LXXVI. HAEMOCYANIN.

PART IV. THE DEPENDENCE OF THE SHAPE OF THE OXYGEN DISSOCIATION CURVE ON THE STATE OF IONISATION OF THE PROTEIN.

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IN the studies on the influence of p_H on the oxygen dissociation curves of the haemocyanins from *Homarus* and *Cancer* which were described in Parts II and III [Stedman and Stedman, 1926] of this investigation the shape of the dissociation curve was found to depend upon the relative amounts of pigment present as salt and in the uncombined form. The precise nature of this dependence was, however, obscure. This was largely due to the uncertainty which existed respecting the values of the isoelectric points of the pigments. The values determined by the method of optimum precipitation did not appear to correspond with the above relationship. Before further progress could be made it therefore became necessary to determine the isoelectric points by an independent method. This has now been done; the results obtained, which are in substantial agreement with those previously recorded, are presented in this communication.

This confirmation of the values obtained by the precipitation method has led to an extension of the investigation which is described below and which, the authors believe, has brought certain aspects of the influence of p_H on the dissociation curves into complete correspondence with the state of ionisation of the protein.

EXPERIMENTAL METHODS.

The serum employed was collected, concentrated and dialysed in the manner described in Part II; determinations of the oxygen capacity were made by the technique outlined in Part III; while p_H determinations were made electrometrically under the conditions described in Part II.

Determination of isoelectric point by cataphoresis. The apparatus employed for this purpose was that described by Michaelis [1925]. The dialysed serum (20 cc.) was mixed with 25 cc. of an acetic acid-sodium acetate buffer of known p_H and, in those cases where no precipitation occurred, introduced directly into the U-tube of the apparatus. When the buffer solution caused partial precipitation of the protein, the precipitate was removed by centrifuging and the supernatant liquid employed for the experiment. The large stopcocks of

the U-tube were then closed and the upper portion of the apparatus, after being thoroughly washed with distilled water, was filled with a mixture of 25 cc. of the same buffer solution with 20 cc. of water. Connection with reversible electrodes, consisting of copper wire dipping in solutions of copper sulphate, by means of small U-tubes containing potassium chloride in agar-agar was next made in the manner described by Michaelis. The subsequent operations were also made in accordance with the details given by this author. As source of E.M.F. an ordinary lighting circuit with a carbon filament lamp in series was employed. The direction of migration of the haemocyanin could be directly observed owing to the colour of this protein. The exact p_H of the protein-buffer mixture was determined electrometrically.

Viscosity measurements. The usual type of Ostwald viscosimeter was employed. The solutions for a given series of experiments were prepared in the following manner. A definite volume (usually 10 cc.) of the dialysed serum under examination was measured with a pipette into a graduated flask, to which were then added dilute hydrochloric acid or potassium hydroxide, in amount necessary to produce the desired change in p_H , and distilled water to the mark. Changes in p_H were, in general, effected by the addition of alkali only when the serum was on the alkaline side of the isoelectric point, and by acid only when on the acid side. The series of solutions thus prepared were allowed to stand for 15–20 hours in the ice-chest, from which they were removed individually as required for viscosimetric measurement. A definite volume (usually 8 cc.) of the haemocyanin solution was introduced into the viscosimeter which was then placed in a thermostat maintained at a temperature of 23°. The time of flow was determined after about 10 minutes.

RESULTS.

Isoelectric point of haemocyanin from Cancer.

The results of the cataphoresis experiments are shown in Table I.

Table I.

p_H of serum- buffer mixture	Direction of migration	Remarks
6.20	To anode	Movement rapid
4.94	"	" slow
4.81	Mainly to anode	" very slow
4.53	To cathode	No movement to anode
4.34	"	Movement fairly rapid
4.05	"	" "

The isoelectric point of haemocyanin from *Cancer* thus clearly corresponds with a p_H value of about 4.7. The point of optimum precipitation has again been determined by the method described in Part II with the same result, namely, maximum precipitation occurred in the buffer solution at p_H 4.8. On allowing the precipitate to settle, however, the supernatant liquid was found to have a p_H value of 4.88. The results of the two methods are thus in substantial agreement.

