

SOME OBSERVATIONS ON THE DISSOCIATION OF HÆMOCYANIN BY THE COLORIMETRIC METHOD.

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CONTENTS

	PAGE		PAGE
1. Introduction	225	4. The effect of salts	235
2. The Effect of temperature	226	5. Summary	237
3. The Effect of hydrogen-ion concentration	230	6. References	238

I. Introduction.

DETERMINATIONS by Winterstein (1908) on the oxygen capacity of the blood of invertebrates endowed with and devoid of hæmocyantin in their blood (together with the more recent investigations of Dhéré (1916-20) who has correlated the oxygen capacity and copper content of the hæmocyantin-containing blood of a number of genera), leave no doubt that hæmocyantin is a reversibly oxidisable pigment which in the oxidised form gives up its oxygen at tensions commensurate with the call of the tissues for oxygen. Recently the Stedmans (1925) have performed a great service towards an understanding of the physical chemistry of the hæmocyantins by recording dissociation curves of blood of several species of crustacea based on direct measurement with Van Slyke's technique. In an attempt to devise a method of illustrating the properties of this substance suitable for class work, some preliminary observations on the effect of various factors on the dissociation curve were recorded in a note describing a colorimetric technique (Pantin and Hogben, 1925). The data recorded below are mainly in extension and confirmation of those previously published with the introduction of some refinements not described elsewhere.

Lancelot T. Hogben

2. The Effect of Temperature.

In the note referred to above two curves were published, one at 1.2° C. and the other at 24° C. to illustrate the effect of temperature on the dissociation of the oxyhæmocyanin of *Palinurus*. From this experiment it was apparent that the affinity for oxygen at low tensions is diminished in the case of crustacean hæmocyanin, as is well known to be true of hæmoglobin. Later experiments on the blood of *Maia* and *Cancer* confirmed this conclusion, and showed that this relation holds continuously over a range of temperatures between 0° and 52° C.

The results of these initial observations repeated several times encouraged the attempt to improve on the method adopted in carrying out the assay with a view to expressing the relation between temperature and dissociation in quantitative form. It was soon found that the colorimetry itself was more satisfactory, when the orange-coloured lipochrome present in varying amount in normal serum is removed. On shaking with chloroform and subsequent separation of the chloroform layer by centrifuging, there is obtained a preparation which, when reduced, is quite colourless; so that colour standards can be prepared, as indicated elsewhere, by dilution of the oxidised serum with water made opalescent by addition of a small quantity of egg albumin. With this improvement in the colorimetric technique, an important source of error presented itself owing to decomposition of bicarbonates. The blood of crustacea compared with that of the mammal is poorly buffered (*cf.* Parsons and Parsons, 1923); and even at normal temperatures, unless the serum has been well shaken at zero pressure before the assay begins, there may result from the reduced CO₂ pressure inherent in the method of carrying out the experiment, sufficient change in *p*H to produce an appreciable displacement of the dissociation curve. By passing through serum placed in a thermostat at 52° C. (the highest temperature on which observations were made) a stream of CO₂-free air and subsequent addition of an appropriate quantity of Na₂HPO₄ acidified to give the *p*H required, it was found possible to maintain the serum at constant *p*H during the manipulations involved in the determination of the effect of temperature on the dissociation

Dissociation of Hæmocyanin

of hæmocyanin. The data referring to *Maia* in figs. 1 and 2 are based on phosphate-buffered blood treated in this way. Those on the other hand relating to *Cancer* in fig. 2 were obtained from an earlier experiment on normal serum, treated for removal of chloroform-soluble pigment alone.

The data represented graphically in the first two figures illustrate the fact that between the temperatures of 0° and 50° C. hæmocyanin, like hæmoglobin, takes up less oxygen at low tensions continuously with rise in temperature. Assuming that hæmocyanin functions as a respiratory pigment, the considerations put forward by Krogh and by Hill (*cf.* Brown and Hill, *op. cit.*) with reference to the relation of climate and metabolism apply equally. However, it should be borne in mind that while hæmocyanin is reducible at tensions appropriate to the call of the tissues for oxygen, we do not yet know that the circulation of blood to the respiratory surfaces (the gills presumably) is so efficient in the crustacean as to ensure that hæmocyanin exists in the blood to any appreciable extent in the oxidised form. These remarks do not apply to the cephalopod, in which hæmocyanin, as Winterstein proved, acts as an oxygen-carrier from the ctenidia to the tissues.

