A COMPARISON BETWEEN THE DISSOCIATION OF THE HAEMOCYANINS OF *HELIX* AND CRUSTACEA

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(With Six Text-figures.)

1. INTRODUCTION.

In a previous communication by one of us (Hogben, 1926) an attempt has been made to explore the properties of haemocyanin (crustacean) as a stoichiometrical reversible oxidation-reduction system. From this standpoint the behaviour of haemocyanin in relation to hydrogen-ion concentration and the presence of salts especially requires more extensive investigation. Certain points which it seems desirable to place on record separately have arisen in connection with experiments upon the haemocyanin of *Helix pomatia*, as herein set forth.

The present investigation, like the foregoing, is based on a colorimetric method already described. While it may be frankly admitted that a direct confirmation of conclusions so derived by gas analysis is ultimately desirable, the facility with which it is possible to arrive at definite conclusions relating to the physical chemistry of haemocyanin, partly on account of the extreme simplicity of the procedure, and partly because the experiments can be carried out in such a way that the points estimated can be chosen at will to fall upon the most significant part of the curve, the task of exploring the ground by a more direct and rigorous method may be simplified and expedited by investigations of this nature. Further, it is hoped that some of the conclusions elucidated may suggest some points in relation to the analogous problem of haemoglobin dissociation. With reference to the colorimetric procedure, the authors wish to re-emphasise the high order of consistency obtained. In every experiment which has been performed, the colorimetry has been carried out by one of us ignorant at the time of the oxygen partial pressure to which the sample was subjected by the method described elsewhere (*loc. cit.*).

The object of the present communication is to set forth certain outstanding particulars in which the behaviour of the haemocyanin of *Helix* definitely contrasts with that of the Crustacea. A characteristic difference was already adumbrated in an earlier note (Pantin and Hogben, 1925). Owing to the small yield per individual (about 1 c.c.) and the large quantity of blood required for accurate determination in a single experiment such as that recorded in Fig. 1, it has not been possible to

carry the investigation, from the theoretical standpoint, beyond the point reached in the study of crustacean haemocyanin.

As regards the preparation of the serum, the following points may be mentioned: reduced serum has a yellowish tint, which cannot be removed like that due to the lipochrome of crustacean blood, by chloroform extraction. However, chloroform treatment of the blood of *Helix* is advisable for two reasons: firstly, after vigorous shaking with chloroform, a good deal of protein material is precipitated, leaving the serum less viscous; secondly, serum treated with chloroform will keep in the refrigerator (about 4° C.) for at least a week, so that successive experiments may be done with the same colour standards. This involves considerable economy of material. The untreated haemocyanin-containing blood of *Helix*, like that of the Crustacea, undergoes spontaneous reduction if kept in a warm room, but regains its colour, again like the latter, on being shaken with air. In these experiments on *Helix pomatia*, as with earlier experiments on *Helix aspersa*, the impression was gained that the blood of these animals is very much more highly buffered than that of the Crustacea, a point which would well repay investigation from the standpoint of studies by Parsons and Parsons (1923) on CO_2 transport in invertebrates. For this reason, in experiments on the effect of hydrogen-ion concentration, the blood was previously dialysed, and the same procedure was adopted in the case of experiments on the action of salts for an ulterior and obvious reason.

The yellowish tint of the reduced serum is easily and satisfactorily matched in preparing the colour standards in the usual way by the addition of a small quantity of an artificial and inert pigment. The intensity of the blue colour of the blood of *Helix* is such that it can be conveniently diluted to an extent which is rarely possible in the case of crustacean blood, indeed, in our experience, it is best for colorimetry to dilute it half-and-half.

For equilibrating the experimental sample of serum, a device has been employed which was not found to be essential in previous experiments. The blood of *Helix* does not coagulate, and seems to froth distinctly more than crustacean blood when shaken at low pressures. A tube of the same calibre as those employed for the colour standards was fused on to a large Pyrex boiling tube, into which the contents of the former could be liberated, thereby exposing a relatively larger surface during equilibration. In order to reduce to a minimum error due to the uptake or evolution of oxygen from the blood itself, two additional precautions have been taken: first, a water trap of larger size was connected to the manometer, so that the gas capacity of the system was considerably increased; by the use of an efficient rotary vacuum pump, for the loan of which we are indebted to Prof. R. L. Stehle, the system could be evacuated rapidly; secondly, in equilibrating each sample, after shaking for some time at constant temperature at a given oxygen partial pressure, mechanical agitation was interrupted while air was readmitted, so breaking the bubbles of gas on the surface of the fluid. This procedure was repeated several times before each estimation.

