LXXXI. HAEMOCYANIN. PART I.

THE DISSOCIATION CURVES OF THE OXYHAEMOCYANIN IN THE BLOOD OF SOME DECAPOD CRUSTACEA.

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THE blue colour of the blood of certain arthropods and molluscs is due to the presence of a protein, containing copper, which exists in oxidised and reduced forms; the former, *oxyhaemocyanin*, has a blue colour, whilst the latter, *haemocyanin*, is colourless. The transformation of the oxidised into the reduced form is readily accomplished by exposing blood containing oxyhaemocyanin to a vacuum or by saturating it with an inert gas; the reverse change takes place spontaneously on again bringing the blood into contact with air. Oxyhaemocyanin has been isolated in crystalline condition from the blood of certain species. [For bibliography see Dhéré, 1915.]

The properties of haemocyanin immediately suggest that this pigment exercises respiratory functions analogous to those of haemoglobin. This view has, in fact, been almost generally accepted and has received support from determinations, by various authors, of the oxygen capacities of the bloods of a number of marine invertebrates. In the case of those species which contain haemocyanin, the values obtained, although differing widely from species to species and even from individual to individual of the same species, were considerably higher than the oxygen capacity of the sea-water in which the animals lived. With those species containing no haemocyanin, on the other hand, the values approximated to that of sea-water. This is illustrated by the following figures, which are quoted from Winterstein [1909].

Containing haemocyanin			Not containing haemocyanin		
<i>Octopus</i>	<i>Palinurus</i>	<i>Maia</i>	<i>Ascidia</i>	<i>Pinna</i>	<i>Patella</i>
4.2-5.0	1.48-1.43	1.13-0.84	0.48-0.38	0.57	0.68 vols. %

Alsberg and Clark [1914], however, state that the blood of *Limulus*, when exposed to a vacuum, yields an amount of oxygen which is not significantly greater than that obtained under the same conditions from sea-water. Whilst not denying that haemocyanin possesses the power of combining with oxygen, these authors conclude from their results that this pigment can only function

as a carrier of oxygen by a mechanism other than that of the physical dissociation of oxyhaemocyanin. The same authors [1910] had previously concluded, on other grounds, that haemocyanin from *Limulus* differs from haemocyanin from *Octopus*.

It is evident that the proof that haemocyanin exercises respiratory functions in virtue of its power to dissociate would be greatly strengthened if it could be shown that the conditions which govern the equilibrium between the oxidised and reduced forms were adapted to the transport of oxygen within the organism. A comparative study with different species would, moreover, throw light on the question of the possible existence of different haemocyanins. The present investigation, which forms part of a more comprehensive one, was undertaken with this object and deals with the oxyhaemocyanin dissociation curves of the blood of the four decapods, *Homarus*, *Palinurus*, *Maia* and *Cancer*.

EXPERIMENTAL.

The determinations of the oxygen content of bloods containing haemocyanin which have hitherto been carried out have been made either by a titration method, involving the use of sodium hydrosulphite ($\text{Na}_2\text{S}_2\text{O}_4$), or by analysis of the gases removed from the blood by evacuation. Of these methods, the former is open to obvious objections and would, moreover, not be applicable to determinations at low oxygen tensions; the latter is capable of yielding extremely accurate results but requires a special mercury pump which was not available. It therefore appeared desirable to adopt one of the methods normally employed in the case of vertebrate blood. These depend upon the liberation of oxygen from oxyhaemoglobin by the use of potassium ferricyanide. A preliminary experiment carried out with the Barcroft differential apparatus indicated, however, that this reagent did not act similarly towards oxyhaemocyanin. In the absence of the discovery of a substitute for the ferricyanide, the use of the Barcroft or Haldane apparatus for the determination of the oxygen capacity of blood containing haemocyanin is thus impossible. On the other hand, the apparatus devised by Van Slyke and Neill [1924] combines the action of ferricyanide with vacuum extraction, and in its most recent modification [Harrington and Van Slyke, 1924] permits the removal of the extracted blood from the apparatus under conditions which preclude recombination with the extracted gases. It therefore appeared that this latter modification would be applicable to the problem under discussion provided that extraction alone was sufficient to cause the complete dissociation of the oxyhaemocyanin contained in the blood. Trial experiments with this apparatus showed that extraction for 1-2 minutes with constant shaking in contact with mercury caused the complete disappearance of the blue colour. Moreover, determinations of the oxygen capacities of the bloods of *Maia* and *Palinurus*, after equilibration with air, gave values of 1.29 and 1.80 vols. % respectively, which are of the same order as those obtained by Winterstein and quoted

above. These results were considered to demonstrate the applicability of the Van Slyke apparatus, which was accordingly employed for the measurements recorded in this paper.