It may now be asked whether this method allows us to draw any conclusions of a quantitative nature with reference to this aspect of the physical chemistry of the hæmocyanins. Barcroft and Hill (1910) and Brown and Hill (1923) have investigated the effect of temperature on the dissociation of oxyhæmoglobin from the standpoint of mass action, applying Vant Hoff's isochore by taking the equilibrium constant of the reaction as proportional to the ratio of reduced and oxidised hæmoglobin at a fixed oxygen concentration. The same problem might be treated by taking K as proportional to the oxygen concentration corresponding to a fixed ratio of oxidised and reduced pigment, if complete curves were available for all temperatures. If the fixed ratio of oxidant-reductant is such *e.g.* 50 per cent. saturation, as to fall near the point of inflexion on the S-shaped curve, the effect of an error in the determination of the adjacent observations is reduced to a minimum. To obtain such curves all that is necessary is a thermostat in which the equilibration of sample tubes can be carried out.

Lancelot T. Hogben

Assuming the most general relation of a stoichiometrical nature between reduced (Cy_r) and oxidised (Cy_0) hæmocyanin, viz.:



Then, if the law of mass action holds for the system under discussion:

$$\frac{(Cy_r)^m \cdot (O_2)^n}{(Cy_0)^l} = K \quad . \quad . \quad . \quad . \quad (2)$$

For the condition Cy_r / Cy_0 is constant, say $Cy_r = m/2$ and $Cy_0 = 1/2$ (50 per cent. saturation), we have

$$(O_2)_{50}^n \propto K.$$

So long as there is no change in factors affecting the solubility of oxygen in water, by Henry's Law,

$$(O_2)_{50}^n \propto x_{50} \propto K,$$

and

$$n(\delta \log x_{50})_t = \delta \log K, \quad . \quad . \quad . \quad . \quad (3)$$

where x_{50} is the partial pressure of oxygen corresponding to 50 per cent. saturation. If a represents a factor for the solubility of oxygen by weight at different temperatures in water,

$$n \cdot d \log ax_{50} = d \log k \quad . \quad . \quad . \quad . \quad (4)$$

The value of a for any temperature can be obtained by graphical interpolation from data given in tables of physical constants. In fig. 2 ax_{50} is calculated on the basis, $a = 1$, $T = 273^\circ$.

Now applying the Vant Hoff isochore,

$$\frac{d \log K}{dT} = \frac{Q}{2} \cdot \frac{1}{T^2}$$

in the indefinite integral form, viz.

$$\log K = -\frac{Q}{2} \cdot \frac{1}{T} + C,$$

it follows from (4) that $\log ax_{50}$ is a linear function of the reciprocal of the absolute temperature, if the law of mass action is applicable to the dissociation of hæmocyanin. That this is indeed the case appears to be indicated in fig. 2 based on the data of one experiment on phosphate-buffered blood of *Maia* (fig. 1) and another series of observations on normal serum of *Cancer*.