Isoelectric point of haemocyanin from Homarus.

The isoelectric point of haemocyanin from *Homarus* could not be determined very accurately by cataphoresis owing to the fact that it is almost completely insoluble in acetate buffers in the region of the isoelectric point. Thus complete precipitation occurred in buffers of p_H 4.4, 4.6 and 4.8. The results of the cataphoresis experiments actually carried out are shown in Table II.

Table II.

p_H of serum- buffer mixture	Direction of migration	Remarks
4.17	To cathode	Movement fairly rapid
4.37	Mainly to cathode	„ slow
5.47	To anode	„ „

The isoelectric point thus lies between the p_H values of 4.37 and 5.47. When redetermined by the method of optimum precipitation, the greatest precipitate formed in the buffer at p_H 4.8. The supernatant liquid had a p_H of 4.84. It may be concluded that the isoelectric point of haemocyanin from *Homarus* is at about p_H 4.7.

Isoelectric point of haemocyanin from the snail (Helix pomatia).

Haemocyanin from the snail differs from the pigments from *Homarus* and *Cancer* in being relatively soluble in the region of the isoelectric point. Its solubility is not sufficiently great to prevent its precipitation from dialysed serum on the addition of acid, but is large enough to render inapplicable in its simple form the method of optimum precipitation for the determination of its isoelectric point. On the other hand, this greater solubility facilitates cataphoresis experiments. Using this method, the isoelectric point of haemocyanin from snail has been found to lie at about p_H 5.3, as can be seen from Table III.

Table III.

p_H of serum- buffer mixture	Direction of migration	Remarks
5.56	To anode	Movement slow
5.39	Mainly to anode	„ very slow
5.16	To cathode	„ „

This value is confirmed by the viscosity experiments described below.

Influence of p_H on the viscosity of solutions of haemocyanin from Cancer.

Tables IV and V relate to experiments carried out on the alkaline and acid sides of the isoelectric point respectively.

Influence of p_H on the viscosity of solutions of haemocyanin from Homarus.

Measurements have only been carried out on the alkaline side of the isoelectric point. The experimental data are given in Table VI.

Table IV.

Vol. of dialysed serum (cc.)	Vol. of 0.110N KOH (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	6.29	60.5	1.88
10	0.3	15	6.68	65.0	2.02
10	0.6	15	7.01	67.4	2.09
10	0.9	15	7.30	70.0	2.17
10	1.2	15	7.57	70.0	2.17
10	1.5	15	7.82	68.6	2.13
10	1.8	15	7.92	66.8	2.07
10	2.1	15	8.10	65.5	2.03
10	2.4	15	8.29	62.4	1.94
10	2.7	15	8.39	60.6	1.89
10	3.0	15	8.49	59.8	1.86
10	3.3	15	8.49	60.0	1.86
10	3.6	15	8.75	62.6	1.94

Time of flow for water = 32.2.

Table V.

Vol. of dialysed serum (cc.)	Vol. of 0.080N HCl (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	4.39	48.4	1.50
10	0.3	15	4.05	48.8	1.52
10	0.6	15	4.01	49.6	1.54
10	0.9	15	3.97	51.0	1.55
10	1.2	15	4.01	52.6	1.63
10	1.5	15	4.00	54.6	1.70
10	1.8	15	3.94	57.2	1.78
10	2.1	15	3.90	60.8	1.89
10	2.4	15	3.87	65.6	2.04

Time of flow for water = 32.2.

Table VI.

