reasons for believing that it is molecularly dispersed in solution. It is possible that this discrepancy is due to the presence of inorganic salts, which might produce aggregation of the molecules of the protein, in the solutions examined by these authors. This possibility is supported by the fact that the oxygen dissociation curve of haemocyanin from *Limulus*, as determined by the same authors, is, in the presence of salts, of an S-shape; if the pigment were molecularly dispersed it should be of the hyperbolic type similar to that given by haemocyanin from *Helix*.

SUMMARY.

The oxygen dissociation curve of haemocyanin from the snail (*Helix pomatia*) has been determined at a temperature of 23° and at p_H values ranging from 4.04 to 9.04. No detectable change in the curve with change in p_H was observed. Analysis of the curve indicates that it is of the hyperbolic type.

The following conclusions are drawn from this result. (1) The combination between haemocyanin from *Helix* and oxygen takes place according to the equation $Hcy + O_2 = HcyO_2$. (2) Each molecule of haemocyanin contains two atoms of copper. (3) The haemocyanin is molecularly dispersed in its solutions.

The bearing of these results on the different phenomena observed in the case of other haemocyanins is discussed.

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