Dissociation of Hæmocyanin

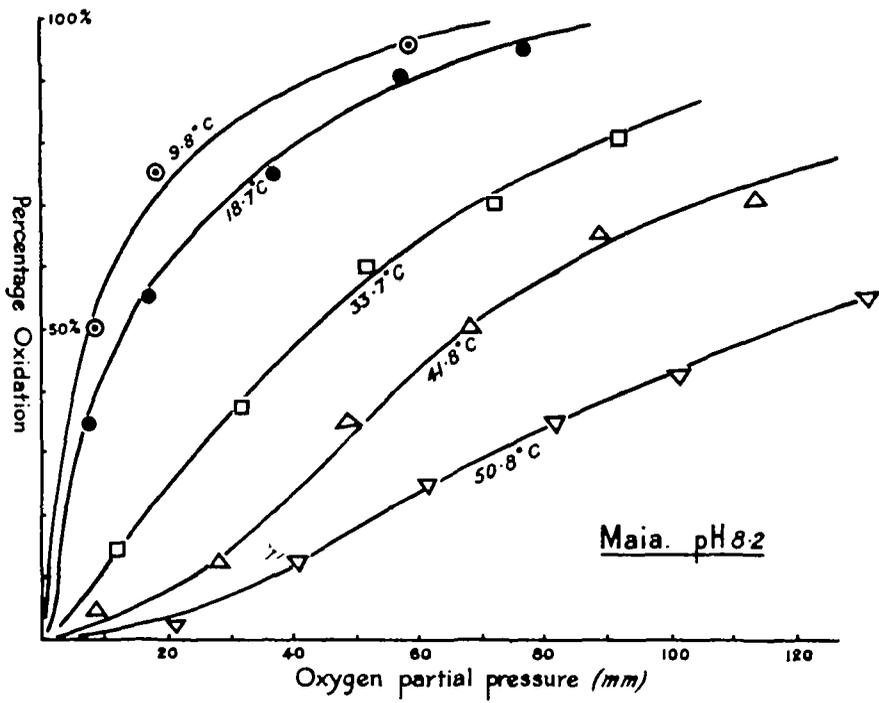


FIG. 1.

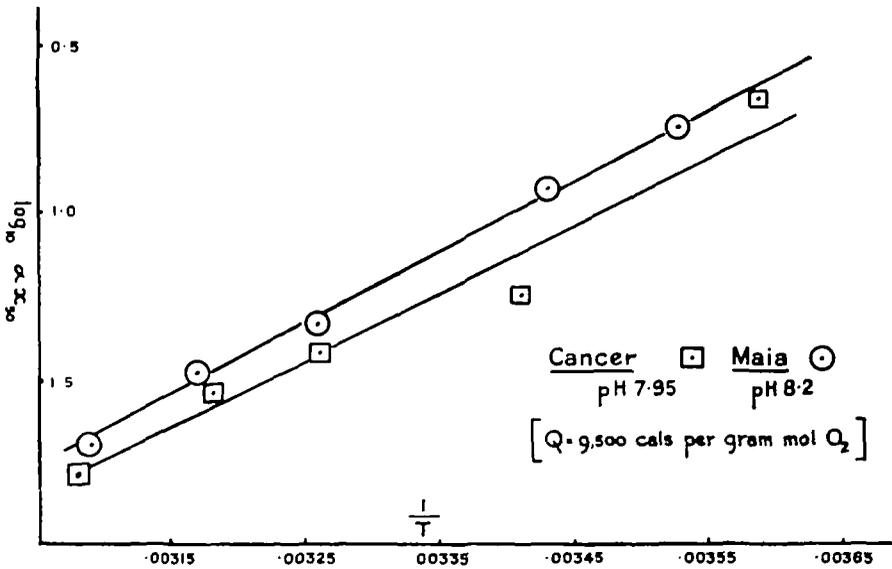


FIG. 2.

Lancelot T. Hogben

To calculate Q , the graphical method is simplest, $\log_{10} ax_{50}$ in fig. 2 being reduced to natural logarithms the solution is,

$$Q/n = 2.2.303 \cdot \tan \theta$$

where $\tan \theta$ is the slope of the line. In equation (1) n is the *least number* of molecules of oxygen which enter into the reaction whereby reduced hæmocyanin is converted into oxidised hæmocyanin. The heat of reaction per gram molecule of oxygen on this basis is 9500 calories.* So calculated it refers to the reaction of hæmocyanin with dissolved oxygen in the water phase. The expected value for an experiment carried out in the usual way would differ from this (*cf.* Brown and Hill) by an amount equivalent to the heat of solution of oxygen.

As regards the value of n , examination of Dhéré's data brings out an interesting fact. For crustacean blood figures for the oxygen capacity (per cent.) per mg. copper for *Astacus*, *Homarus*, and *Cancer* are respectively 0.30, 0.295, and 0.291. It would therefore appear that one gram atom of copper combines with one mol. of oxygen. It should be noted that the amount of oxygen equivalent to one gram atom of copper is significantly different in the case of crustacean and molluscan blood.

3. The Effect of hydrogen-ion concentration.

Previous experiments elsewhere published (*op. cit.*) have shown that over a certain range the extent of oxidation of *Palinurus* hæmocyanin at low tensions diminishes with increasing hydrogen ion concentration. More extensive experiments on serum of *Palinurus*, *Homarus*, *Cancer*, and *Maia* show that the observations previously recorded do not form a continuous series, as might at first sight appear.

