CXVI. HAEMOCYANIN.

PART III. THE INFLUENCE OF HYDROGEN ION CONCENTRATION ON THE DISSOCIATION CURVE OF THE OXYHAEMOCYANIN FROM THE BLOOD OF THE EDIBLE CRAB (*CANCER PAGURUS*).

BY ELLEN STEDMAN AND EDGAR STEDMAN.

From the Department of Medical Chemistry, University of Edinburgh.

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THE experiments on the effect of p_H on the oxygen dissociation curve of the haemocyanin from *Homarus* described in the preceding paper have now been extended to the pigment from *Cancer*.

EXPERIMENTAL METHODS.

The technique employed was essentially the same as that used in Part II and need not, therefore, be described in detail. The following modifications, or additions have, however, been introduced.

Preparation of solutions of haemocyanin. The experiments on *Homarus* were confined to $[H^+]$ on the alkaline side of the isoelectric point, the more acid solutions of the pigment being obtained by dialysing the serum for a sufficient length of time against distilled water. In the present series of experiments on the haemocyanin from *Cancer*, it was desired to extend the observations to solutions on the acid side of the isoelectric point as well as to those which approached this point more closely on the alkaline side. As previously recorded, the isoelectric point of haemocyanin from *Cancer* is, according to the method of optimum precipitation, at about p_H 4.8. To approach or pass through this point, addition of acid is obviously necessary. Trial experiments showed, however, that the precipitate of haemocyanin formed by addition of acid could not be re-dissolved without such dilution of the solution as to render it useless for the experiments. The following method, which proved extremely effective, was therefore adopted. The concentrated serum was dialysed in closed collodion membranes against distilled water until free from salts. The distilled water was then replaced by an acid solution of suitable strength. With very dilute acids the p_H of the dialysed serum was reduced without the occurrence of precipitation. With more concentrated acids, the haemocyanin was precipitated as a coherent mass which filled the collodion membrane.

By allowing dialysis against the acid to proceed, this mass slowly re-dissolved and a clear solution was finally obtained. Excessive dilution of the solution and denaturation of the protein were thus obviated; further, any salts which might be formed during the process would be removed by dialysis.

In studying the effect of p_H on the dissociation curves, it has been the authors' aim in the present experiments, as well as in those of Part II, to work with solutions as free from salt as possible. In conformity with this aim, the addition of salts (*e.g.* in the form of buffer solutions) to the dialysed serum has been avoided; when adjustments in p_H have been necessary after removal of the solutions from the collodion sacs, these have, in general, been made by the addition of alkali when the solution was on the alkaline side of the isoelectric point and of acid when on the acid side. In only one instance has this procedure been departed from, namely, in the case of the curve at p_H 5.60 (see Fig. 2). The solution employed for the series of estimations represented by this curve was brought from about p_H 6.0 to 5.60 by the addition of a small amount of acid, although the solution was on the alkaline side of the isoelectric point. It is thought, however, that any salts which may possibly have been formed in this process would be so small in amount that any influence which they might exert on the shape of the dissociation curve would be negligible.

Determination of the oxygen content of solutions. The dissociation curves of haemocyanin from *Cancer* on the extreme acid and alkaline sides of the isoelectric point are so steep that the Van Slyke technique as hitherto employed by the authors is inapplicable without modification to facilitate the extraction of the oxygen. For information leading to a solution of this problem the authors are indebted to Mr A. C. Redfield, who informed them in a private communication that potassium cyanide completely displaces the oxygen from the oxyhaemocyanin from *Limulus*. This reagent also acts similarly towards oxyhaemocyanin from *Cancer*, but unfortunately it cannot be employed for the determination of those curves where the use of such a reagent is essential owing to the fact that its solution is so strongly alkaline that it would cause the precipitation of the protein in the pipette of the apparatus. On the assumption that the cyanogen ions were responsible for the displacement of the oxygen, it appeared that another cyanide would be equally efficient. The only soluble cyanide available which does not suffer from the same disadvantages as potassium cyanide is, however, mercuric cyanide, and this is practically unionised. Nevertheless, it was thought that solutions of this salt might contain sufficient cyanogen ions to displace the oxygen from the oxyhaemocyanin, while the concentration of mercuric ions would be so small as to be without effect on the protein. Some tests which were carried out showed that the amount of oxygen extracted from alkaline haemocyanin solutions was, in fact, greater in the presence than in the absence of mercuric cyanide. That mercuric cyanide is less efficient than either potassium cyanide or hydrocyanic acid in this respect is, however, shown by the fact that whereas the two latter reagents completely decolorise haemocyanin when in contact with air, mer-