2. EFFECT OF TEMPERATURE.

Qualitatively, the effect of temperature on the dissociation of the haemocyanin of *Helix* is similar to the effect described in the case of crustacean haemocyanin, *i.e.* rise of temperature diminishes the affinity for oxygen at low tensions as seen from

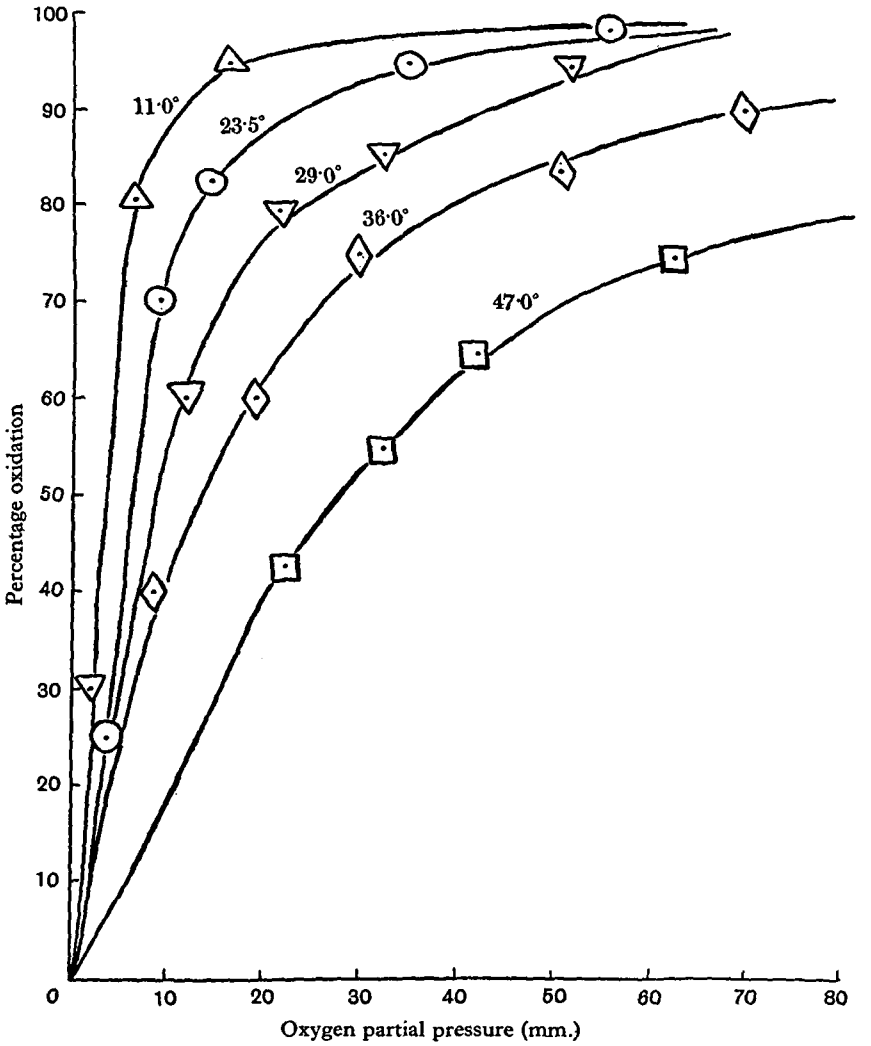

Fig. 1.

the experiment represented graphically in Fig. 1. The sample of blood there used was diluted with 1/5 saturated Na_2HPO_4 (5 to 1). CO_2-free air was bubbled through one hour at 47° C. before use. The sample was not dialysed.

In analysing the results of the experiment, the same reasoning adopted for crustacean haemocyanin may be employed. Assuming the most general relation

of a stoichiometrical nature between reduced (Cy_r) and oxidised (Cy_o) haemocyanin, viz.:

$$l Cy_o = m Cy_r + n O_2 \qquad \text{.....(1).}$$

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

$$\frac{(Cy_r)^m . (O_2)^n}{(Cy_o)^l} = K \qquad \text{.....(2).}$$

For the condition Cy_r/Cy_o is constant, say $Cy_r = m/2$ and $Cy_o = l/2$ (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

$$n\,(\partial \log x_{50})_t = \partial \log K \qquad \text{.....(3),}$$

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,

$$n . d \log a x_{50} = d \log K \qquad \text{.....(4).}$$

The value of a for any temperature can be obtained by graphical interpolation from data given in tables of physical constants.