Vol. of dialysed serum (cc.)	Vol. of 0.110N KOH (cc.)	Water to (cc.)	p_H	Time of flow (secs.)	Relative viscosity (water = 1)
10	0	15	7.59	61.2	1.90
10	0.3	15	7.95	58.7	1.82
10	0.6	15	8.22	56.7	1.76
10	0.9	15	8.42	54.8	1.70
10	1.2	15	8.69	53.6	1.66
10	1.5	15	8.84	51.6	1.60
10	1.8	15	9.04	50.6	1.57
10	2.1	15	9.26	50.2	1.56
10	2.4	15	9.58	51.4	1.60
10	2.7	15	9.88	52.4	1.63
10	3.0	15	10.07	58.2	1.81

Time of flow for water = 32.2.

*Influence of p_H on the viscosity of solutions of haemocyanin from snail
(Helix pomatia).*

The difficulty experienced in dissolving precipitated haemocyanin from *Cancer* in acid commented upon in Part III was not encountered in the case of haemocyanin from snail, presumably owing to its greater solubility and possibly, also, to its greater stability in acid solution. It was thus possible

to carry out viscosity measurements on both sides of the isoelectric point with the same sample of serum. These measurements are recorded in Table VII. In the first half of the Table the p_H of the dialysed serum was changed by the addition of alkali and in the second half by the addition of acid.

Table VII.

Vol. of dialysed serum (cc.)	Vol. of 0.110 <i>N</i> KOH or 0.098 <i>N</i> HCl		Water to (cc.)	<i>p</i> _H	Time of flow (secs.)	Relative viscosity (water = 1)
	(cc.)	(cc.)				
10	1.5 (alkali)	15	10.53	80.0	2.39	
10	1.2	15	9.33	64.8	1.99	
10	0.9	15	8.88	67.4	2.01	
10	0.6	15	8.70	68.0	2.03	
10	0.3	15	8.03	54.7	1.63	
10	0	15	7.46	47.2	1.41	
10	0.6 (acid)	15	4.76	41.8	1.25	
10	0.9	15	4.27	47.8	1.43	
10	1.2	15	3.98	55.4	1.65	
10	1.5	15	3.55	64.3	1.92	

Time of flow for water = 33.5.

Determination of the p_H at which the affinity of haemocyanin from Cancer for oxygen is at a minimum.

The variations which occur in the affinity of haemocyanin from *Cancer* for oxygen were discussed in Part III. It became necessary in connection with the viscosity experiments described above to determine as accurately as possible the p_H at which there is minimal affinity. For this purpose advantage was taken of the fact demonstrated in Part III that this particular haemocyanin is far from completely saturated with oxygen when in equilibrium with air at a temperature of 23° and at a p_H in the neighbourhood of neutrality, and a series of experiments was carried out similar to those made in Part II in another connection and illustrated in Fig. 3 of that paper. The data, which are self-explanatory, are given in Table VIII.

Table VIII.

Vol. of dialysed haemocyanin sol (cc.)	15	15	15	15	15
Vol. of 0.110N KOH (cc.)	...	0	0	0.3	1.0
Vol. of 0.008N HCl (cc.)	...	3.0	1.5	0	0
Distilled water to (cc.)	...	20	20	20	20
p_H	...	6.82	7.02	7.22	7.46
Oxygen content (vols. %)	...	1.336	1.256	1.212	1.262
Combined oxygen (vols. %)	...	0.747	0.667	0.623	0.673
					0.921

At an oxygen pressure corresponding with that in the atmosphere (151 mm. in the experiments recorded in Table VIII) and a temperature of 23° haemocyanin from *Cancer* thus has a minimal affinity for oxygen when at a p_H of about 7.3.

It is convenient to point out here that the expression "minimal affinity" is somewhat ambiguous. It was shown in Parts II and III that certain of the oxygen dissociation curves determined at different $[H^+]$ intersected. Obviously,

when two such curves intersect, one cannot state at which acidity the affinity of the haemocyanin is the greater unless one defines the oxygen pressure at which the affinities are being compared. In the case of the pigments from *Cancer* and *Homarus* this difficulty disappears at $[H']$ in the neighbourhood of neutrality, as will be evident from an inspection of the curves referred to above. When in the following pages affinities at different $[H']$ are compared without reference to the oxygen pressure under consideration, it must be understood that the comparison is limited to the initial portions of the curves, *i.e.* if two curves intersect the comparison is limited to oxygen pressures lower than that at which the intersection occurs.