In order to correct for protein and salt error the following procedure was adopted. The serum is colourless by transmitted light when diluted one in five. Since the buffer action of the serum is poor, dilution to the same extent with a strong buffer may be assumed to produce an insignificant shift in the

* The above method of analysis makes no assumptions regarding the values of m and l in equation (1): the advantage of this is evident from Adair's criticism of Hill's equation for hæmogoblin.

Dissociation of Hæmocyanin

pH of the latter. The pH of the serum being determined roughly by addition of indicator and comparison with a standard buffer, it is then compared with standards ± 0.2 and ± 0.4 of the apparent value, prepared as indicated by dilution of the fresh serum with strong buffer of known pH . It is assumed that the small correction thus made is due to protein-salt error. This procedure is admittedly an approximation,

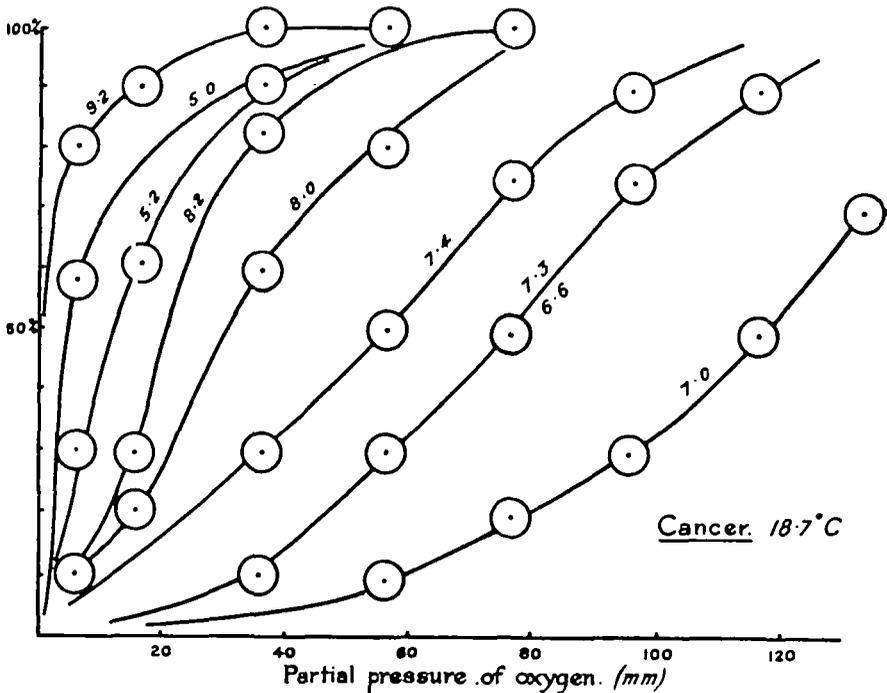


FIG. 3.

but in any case the corrections so made do not affect the main conclusions stated below.

A typical experiment on the blood of *Cancer* is seen in fig. 3.

The results of two series of experiments carried out respectively on sera of *Cancer* and *Palinurus* are summarised in fig. 4, in which the oxygen pressure corresponding to 50 per cent. saturation (x_{50}) is plotted against pH . The hydrogen ion concentration was varied as indicated in the previous paper. From fig. 4 it will be seen that in each case the uptake of oxygen at low tensions diminishes up to a certain critical

Lancelot T. Hogben

value as the hydrogen ion concentration is increased : on further increase of the latter the value of x_{50} increases also.

If affinity is defined with reference to conditions for which the arbitrary terms involving initial and final states in the