Now, applying the van't 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, if the law of mass action is applicable to the dissociation of haemocyanin, $\log a x_{50}$ is a linear function of the reciprocal of the absolute temperature.

To calculate Q, the graphical method is simplest, $\log_{10} a x_{50}$ being reduced to natural logarithms the solution is

$$Q/n = 2.2.303 . \tan \theta,$$

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 haemocyanin is converted into oxidised haemocyanin. So calculated, Q refers to the reaction of haemocyanin 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.

The value of Q per n gram molecules of oxygen calculated from this experiment (see Fig. 2) graphically, is approximately 8000 calories. Owing to an error in calculating the absorption coefficient of oxygen, in Fig. 2 of the previous communication (Hogben, 1926), the value for Q there given (9500) is a little higher than it should

be. The experiment on *Maia* has been recalculated, and reproduced graphically in Fig. 2 (present paper). For purposes of convenient graphical representation, $a = 10$ at $T = 273$ for *Helix*, and unity at $T = 273$ for *Maia*. With the correction now made, Q for the haemocyanin of *Maia* would appear to be approximately 9000. The two values are of the same order of magnitude, but though it would be inadvisable to be dogmatic, it would seem that the figure for Q/n in the case of the

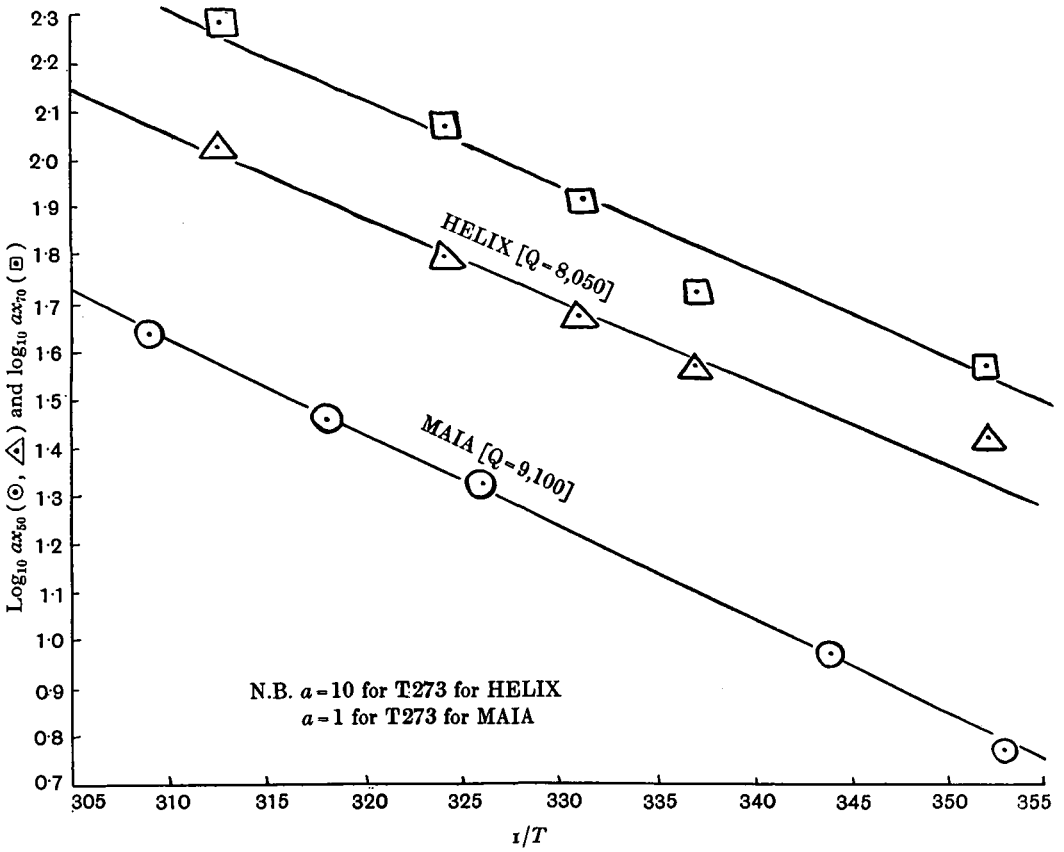

Fig. 2.

haemocyanin of *Helix* is significantly lower. If the law of mass action is applicable to the system under consideration, it is clear from the reasoning given above that the values calculated from partial pressures corresponding to 50 per cent. and 70 per cent. saturations should be consistent with one another, and a satisfactory measure of consistency seems to be indicated by the two sets of points plotted for *Helix* in Fig. 2.