DISCUSSION.

The experiments described above render necessary a re-examination of the dissociation curves determined in Parts II and III. Of these, the series of curves illustrated in Figs. 1 and 2 of Part III, which relate to the haemocyanin from *Cancer*, extend over the greater p_H range and will therefore be considered in rather more detail.

It was pointed out in Part III that there were indications that the haemocyanin employed for the curve at p_H 3.84, which was the most acid solution examined, had been partly converted into a form which no longer combined reversibly with oxygen. That some drastic change in, or decomposition of, the pigment takes place at or near an acidity corresponding with this p_H receives definite confirmation from the p_H -viscosity experiments recorded above in Table V. Whilst addition of acid to the dialysed serum at p_H 4.39 produced continuous increases in its viscosity, no such change occurred in the p_H . Thus, the addition of the first 0.3 cc. of acid caused the p_H to decrease to 4.05, but the further addition of 2.1 cc. scarcely produced any appreciable effect. Evidently some peculiar change occurs in the protein at a p_H of about 4.0. Changes in the external characteristics of the solution accompany this change. Thus, the initial solution (p_H 4.39) employed for the experiments recorded in Table V exhibited a marked blue opalescence (Tyndall phenomenon) which diminished progressively with increasing content of acid, giving place in the mixture at p_H 3.87 to a clear greenish-yellow solution. The intervening mixtures had a greenish appearance which was the more pronounced the greater the blue opalescence. One is forced to the conclusion that the loss in opalescence is indicative of a loss of power to combine reversibly with oxygen, a conclusion which is reinforced by the observation, made during the p_H determinations, that the opalescence disappears entirely when hydrogen is bubbled through the solutions whereas the greenish-yellow colour is not affected by this treatment. Since, further, the loss of opalescence following the addition of acid takes place slowly, it is clear that the dissociation curve at p_H 3.84 may be subject to errors arising from this cause. Such a possibility was tacitly suggested in Part III, and in view of the above results the position of the curve must be regarded as doubtful. There then remain in the immediate

neighbourhood of the isoelectric point, which has now been definitely shown to lie at about p_H 4.7 (see Table I), two curves, one on the acid side (p_H 4.19) and one on the alkaline side (p_H 5.60). The former is steeper than the latter, but in view of the fact that the p_H interval between it and the isoelectric point is the smaller, this is not inconsistent with the occurrence of a maximal affinity of the pigment for oxygen at the isoelectric point. Unfortunately it is doubtful, owing to the insolubility of the pigment in the zone surrounding the isoelectric point and to the above-mentioned change which occurs at p_H 4, whether the existence of such a maximal affinity could be directly demonstrated. Nevertheless, the probability that it does exist seems very great, although the occurrence of a maximal affinity on the acid side of, and not at, the isoelectric point would not be inconsistent with the results discussed below.

Fig. 1.

The variations in affinity which occur on the alkaline side of the isoelectric point must now be considered in connection with the viscosity determinations. For this purpose the results given in Tables IV and VIII have been plotted in Fig. 1. It can be seen that not only does the decrease in affinity which takes place as one recedes from the isoelectric point correspond, as was to be expected, with an increase in viscosity, but, what is more significant, the p_H (7.3) at which the affinity is at a minimum coincides with that at which the viscosity is at a maximum. Further, if one examines the dissociation curves illustrated in Part III it is evident that the curves (Fig. 2) determined at p_H 's more acid than 7.3 are not the exact counterpart of those (Fig. 1) determined at more alkaline p_H 's. On the contrary, while the latter are definitely of the familiar S-shape, the former do not show a marked point of inflexion. In fact, the first few points of the curves at p_H 4.19, 5.60 and 6.21 lie on straight lines passing through the origin. Whilst the possibility of a slight inflexion in the curves, such as that actually illustrated, cannot be definitely excluded it would seem that between the isoelectric point and the point of maximal

viscosity the amount of oxygen taken up in combination is, within the limits of experimental error, initially proportional to the oxygen pressure.