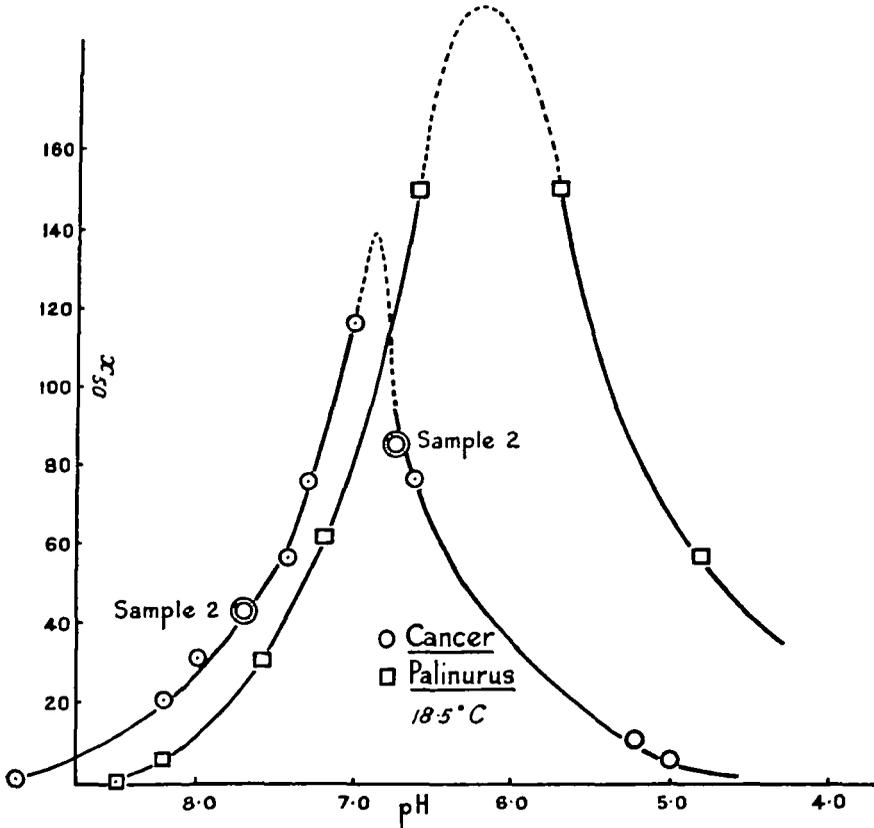


FIG. 4.

Vant Hoff isotherm vanish, A being the affinity of hæmocyanin for oxygen and K the constant in equation (2),

$$A = -RT \log K$$

$$A \propto -\log x_{50} \quad \dots \quad (5)$$

In fig. 5 three sets of observations on *Maia* are represented. The ordinate in this case is $\log_{10} x_{50}$ which is thus proportional to the affinity of hæmocyanin for oxygen. Plotted in this way the points lie distributed in almost linear fashion about two straight lines inclined with gradients of opposite sign with reference to the abscissa.

The behaviour of hæmocyanin in this respect agrees with

Dissociation of Hæmocyanin

that of hæmoglobin as described by Rona and Ylppo (1916) who have shown that on making a solution of hæmoglobin more acid than pH 6.0 (the value for which affinity for oxygen is a minimum) the amount of oxidation at low tensions increases.