3. HYDROGEN-ION CONCENTRATION.

The *p*H of the normal blood of *Helix pomatia* is $8{\cdot}55 \pm 0{\cdot}1$. In a single experiment recorded in a communication describing the colorimetric method (Pantin and Hogben, 1925), attention was directed to the fact that the dissociation of the

haemocyanin of the snail (*Helix aspersa*) is, as compared with crustacean haemocyanin, extraordinarily little affected by changes in hydrogen-ion concentrati and with the somewhat cruder procedure initially adopted, no effect could be detected over a considerable range. In this investigation the matter has been more carefully probed. A typical experiment is epitomised graphically in Fig. 3,

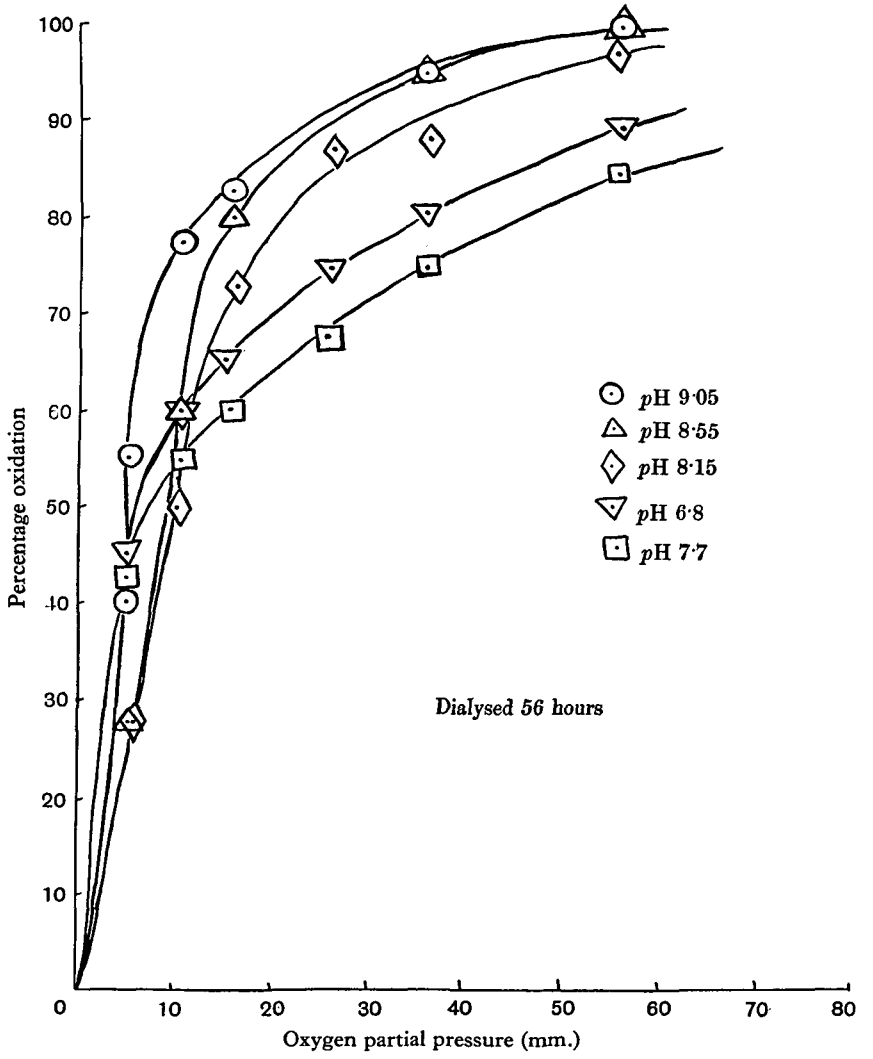

Fig. 3.

where the *p*H of the dialysed serum diluted 7 to 3 was modified by the addition of two drops of standard buffers of different strengths to 10 c.c. serum. The authors are indebted to Mr A. Zoond for assistance in checking electrometrically the *p*H of the samples, which were also determined colorimetrically, with a discrepancy $< 0{\cdot}1$.