The viscosity experiments (Table VI) on haemocyanin from *Homarus*, the results of which are plotted in Fig. 2, are in conformity with the above relationship. Owing, however, to the more extensive zone of insolubility exhibited by this pigment in the region of the isoelectric point the authors have not hitherto succeeded in demonstrating the occurrence of either a point of maximal viscosity or one of minimal affinity. Nevertheless, it is clear from Fig. 1 in Part II that if a point of minimal affinity exist it will occur at a p_H of less than 7.76. By analogy with other proteins it is also evident from Fig. 2 that a point of maximal viscosity will likewise occur at a p_H somewhat lower than 7.59. In view of the results obtained with haemocyanin from *Cancer* there seems little doubt that these two points will coincide. One further point merits mention in connection with *Homarus*. It will be recalled that at p_H 9.65 the combination with oxygen of the pigment from this species follows closely, if

Fig. 2.

not completely, the laws applicable to homogeneous solutions. Reference to Fig. 2 shows that at this p_H the viscosity lies on the alkaline side of a minimum in the p_H -viscosity curve. The increase in viscosity which is taking place evidently represents the commencement of those processes which finally produce, in more alkaline solutions, gelation.

Although the determinations of the viscosity of solutions of haemocyanin from snail (Table VII) were made primarily with the object of confirming the position of the isoelectric point, an inspection of Fig. 3, in which the results are plotted, shows that a maximal viscosity occurs on the alkaline side of the isoelectric point at a p_H of about 8.5. It is thus possible to predict that the affinity of this haemocyanin will be at a minimum in the neighbourhood of this p_H .

The examination of further respiratory pigments will doubtless reveal relationships between viscosity and affinity similar to that demonstrated in this communication. Whilst it is to be expected that the relationship will be

of the same precise nature, it can be foreseen that it will be less marked with certain pigments. Loeb [1922] has shown that the viscosity of solutions of proteins, such as egg-albumin, which are readily crystallisable, is influenced by changes in p_H to a smaller degree than, for example, that of a gelatin solution. Whilst, as shown by Pauli and his co-workers [1925, 1926], changes of the same nature do actually occur with both types of protein, it is evident from Loeb's work that they are less pronounced in the case of the crystallisable proteins. In other words, the more readily a protein can be crystallised the less marked are its colloidal properties. The haemocyanins from *Cancer* and *Homarus* are examples of proteins with pronounced colloidal properties. As a result, the influence of p_H on both the viscosity and the power of combination with oxygen of these proteins is very great. The haemoglobins are less colloidal in nature; hence the relationship between viscosity and oxygen-combining power will not be so evident although it should be no less precise than in the case of the haemocyanins.

Fig. 3.

The ultimate bearing of the relationship discussed above on the mechanism of the combination between oxygen and respiratory pigments cannot yet be foreseen, but certain factors appear to be quite evident. Maximal affinity for oxygen probably corresponds with minimal ionisation and minimal viscosity, *i.e.* occurs at the isoelectric point; the alternative view that the point of maximal affinity occurs on the acid side of the isoelectric point and corresponds with maximal ionisation of the protein as cation cannot, however, be entirely excluded. As stated above, it is unfortunately scarcely possible to distinguish between these two possibilities in the case of haemocyanin from *Cancer* owing to the limits of stability of this pigment. According to Pauli [1926], maximal viscosity in proteins indicates maximal ionisation; hence, since in the case of certain haemocyanins the former corresponds with minimal affinity, it would appear that the greater the degree of ionisation (as anion) of the pigment the smaller is its affinity for oxygen. As the ionisation is repressed by increased alkalinity of the solution the affinity again increases. Finally, at $[H']$ at which