The blood was collected by the following methods, for instruction in which the authors are indebted to Dr L. T. Hogben:

Homarus vulgaris (Common lobster). An incision was made between the terminal and adjoining terga of the abdominal segments and was accompanied by section of the abdominal artery. This caused a ready flow of blood which was collected and placed in the ice chest until the clotting process was complete. The freshly drawn blood was pale pink in colour but rapidly became purplish blue on exposure to air. The clotted blood was defibrinated by grinding with a small quantity of powdered glass and filtering through bolting silk.

Palinurus vulgaris (Rock lobster). An identical method was employed. The blood of this animal differed from that of *Homarus* in containing a greater amount of pink pigment and in clotting with much greater rapidity.

Maia squinado (Spider crab). The carapace was opened over the heart and an incision made in this organ. The outflowing blood, which contained only a trace of pink pigment, quickly became blue. Apart from agglutination of the leucocytes, which were removed by filtration, no clotting occurred.

Cancer pagurus (Common crab). The method employed was identical with that used in the case of *Maia*. The blood did not clot, was practically colourless when drawn, and assumed a rich blue colour, unmasked by pink pigments, when in contact with air.

The following technique was employed for the determinations. About 8 cc. of blood were drawn into a tonometer, equilibrated at room temperature (15°) with an analysed mixture of oxygen and nitrogen, and then transferred by displacement with mercury to an Ostwald pipette provided with a stop-cock. 5 cc. were introduced into the Van Slyke apparatus, two drops of octyl alcohol added to prevent foaming, and the blood extracted by shaking in the chamber for 3 minutes. The blood was then removed through the trap and the gas analysed by the method described by Harington and Van Slyke, the manometer readings being made at a volume of 0.5 cc. The method employed for equilibration was tested by equilibrating two samples of the same blood, one of which was in equilibrium with the air whilst the other had been freed from oxygen by evacuation, with a low oxygen tension. Analyses of the two samples yielded identical results, thus showing that the method permitted equilibrium to be reached reversibly.

All analyses were made within 12 hours of drawing the blood, which was stored in the ice chest when not in use. It was proved by determinations on the blood of *Maia* that, under these conditions, no loss in oxygen-combining power occurred during that period. When sufficient blood was required for a series of determinations, a uniform specimen was obtained by mixing the blood of two or more animals. The results were calculated according to Van Slyke's equation and are given in the following table:

Species	Number of animals	Oxygen tension (mm.)	Oxygen capacity of blood (vols. %)	Oxygen capacity of haemocyanin (vols. %)*	% saturation of haemocyanin
<i>Maia</i>	2	8.0	0.31	0.28	24.5
		19.8	1.05	0.94	82.5
		77.9	1.40	1.12	98.2
		149.8	1.75	1.22	100
		(347.0)†	(2.39)	1.14	—
		741.0	3.66	—	—
<i>Palinurus</i>	2	11.0	0.47	0.43	35.6
		20.8	1.13	1.05	86.0
		79.0	1.49	1.20	99.9
		148.6	1.80	1.25	100
		(344.1)	(2.49)	1.22	—
		735.0	3.88	—	—
<i>Cancer</i>	3	13.5	0.90	0.86	46.5
		21.7	1.69	1.63	88.1
		80.0	2.09	1.85	100
		149.8	2.29	1.85	—
		(289.0)	(2.74)	1.85	—
		741.0	4.02	—	—
<i>Homarus</i>	3	16.5	0.61	0.56	76.6
		22.5	0.84	0.76	—
		80.3	0.99	0.73	100
		147.8	1.22	0.73	—
		(342.5)	(1.88)	0.73	—
		732.5	3.22	—	—
<i>Maia</i>	3	Air	1.68	—	—
"	3	"	1.29	—	—
<i>Homarus</i>	1	"	1.18	—	—
"	1	"	1.09	—	—
<i>Cancer</i>	1	"	0.96	—	—

* Obtained from curves in Fig. 1.

† The figures in brackets were obtained by interpolation.

The results of the first four experiments have been used to plot the oxyhaemocyanin dissociation curves shown in Fig. 1. The method employed for this purpose is illustrated by Fig. 2, which refers to the blood of *Cancer*. Curve *A* has been plotted from the experimental values and hence represents the relation between the oxygen capacity of the blood and the oxygen tension. *B* represents the physically dissolved oxygen and has been obtained by drawing a line through the origin parallel to the region of *A* in which the capacity is proportional to the oxygen tension. The differences between the ordinates of *A* and *B* give the amounts of oxygen bound to haemocyanin at any particular tension. These have been plotted in *C*, which thus represents the dissociation curve of the oxyhaemocyanin in the blood. *D*, the oxygen capacity curve for sea-water at the temperature of the experiments has been added for comparison. All the curves in Fig. 1 have been obtained from the experimental results in this manner.

DISCUSSION.