There is only a very slight difference in partial pressure corresponding to 50 per cent saturation at a *p*H of 9·0 and at 6·8. By careful analysis of the curves in Fig. 3 the following values were found:

*p*H	x_{50}
9·0	5·0
8·55	9·0
8·15	10·0
7·70	7·5
6·8	6·0

Accepting these values, 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 affinity for oxygen, and beyond that point to increase it. The point of minimal value for x_{50} corresponds to a *p*H of $8{\cdot}0 \pm 0{\cdot}1$. 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. In studying the dissociation of crustacean haemocyanin, it is noteworthy that the gradient $\frac{d \log x_{50}}{d \log H}$ is not the same on either side of the point of minimal affinity. On re-examination of the crustacean curves, it is at once seen that the acid curves for crustacean haemocyanin are relatively flatter at the top than the alkaline curves, though the distinction is not so striking as in the case of *Helix*, illustrated in Fig. 3.

It has been suggested in the previous communication that the haemocyanins of different species might each exist in two tautomeric forms, depending on the *p*H, and the new observations here recorded are fully consonant with such a possibility*. As already mentioned, the work of Rona and Ÿllpo indicates that beyond *p*H 6·0 on the acid side, the affinity of haemoglobin for oxygen increases instead of decreasing further. Though their investigation was not carried sufficiently far to permit a detailed comparison of the behaviour of haemoglobin with these and previous studies on the behaviour of haemocyanin, it is tempting to extend the same suggestion further afield, and perhaps some additional plausibility is gained from the consideration that haematin is known to exist in alkaline and acid solutions in two distinct modifications. The fact that in *Helix* the critical *p*H is so near to, though still on the acid side of, the hydrogen-ion concentration of normal blood, suggests an explanation of a result recorded by Redfield and Hurd (1924) in a preliminary communication on the haemocyanin of *Limulus*, wherein it is stated that increasing CO_2 tension resulted in the increase of oxygen uptake at low tensions; and it is

* Or at least indicate a different *order* of reaction.

legitimate to predict the likelihood, on the basis of experiment on the haemocyanins of *Helix* and Crustacea, that with more extended observations, the haemocyanin of *Limulus* will not be found to contrast in behaviour in this respect with that of haemoglobin as described by Rona and Ÿllpo and with the haemocyanins so far investigated.

One other point deserves mention. The effect of increasing hydrogen-ion concentration has been discussed in its physiological bearing in relation to the response of haemoglobin to the call of the tissues for oxygen. In the absence of more definite evidence that haemocyanin actually performs in the metabolism of *Helix* the rôle of a respiratory pigment in the physiological sense, it would not be permissible to draw any conclusions of a definite nature in this case, especially as the influence of the hydrogen-ion is so slight, but it is interesting to note that if the value of x_{50} is plotted against pH as in Fig. 4 of the previous communication (Hogben, 1926), it is found that the pH of the normal serum of *Helix* lies in the very restricted region where there is any appreciable shift due to increasing hydrogen-ion concentration.

4. THE EFFECT OF SALTS.

From the standpoint of oxidation-reduction equilibrium, perhaps the most interesting aspect of the physical chemistry of haemocyanin is the nature of the salt effect, a question which has never been adequately explored from this angle in the analogous case of haemoglobin; and it was with this end in view that *Helix*, which, on account of the intense blue colour of its blood, provides most ideal material for study in relation to the action of salts by the technique employed for crustacean haemocyanin, was selected for this research. In relation to this objective, however, the results obtained were somewhat disappointing, though not perhaps uninstructive.

The nature of the problem has been indicated previously. The effect of adding neutral chloride both to haemoglobin and crustacean haemocyanin is to increase the affinity for oxygen. Now, in proteins generally, the effect of adding a neutral salt with a common kation on the alkaline side of the isoelectric point is qualitatively similar in a general way to the effect of increasing the hydrogen-ion. It does not seem by any means certain—and the effects to be described in the case of *Helix* haemocyanin reinforces this consideration—that the well-known effect of chlorides on other respiratory pigments is a kation effect. This raises the possibility that the phenomenon described in crustacean haemocyanin is due rather—and the possibility is set forth tentatively in an earlier communication—to an equilibrium in which a complex haemocyanin anion competes with other anions characterised by a difference of affinity with respect to their electric charges; and if there is anything in this possibility, further light might be obtained by a comparison of the effects of, say, sodium iodide and sodium chloride. On the other hand, seeing that haemoglobin and haemocyanin are proteins, it would not seem likely that the action of a common kation should have no influence. Hence it is not necessarily surprising that, in all experiments which have been carried out on *Helix*,

the effect of adding neutral chlorides in a relatively alkaline medium has been—in contrast to the phenomenon recorded in the case of crustacean haemocyanin—to depress the dissociation curve.