the hydration processes which ultimately result in gelation have commenced the combination with oxygen takes place according to the laws for true solutions. So much seems clear from the combined experiments on *Cancer* and *Homarus* haemocyanins. Why the affinity should vary with the ionisation is not so clear, nor is it apparent why the curves on the acid and alkaline sides of the point of maximal viscosity should be of such a markedly divergent character. One factor which must certainly be taken into account in this connection is the hydration of the micellae. According to Pauli's views the point of maximal viscosity corresponds with maximal hydration of the micellae, due to the greater affinity of the ions for water, from which one might infer that the affinity for oxygen is influenced primarily by hydration and is only affected by ionisation in so far as this influences the hydration of the micellae. But the question arises: is the viscosity curve an accurate indicator of the state of hydration of the micellae? According to Smoluchowski [see Freundlich, 1926] the presence of a charge on the micellae of a sol produces an increase in the viscosity of the sol. Since maximal viscosity in a protein sol corresponds with maximal ionisation, *i.e.* with maximal charge on the micellae, it is conceivable, indeed probable, that the variation in viscosity is conditioned largely, if not mainly, by the variation in the charge on the micellae and not by variation in the state of hydration. On this basis it would be necessary to assume that the hydration of the micellae increases uniformly, or at any rate without passing through a maximum, as one recedes from the isoelectric point until finally the sol undergoes gelation. The affinity would then be directly controlled in, it must be admitted, an unknown manner by the magnitude and perhaps also the sign of the charge on the micellae, whilst the actual contour of the curve would be determined by the degree of hydration of the micellae. (The sign of the charge could only be considered of influence if it were shown that maximal affinity occurred on the acid side of the isoelectric point and corresponded with maximal ionisation of the protein as cation; such a result would be of great theoretical interest, but its consideration must be postponed until it has been found possible to distinguish experimentally between the two possibilities.) This would account for the different shapes of the curves on the two sides of the point of maximal viscosity. It would further account for the observation that with increasing alkalinity the curve approximates more and more closely to the hyperbolic type, for it is recognised that a protein gel resembles in many of its properties a true solution much more nearly than does a protein sol; this is presumably due to the facts that the micellae are highly hydrated and that the volume occupied by the intermicellar liquid is negligible in comparison with that occupied by the micellae.

SUMMARY.

The isoelectric points of the haemocyanins from *Cancer* and *Homarus* are at p_H 4.7; that of haemocyanin from the snail (*Helix pomatia*) is at p_H 5.3.

The viscosity of solutions of haemocyanin from *Cancer* passes through a

maximum at p_H 7.3; at the same p_H the affinity of the pigment for oxygen passes through a minimum. Viscosity determinations with solutions of haemocyanin from *Homarus* suggest that a similar relationship holds in the case of this pigment.

The above results taken in conjunction with the shape of the oxygen dissociation curves determined at different p_H lead to the following conclusions. The affinity of the pigment for oxygen is probably at a maximum at the isoelectric point; alternatively, maximal affinity occurs on the acid side of the isoelectric point at a p_H corresponding with maximal ionisation of the protein as cation. It is not at present possible to differentiate between these two possibilities owing to an abnormal change which haemocyanin from *Cancer* undergoes at p_H 4. As the pigment ionises as anion its affinity for oxygen decreases, passes through a minimum at a p_H at which ionisation is at a maximum, and increases again as the ionisation is repressed by the presence of higher concentrations of alkali. Finally, as the p_H approaches more and more closely to that at which gelation occurs, the dissociation curve approximates more and more closely to the type to be expected if the combination obeyed the law of mass action. The shape of the curve is controlled by two factors: the charge on the micellae, and their state of hydration.

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