It is at once evident from the curves reproduced in Fig. 1 that not only does oxyhaemocyanin yield oxygen to low tensions of this gas but that it does so under conditions which render it capable of exercising real respiratory functions within the organism. Corresponding with the low oxygen tension

of the medium in which the animals live, the haemocyanin is, under temperature conditions which approximate to the normal, about 85 % saturated when in equilibrium with oxygen tensions of 20 mm. At the lower tensions which doubtless exist in the tissues, dissociation would occur, thus providing the latter with oxygen. Haemocyanin may thus be regarded as a respiratory pigment which functions in a manner analogous to that of haemoglobin.

Fig. 1.

Fig. 2.

Whilst a comparison of these two proteins must be deferred until further experimental work has been carried out on the various conditions modifying the affinity of haemocyanin for oxygen, attention may be directed to the tendency of the curves in Fig. 1 to assume the S-shaped form characteristic

of the curve for haemoglobin. Should this form of the curve be confirmed by further work, an interesting analogy between the two proteins will appear and experiments will be carried out to ascertain if it is dependent on the same factors which are responsible for the double inflexion in the haemoglobin curve.

Fig. 3.

When a comparison is made between the oxyhaemocyanin dissociation curves for the four species examined it is found that whilst the oxygen capacities of the bloods differ widely in magnitude, the shapes of the curves show a close resemblance. If, further, the oxygen tension at which the haemocyanin is 50 % saturated is selected as a measure of its affinity for oxygen it is found, within the limits of experimental error, that identical values (14 mm.) are yielded by the four curves. The significance of these results becomes more evident when a composite curve, in which the percentage saturation is plotted against the oxygen tension, is drawn. This has been done in Fig. 3, omitting one reading for *Homarus* which is obviously erroneous (see Fig. 1) and also the higher oxygen tensions at which the haemocyanin is 100 % saturated. The points all lie on a smooth curve which retains, and in fact emphasises, the S-shaped form mentioned above. Whilst, in the absence of corroboration of a different nature, we should hesitate to assert the identity of the haemo-

cyanins present in these species, these results, nevertheless, are very strong evidence in its favour.

In this connection, reference may be made to a paper by Redfield and Hurd [1925] which appeared while this work was in progress. These authors utilised the blue colour of the oxyhaemocyanin itself for the colorimetric estimation of the percentage saturation, at various oxygen tensions, of the haemocyanin present in the bloods of *Limulus* and *Loligo*. The shapes of the two dissociation curves obtained in this manner show little resemblance, but when the curve for *Limulus* is compared with those obtained in this investigation an unmistakable similarity is found to exist. This similarity extends, moreover, to the oxygen tensions at half saturation, the value for *Limulus* approximating to that given above. This corresponds not only with the fact that the animals are members of the same phylum, but also with the similarity in their habits and environment.

It will be observed that the oxygen capacities as determined by the Van Slyke apparatus are somewhat higher than those obtained by Winterstein using a mercury pump. This may be connected with the causes which are responsible for the well-known discrepancy between the results obtained with the Van Slyke and the Haldane apparatus in the case of vertebrate blood. Nevertheless it must be emphasised that considerable variations in the haemocyanin content of the blood of different individuals of the same species do actually occur. This is evident, not only from the values which are given in the table, but also from the differences in the intensities of the colour of the blood.

Curves *B* and *D* in Fig. 2 show that the amount of oxygen physically dissolved in the blood of *Cancer pagurus* is almost the same as that dissolved in sea-water when in equilibrium with the same oxygen tension. With the other three species the agreement was even closer. This accords with the observations of various workers [Höber, 1922] that the freezing points and consequently the salt concentrations of the blood of marine invertebrates are practically identical with those of sea-water.

SUMMARY.

1. The dissociation curves of the oxyhaemocyanin present in the blood of *Maia*, *Palinurus*, *Cancer* and *Homarus* have been plotted from determinations of the oxygen capacities of blood equilibrated with oxygen at various tensions.
2. The affinity of the haemocyanin for oxygen is the same for all four species.
3. The results show that haemocyanin is capable of exercising respiratory functions in a manner analogous to haemoglobin, and suggest that the haemocyanins present in the species examined are identical.

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REFERENCES.

- Alsberg and Clark (1910). *J. Biol. Chem.* **8**, 1.
—— — (1914). *J. Biol. Chem.* **19**, 503.
Dhéré (1915). *J. Physiol. Pathol. Gen.* **16**, 985.
Harington and Van Slyke (1924). *J. Biol. Chem.* **61**, 575.
Höber (1922). *Physikalische Chemie der Zelle und der Gewebe*, 36
Redfield and Hurd (1925). *Proc. Nat. Acad. Sci.* **11**, 152.
Van Slyke and Neill (1924). *J. Biol. Chem.* **61**, 522.
Winterstein (1909). *Biochem. Z.* **19**, 384.