Two series of experiments were carried out. In the first series, the actions of sodium, lithium, and magnesium chlorides are recorded (Figs. 4 and 5).

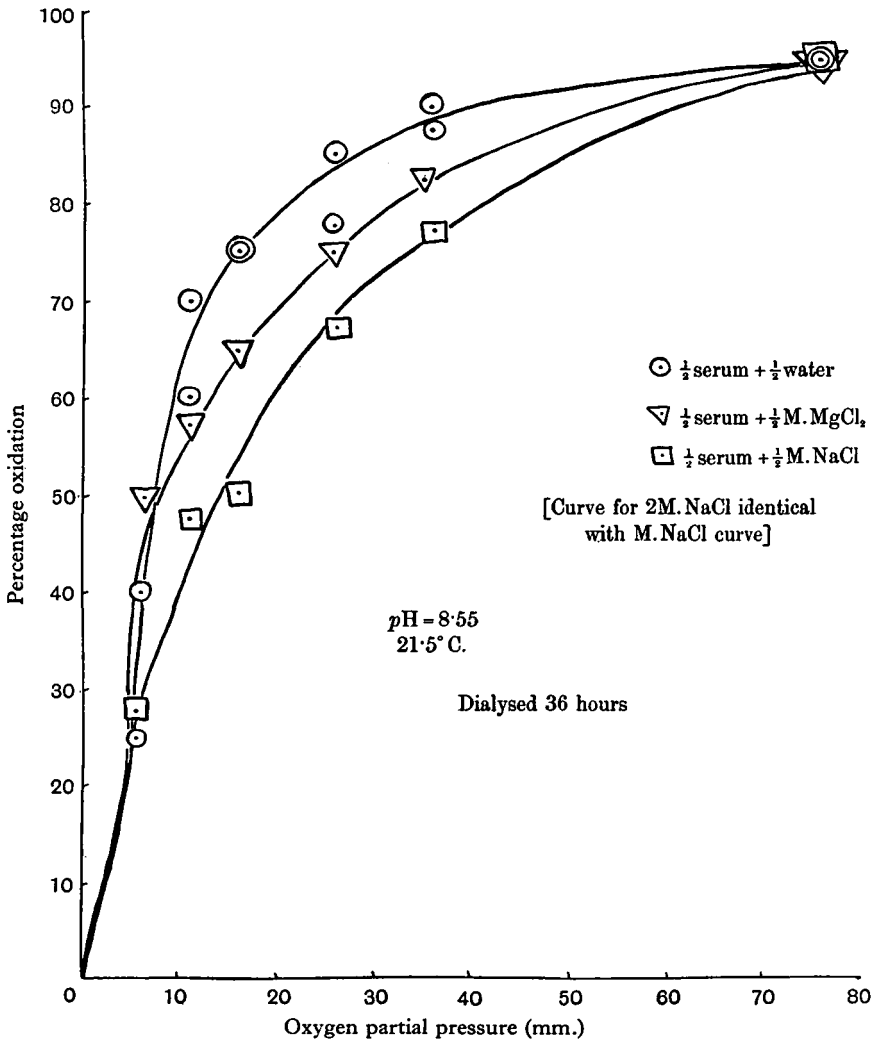

Fig. 4.

When the dissociation curve of the sample of dialysed serum—*p*H 8·55—diluted with an equal quantity of distilled water, is compared with a sample of dialysed serum of the same *p*H to which an equal quantity of molar NaCl was added, it is seen that there is a very striking depression of the extent of oxidation at low tensions. Double this quantity of NaCl (½ serum, ½ 2M. NaCl) does not further depress the curve.

The effect of a corresponding quantity of LiCl ($\frac{1}{2}$ serum, $\frac{1}{2}$ M. LiCl) is the same; but the addition of $MgCl_2$ in equivalent quantities produces a significantly smaller effect. Thus, taking the 50 per cent. and 75 per cent. saturation pressures from a series of curves, with serum *p*H 8·5, the results are as follows:

Sample	x_{50}	x_{75}
$\frac{1}{2}$ serum, $\frac{1}{2}$ water (1)	7·5	16·0
,, ,, (2)	8·0	17·5
$\frac{1}{2}$ serum, $\frac{1}{2}$ M. NaCl	15·0	34·0
$\frac{1}{2}$ serum, $\frac{1}{2}$ M. LiCl	17·5	32·5
$\frac{1}{2}$ serum, $\frac{1}{2}$ M. $MgCl_2$	8·0	26·0
$\frac{1}{2}$ serum, $\frac{1}{2}$ M./2 $MgCl_2$	10·0	22·0