curic cyanide does not do so although it causes a considerable diminution in the intensity of the colour. These results suggest that one is dealing with two reversible reactions in which cyanogen ions and oxygen are competing for the haemocyanin. If this is so, reduction in the concentration of the oxygen would favour the combination of the haemocyanin with the cyanogen ions and, at the extremely low oxygen pressures existing in the pipette of the Van Slyke apparatus, one would expect the combination with the cyanogen ions to be complete. On the assumption that these views are correct, mercuric cyanide has been employed to facilitate the extraction of the oxygen in the following way. About 7 cc. of a saturated solution of mercuric cyanide are introduced into the Van Slyke apparatus, completely extracted, and displaced into the cup of the apparatus. The sample of haemocyanin to be extracted is then introduced into the pipette and washed in with 3 cc. of the mercuric cyanide solution. The remainder of the estimation is then carried out in the manner previously described. In order to assist the action of the cyanide reagent to the greatest possible degree, the oxygen pressure in the apparatus has been reduced to the minimum consistent with accuracy by varying the volume of the sample according to the oxygen pressure with which it had been equilibrated. Thus, with low oxygen contents 5 cc. samples were employed, while 3 cc. and 2 cc. samples were used with solutions of medium and high oxygen content respectively. Although it has been demonstrated that the mercuric cyanide reagent does facilitate the extraction of the oxygen, the real justification for its use lies in the fact that the values for physically dissolved oxygen obtained from the different series of experiments are all in approximate agreement with the value for pure water.

In calculating the volumes per cent. of oxygen contained in the haemocyanin solutions the complete equation, including the solubility factor, of Van Slyke and Neill [1924] has been employed; the use of the mercuric cyanide has rendered unnecessary and inapplicable the method of graphical correction outlined in Part II.

RESULTS.

Table I contains the data derived from eight series of experiments on haemocyanin solutions from *Cancer*. All samples were equilibrated with the gas mixtures at a temperature of 23°.

Table I.

p_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9.52	3.82	0.292	0.015	0.277	14.4
	7.38	0.610	0.029	0.581	30.2
	13.47	1.104	0.053	1.051	54.7
	22.11	1.421	0.086	1.335	69.6
	79.99	1.958	0.312	1.646	85.7
	151.75	2.378	0.592	1.786	93.0
	256.44	2.864	1.000	1.864	97.2
	375.83	3.375	1.466	1.909	99.4
				1.920	100.0

Table I (continued)