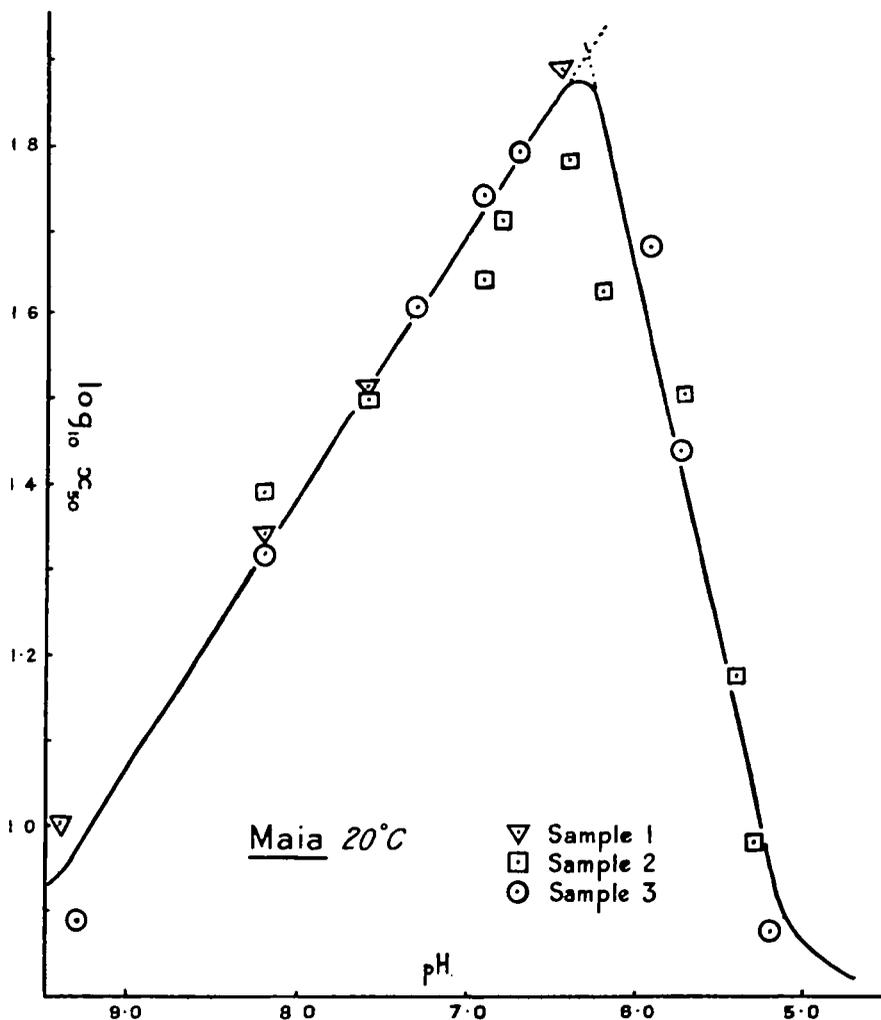


FIG. 5.

For the increase of affinity for oxygen showed by both hæmocyanin and hæmoglobin on either side of a minimal value corresponding to a particular hydrogen ion concentration an explanation might be sought along one of three lines, the ionisation, state of aggregation, or the internal configuration of the protein molecule.

Lancelot T. Hogben

(1) It is well known that many physical properties of proteins have minimal values at the isoelectric point. The isoelectric point of crustacean hæmocyanin is not known. But the fact that the point of minimal affinity of hæmoglobin for oxygen is so far beyond the isoelectric point makes this suggestion by analogy unlikely.

(2) Rona and Ylppo put forward (pp. 216-7) the following explanation in regard to hæmoglobin:—

“Betrachten wir das Hæmoglobin als einen Eiweisskörper von kolloidal Natur, so können wir uns vorstellen, dass sich die einzelnen Hæmoglobinmoleküle zuerst bei geringer Säurezufuhr teilweise zusammenballen. . . . Steigert man aber dann immer die Acidität, so lösen sich die Molekülaggregate nochmals auf.”

There does not seem to be any cogent reason to prefer this interpretation to the following alternative.

(3) There exist two tautomeric forms of the pigment. It may be noted in this connection that according to Anson and Mirsky (1925) the affinity of heliocorubin for oxygen increases with increasing hydrogen ion concentration.

It will be seen that between pH 6.5 and 9.5 the relation between pH and $\log_{10} x_{50}$ in fig. 5 (*Maia*) is approximately linear. A close approximation to a linear relation between $\log K$ and pH was shown to exist on the alkaline side of the isoelectric point of hæmoglobin by Barcroft and Peters. It is tempting to speculate on the possible significance of this relation. In the theory of the oxygen electrode an equilibrium between oxygen pressure and hydroxyl ion concentration is postulated thus:



Assuming then that a reaction of the type defined by equations (1) and (2) coexists with one defined by (6), the equilibrium of the whole system would then be conditioned by a relation of the form:

$$\frac{(C_{y_r})^r (O)^r}{(C_{y_0})^r (OH)^r} = k.$$

And for a fixed ratio of $C_{y_r} : C_{y_0}$

$$\frac{x_{50}^r}{(OH)^r} = k^r$$

Dissociation of Hæmocyanin

Whence $\log x_{50}$ is a linear function of pOH . On this way of looking at the problem no assumptions as to the state of aggregation of the hæmocyanin molecule are made.