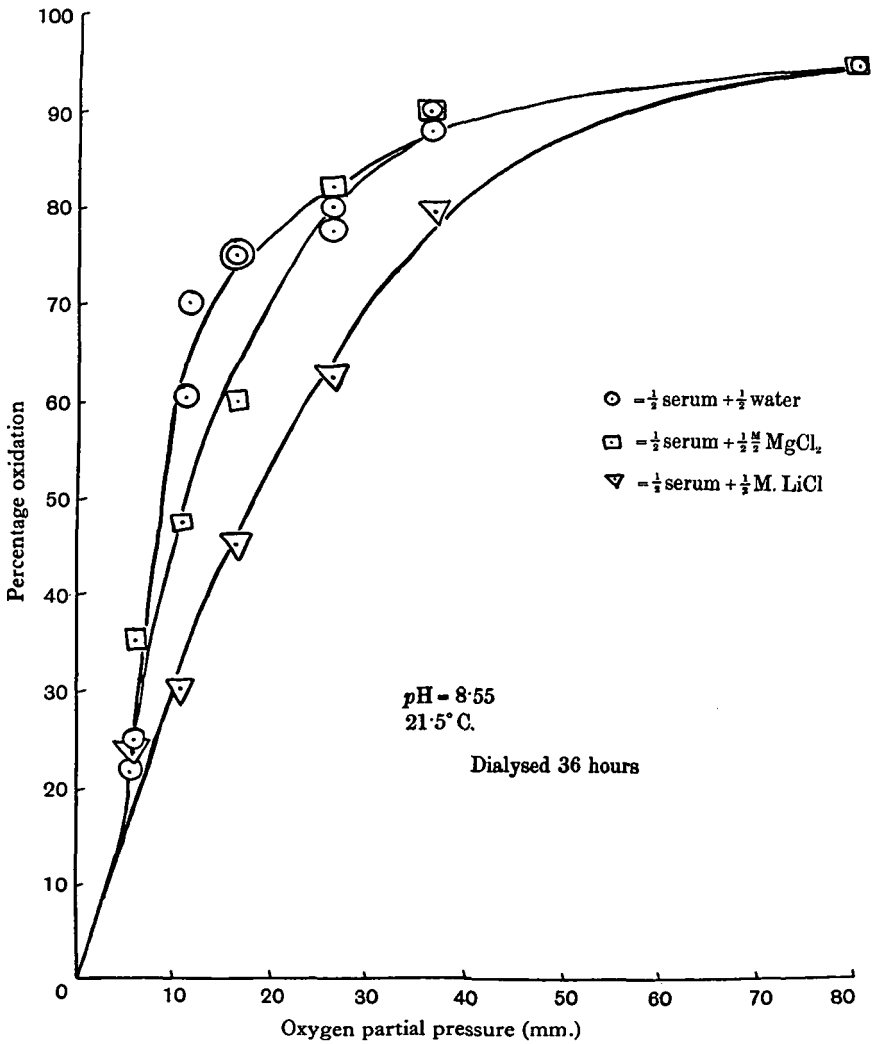

Fig. 5.

From the foregoing observations, it seemed desirable to elucidate the effect of increasing the salt concentration at a higher hydrogen-ion concentration. By the addition of NaH_2PO_4 the serum was brought to *p*H 5·9. Identical dissociation curves were obtained from serum diluted with an equal quantity of water and serum diluted with an equal quantity of molar NaCl. KCl was also without effect.