P_H	Oxygen pressure (mm.)	Oxygen content (vols. %)	Physically dissolved oxygen (vols. %)	Combined oxygen (vols. %)	Percentage saturation
9.25	4.05	0.315	0.016	0.299	12.2
	7.74	0.801	0.030	0.771	32.8
	23.29	1.999	0.091	1.908	81.2
	89.35	2.536	0.348	2.188	89.5
	155.94	2.840	0.607	2.233	95.0
	276.32	3.424	1.078	2.346	100.0
	364.41	3.786	1.421	2.365	100.7
	476.68	4.207	1.859	2.348	100.0
9.17				2.347	100.0
	4.37	0.153	0.017	0.136	6.8
	7.78	0.417	0.030	0.387	19.3
	15.17	1.394	0.059	1.335	66.7
	17.08	1.580	0.067	1.513	75.6
	27.27	1.908	0.106	1.817	90.8
	153.64	2.542	0.599	1.968	98.4
	432.62	3.626	1.687	1.984	99.2
7.35	699.06	4.655	2.726	2.009	100.4
				2.000	100.0
	64.99	0.545	0.253	0.292	13.3
	99.16	1.066	0.387	0.679	31.0
	152.38	1.623	0.594	1.029	46.8
	230.82	2.732	0.900	1.832	83.6
	256.56	2.896	1.001	1.895	86.4
	435.49	3.889	1.698	2.191	99.8
6.21	599.18	4.531	2.337	2.194	100.0
				2.194	100.0
	33.69	0.467	0.131	0.336	16.1
	69.95	0.995	0.273	0.722	34.7
	89.55	1.309	0.349	0.960	46.2
	153.85	2.224	0.600	1.624	78.1
	257.78	2.866	1.005	1.861	89.5
	519.60	4.025	2.026	1.999	96.1
5.60	591.46	4.418	2.307	2.111	101.5
	715.75	4.864	2.791	2.073	99.6
				2.080	100.0
	12.09	0.347	0.047	0.300	17.4
	31.81	0.960	0.124	0.836	48.5
	56.22	1.467	0.219	1.248	72.3
	126.08	2.089	0.492	1.597	92.5
	153.64	2.213	0.599	1.614	93.6
4.19	280.37	2.817	1.093	1.724	99.9
	384.31	3.205	1.479	1.726	100.0
				1.726	100.0
	4.03	0.242	0.016	0.226	11.3
	8.49	0.583	0.033	0.550	27.5
	10.17	0.701	0.040	0.661	33.0
	35.91	1.692	0.140	1.552	77.6
	82.96	2.067	0.324	1.743	87.1
3.84	122.06	2.429	0.476	1.953	97.6
	155.52	2.514	0.607	1.907	95.3
	242.26	2.928	0.945	1.983	99.1
	337.46	3.356	1.316	2.040	102.0
	469.17	3.787	1.830	1.957	97.8
				2.000	100.0
	3.01	0.195	0.012	0.183	24.1
	7.81	0.403	0.030	0.373	49.1
3.84	12.72	0.549	0.050	0.499	65.7
	55.53	0.830	0.217	0.613	80.7
	119.22	1.167	0.465	0.702	92.4
	151.54	1.333	0.591	0.742	97.6
	252.04	1.733	0.983	0.750	98.7
				0.760	100.0

The columns in Table I correspond with those in Table I of Part II except that, for reasons stated above, it has been unnecessary to introduce any correction to be added to the experimentally determined values. It should be stated, however, that in the experiments carried out at p_H 9.17 no mercuric cyanide was employed. In this case a small graphical correction has, therefore, been made and incorporated in the figures representing oxygen content. The physically dissolved oxygen has again been calculated on the assumption that at an oxygen pressure of 159 mm. and a temperature of 23° the haemocyanin solutions dissolve 0.62 volumes per cent. of oxygen. From the data in Table I eight dissociation curves have been plotted and are reproduced in Figs. 1 and 2.

Fig. 1.

Fig. 2.

DISCUSSION.

The four dissociation curves obtained with the more alkaline solutions and shown in Fig 1 cover a p_H range approximating to that investigated in the case of *Homarus*. A comparison of these curves with those in Fig. 1 of Part II of this series indicates that the general influence of p_H on the dissociation curves of the pigments from the two species is the same. The intersection of the curves commented upon in connection with haemocyanin from *Homarus* can again be observed in Fig. 1, indicating that the more alkaline the solution the more does the curve approximate to the hyperbola which would be expected if the combination of the haemocyanin with oxygen followed the laws applicable to homogeneous solutions. The most alkaline solution (p_H 9.52) investigated, however, is not quite of the hyperbolic type, and an attempt to determine the curve for a more alkaline solution (p_H 9.95) failed owing to the haemocyanin slowly undergoing gelation. Incidentally, it should be noted that even in the form of a gel the pigment was still able to combine reversibly with oxygen. Despite this failure to realise the conditions necessary to give a hyperbolic curve, it seems clear from the general trend of the curves in Fig. 1 that if one could increase the alkalinity of the solution above p_H 9.52 and still retain the haemocyanin in the form of a sol such a curve would finally be obtained.

One further point emerges from a comparison of the curves from *Cancer* with those from *Homarus*. Although changes in p_H produce the same general effect, it is clear that the affinity of *Cancer* haemocyanin for oxygen as expressed by the steepness of the dissociation curves, is, within the p_H range investigated, greater than that of *Homarus* haemocyanin at the same p_H . This forms the experimental proof for the statement made in Part II that the pigments from the two species are not identical.