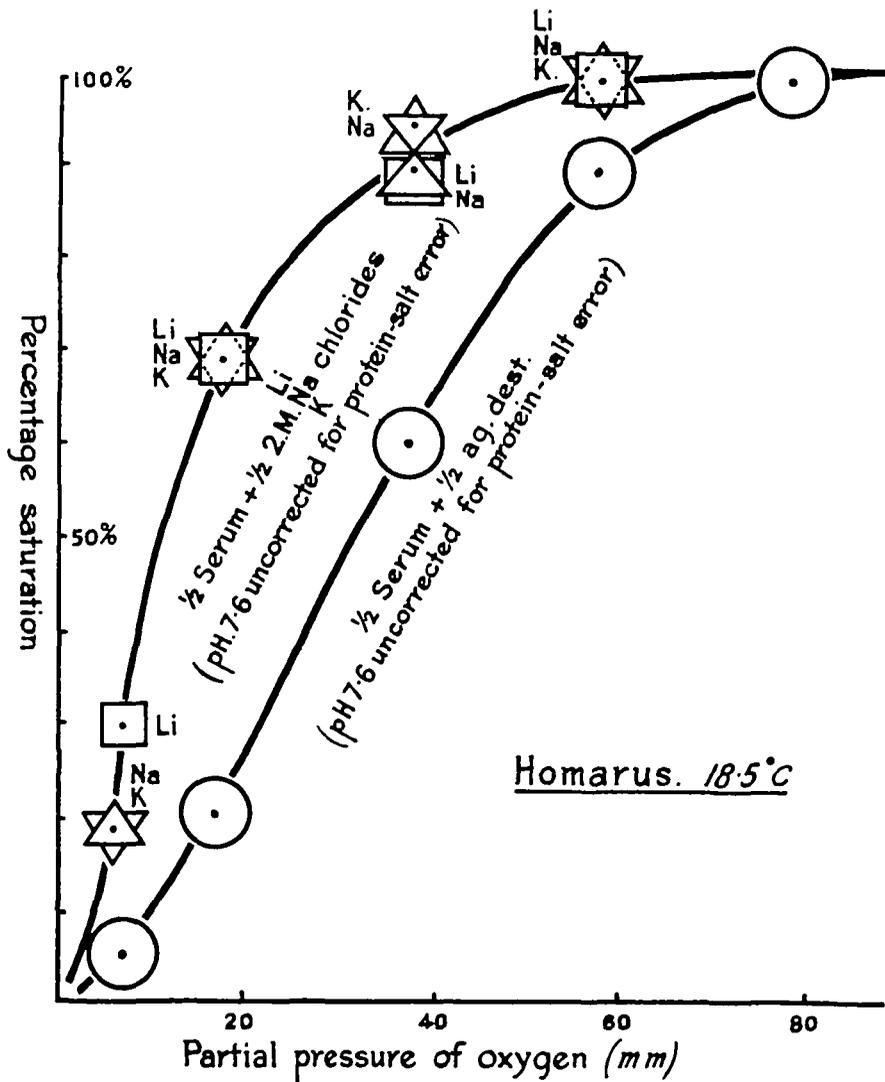


FIG. 6.

4. The Effect of salts.

The action of neutral salts in increasing the steepness of the dissociation curve of hæmoglobin has played an important part in the theory of the latter. On this issue the only new

Lancelot T. Hogben

experiments have been made on the same lines as previously (*op. cit.*), viz., by comparing the dissociation curve of serum diluted respectively with distilled water on the one hand, and