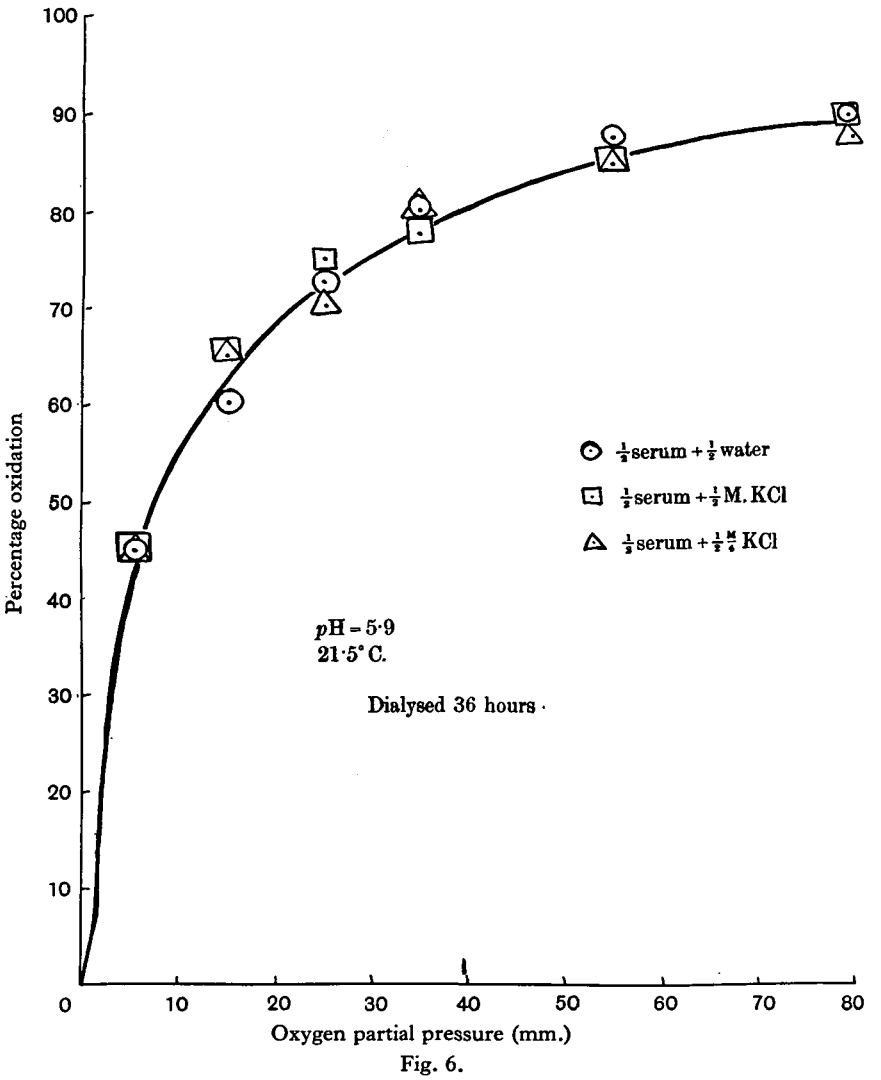

Fig. 6.

In Fig. 6 the dissociation curve of dialysed serum at *p*H 5·9 is compared with the dissociation curves of serum made roughly isotonic with crustacean blood (½ serum, ½ M. KCl) and serum roughly isotonic with the snail's own blood (½ serum, ½ M./4 KCl) by addition of KCl. All the points can be referred to a single curve within the limits of error inherent in the method. The contrast between the

behaviour of crustacean haemocyanin and the haemocyanin of *Helix* in relation to the presence of salts is even more striking than in relation to the effect of *p*H. It is evident that a more searching investigation of the relation of ions other than the hydrogen-ion to the dissociation of crustacean haemocyanin is required before this discrepancy can be viewed in its right perspective, and since it is the intention of the authors to undertake this work, further comment would be out of place at this stage.

5. SUMMARY.

1. The effect of increasing temperature upon the dissociation curve of *Helix* is similar qualitatively to that which has been recorded in the case of haemoglobin and crustacean haemocyanin. The value of Q per n gram molecules of oxygen is about 8000 calories. That is, it is of the same order of magnitude as the value for crustacean haemocyanin, but in all probability significantly less.

2. The effect of increase of hydrogen-ion concentration upon the haemocyanin of *Helix pomatia* is remarkably slight as compared with its effect on crustacean haemocyanin. At low tensions no effect is detectable. Comparing the 50 per cent. and 75 per cent. saturation points, it is seen, that, as with crustacean haemocyanin, increasing hydrogen-ion concentration at first diminishes, but beyond a certain point increases, affinity for oxygen. The curves obtained on the acid side of this point are not identical in shape with the curves obtained on the alkaline side. The significance of this fact in relation to previous observations on crustacean haemocyanin, and to Rona and Ÿllpo's experiments on haemoglobin, is discussed in the text.

3. The behaviour of the haemocyanin of *Helix* as compared with that of crustacean haemocyanin in relation to the presence of neutral chlorides of the alkaline and alkaline earth metals is even more different. In alkaline medium, the addition of neutral chlorides to the serum depresses the dissociation curve; at a point on the acid side of the critical *p*H referred to in section 2, addition of salts was not found to exert any detectable influence.

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