The curves obtained with the more acid solutions are shown in Fig. 2. It is at once evident that they form the counterpart of the curves in Fig. 1. The intersection again appears although, probably owing to the greater p_H interval between the successive curves, not in such a pronounced form. Just as increase in alkalinity causes the curves in Fig. 1 to approximate more and more closely to the hyperbolic form so, in Fig. 2, increase in acidity produces the same effect. The points from which the curve at p_H 3.84 has been drawn, when tested by the method employed in Part II, appear, within the limits of experimental error, to correspond with the combination with oxygen following the law of mass action. Unfortunately, the oxygen content of the solution employed in this series of experiments was relatively low, suggesting that during the addition of acid some of the pigment was converted into a form which no longer yielded oxygen to a vacuum. At a somewhat greater acidity the haemocyanin loses its capacity for combining reversibly with oxygen although it retains a light green colour.

From the foregoing it is obvious that the affinity of haemocyanin for

oxygen passes through a minimum at a certain p_H . This minimum appears, from the experiments here recorded, to occur in the neighbourhood of the neutral point. That such a minimum exists has also been recently shown by Hogben [1926] using a colorimetric method based upon that of Redfield and Hurd [1925]. Its significance is not, however, clear. Hogben appears to reject the view that it corresponds with the isoelectric point and to prefer the explanation that it is due to the pigment existing in tautomeric forms. The experiments described above, as well as those in Part II, suggest that the shape of the dissociation curve is controlled, in some manner as yet undetermined, by the relative amounts of pigment present in the form of salt (either with acid or base) and in the uncombined form. The greater the proportion of salt the greater is the affinity of the haemocyanin for oxygen and the more nearly does the dissociation curve assume a hyperbolic form. From this behaviour a minimum affinity for oxygen would be expected at the isoelectric point. The chief difficulty in the way of this interpretation is the fact that the isoelectric point, assuming that it coincides with optimum precipitation, appears to be at about p_H 4.8, while the minimum affinity occurs at about p_H 7. According to Michaelis [1922], however, the point of optimum precipitation only coincides with the isoelectric point provided that any salt formed remains completely ionised. This question cannot, therefore, be decided until the isoelectric point has been determined by another method.

There is one further point to which attention should be directed. It is clear both from the experiments on *Cancer* and *Homarus* haemocyanin that the pigment is, under the conditions employed, by no means completely saturated with oxygen until oxygen pressures considerably greater than that due to the atmosphere are reached. Thus, at p_H 7.35 and a temperature of 23° *Cancer* haemocyanin is only approximately 50 % saturated when in equilibrium with an oxygen partial pressure corresponding with that in the atmosphere. This result is not without significance for methods of determining dissociation curves based upon colorimetry. Although the colorimetric method might be expected to give accurate results and possesses the advantage of being rapid and simple, it is evident that curves obtained by this method under different conditions of acidity will not be comparable unless the standards are, in each case, maintained at the same degree of saturation. This condition will not be fulfilled in the case of the haemocyanin from *Cancer* and *Homarus* if the standards are in equilibrium with air and the temperature is as high as 23°. It is improbable that they were fulfilled at the slightly lower temperature of 18.7° employed by Hogben in the case of *Cancer* serum, unless, indeed, the salts added in the form of buffer mixtures as well as those present in the serum itself profoundly modified the shape of the curves. Whilst the colorimetric method is undoubtedly capable of indicating the general influence of p_H on a particular haemocyanin, the results so obtained can have no quantitative significance unless care is taken to avoid the sources of error indicated above.

SUMMARY.

The oxygen dissociation curves of haemocyanin from *Cancer* have been determined at p_H values ranging from 3.84 to 9.52. The affinity of the haemocyanin for oxygen, as expressed by the steepness of the dissociation curve, is at a minimum in the neighbourhood of neutrality. As the acidity or alkalinity of the solution is increased the curve approaches more and more closely to the hyperbolic form which would be expected if the combination with oxygen followed the law of mass action as formulated for homogeneous solutions. These results suggest that the shape of the curve is controlled by the relative amounts of pigment present in the form of salt and in the uncombined state.

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