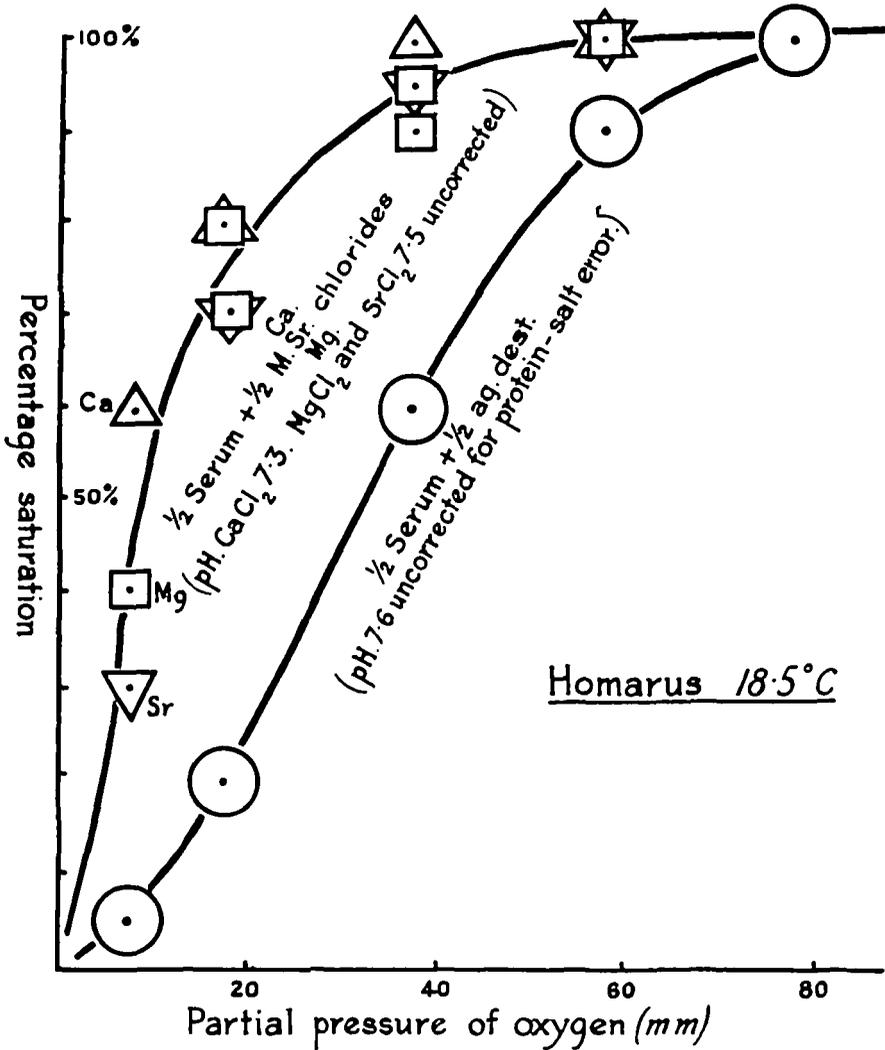


FIG. 7.

with hypertonic solutions of chlorides of the alkaline and alkaline earth series on the other. The results obtained in numerous experiments with blood of *Cancer*, *Maia*, and *Homarus* agree in demonstrating clearly that concentration of the serum with neutral salts increases affinity for oxygen at

Dissociation of Hæmocyanin

low tensions: the result obtained with *Palinurus* serum in a single experiment figured elsewhere was not confirmed.

These experiments illustrated in figs. 6 and 7 were carried out by the method as originally described. To draw more positive conclusions would only be justifiable with data based on more rigid control of pH . However, there are two points which emerge from a study of the curves obtained with *Maia* and *Cancer* blood: to what extent does the effect depend upon the anion and cation of the salt, and to what extent does the valency of the cation alone enter into the result. In these experiments the effect of M solutions of Ca, Sr, and Mg chlorides was in all cases at least as great as that of $2M$ solutions of K, Na, and Li chlorides, when the serum was diluted 50 per cent. with the reagent. And this would suggest that it is not primarily the cation which enters into the question. But, as stated, until more carefully controlled experiments have been made it would be an act of some temerity to put forward a definite conclusion on this point.

5. Summary.

1. By means of the colorimetric method the effect of temperature, hydrogen ion concentration, and salinity upon the dissociation of the hæmocyanin of four species of decapod crustacea (*Maia*, *Cancer*, *Palinurus*, and *Homarus*) has been studied.

2. Rise in temperature depresses the dissociation curve continuously between 0° and $50^{\circ}C$. Reasons are given in favour of the conclusion that this behaviour is consonant with the applicability of the mass action law. On this understanding the heat of reaction between hæmocyanin and oxygen dissolved in the water phase in the case of *Maia* is of the order 9500 calories per n gram molecules of oxygen, n being defined as the least number of molecules of oxygen which can enter into the reaction.

3. In the case of all four crustaceans referred to above, the affinity for oxygen diminishes up to a point as the hydrogen ion concentration is increased: on further increasing the hydrogen ion concentration beyond a critical value for which the affinity of the serum for oxygen is minimal, the amount of oxygen

Lancelot T. Hogben

taken up at low tensions increases and may surpass the values obtained for serum at normal pH . The similarity of this result with the observations of Rona and Yllpo on hæmoglobin is discussed.

4. On concentrating serum with neutral chlorides of the alkali and alkaline earth metals the dissociation curve is made steeper.

6. References.

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