INVESTIGATIONS ON INVERTEBRATE BLOOD

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Thesis submitted for the degree
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by Kathleen Godwin Pinhey
McGill University,

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The matter of this thesis falls under two heads, first, an inquiry into some of the factors influencing the uptake of oxygen by the respiratory pigment haemocyanin, and second, an elucidation of an enzyme system in the blood of decapod Crustacea. It will be more convenient to discuss these two subjects separately, although experiments on the first led directly to the investigation of the second.

PART 1.

Haemocyanin.

Haemocyanin.

1. Historical.

Haemocyanin is a blue pigment, the commonest respiratory pigment in the blood of the Invertebrata, where it occurs in many species of the Arthropoda and Mollusca, and may be considered analagous to the red pigment haemoglobin of the Vertebrata, though it is vastly inferior as an oxygen carrier, and is, in fact, supplemented by haemoglobin in the tissues of some animals where metabolism is most active, as in the snail, where the respiratory pigment in the blood is haemocyanin, and haemoglobin is present in some of the muscles.

To Frédericq (1878) is due the origin of the name, which he gave to the protein causing the blue colour of the blood in Cephalopoda. Later investigators extended the name to the blue protein in other groups. Scattered references to a blue colour in the blood are common in the literature since Ermann, in 1816, noted a bluish appearance in the blood of the snail, which he thought was due to opalescence. In 1824 Carus reported a blue colour in the body fluid of the crayfish. The first extended experiments on the nature of this colour were done on the blood of the snail by Harless and Bibra in 1847, who established the loss of colour of the blood when exposed to CO_2, and its reappearance when oxygen was readmitted. A strange error is made by these authors in their experiments on Cephalopod blood, which they considered to lose its

colour on exposure to oxygen, and regain it again in an atmosphere of CO_2. In both Mollusc and Cephalopod blood they were able to detect copper, but no iron, and suggest that the protein responsible for the blue colour is combined with this element, as the protein of haemoglobin is combined with iron. The presence of copper in the blood was confirmed by Rouget(1859), but he corrects the error of Harless and Bibra as to the behaviour of Cephalopod blood to CO_2, and shows it to be the same as that of the snail. In 1852 Genth had investigated the blood of the king crab, Limulus, showing that its behaviour towards gases was the same as the other blue bloods, and estimating its copper content. Witting in 1858 extended the same observations to the blood of the mollusc, Unio.

Up to this time, though it had been noted that the loss of colour of the protein was correlated with lack of oxygen, no evidence had been put forward as to the action of the protein as a respiratory pigment in the animal. Bert (1867) observed that in the cephalopod Sepia, the blood entering the gills was colourless, and in the vessels leaving the gills was blue, thus showing that the previously reported behaviour of the pigment in vitro actually occurred in the animal. He found that the pigment was in the serum, and not in the corpuscles, so that the serum, contrary to the conditions which hold in the higher animals, was the seat of the respiratory as well as the nutritive function of the

blood.

The succeeding investigators contributed little to the problem, merely confirming the results of their predecessors, and extending the records of the distribution to other species. The early workers were not concerned with the purity of the protein, and confusing records of the blood colour in the decapod Crustacea were proved by Jolyet and Regnard (1877) to be due to the failure to discriminate between the blue colour due to the respiratory protein, and a rose colour due to the presence in the blood of a lipochrome pigment (later shown to be a tetronerythrin). This pigment they were able to separate by ether extraction; The same authors are responsible for the first determinations of oxygen and carbon-dioxide in the blood. The blood held little more oxygen then sea water, but a comparatively large amount of carbon-dioxide, which results they consider to be the expected ones in animals deriving their oxygen from a liquid medium.

In the next year followed the classical work of Fredericq (1878), in which he suggested haemocyanin as the name of the blue protein. The salt concentration in the blood of the Octopus was the same as that of sea water. The blue protein was the only protein in the blood, as determined by heat and alcohol precipitation, and belonged to the globulin gruop. Decolorisation, as had already been noted many times, could be effected by a vacuum, by an atmosphere of CO_2, or by reducing agents such as sodium sulphide, showing haemocyanin in this respect to be analagous to haemoglobin. This Fredericq

further confirmed by repeating and extending the observations of Bert, that the blood in the vessels coming to the gills is colourless, that in the vessels leaving them blue; and that if respiratory movements were hindered by an unfavourable medium for the animal, or by paralysis, no oxygenation of the blood occurred in the gills, as the blood leaving the gills was pale. He was able to find copper in the blood in large quantities.

Krukenberg (1880) extended the knowledge of the distribution of the pigment, made observations on the copper content and the amount of oxygen carried, and noted that in molluscs (the snail) the ~~blood~~ oxygen was held in firmer combination by the haemocyanin than in the crustacea, a conclusion that has been confirmed by the recent work of Pantin and Hogben(1925) Hogben and Pinhey(1926) Stedman and Stedman(1928), all of whom showed that the oxygen held in combination by the blood of the snail is given up only at very low partial pressures. This same difficulty in reducing snail blood by vacuum or CO_2 had been noted by Cuénot (1892 a), and he confirmed Fredericq's contention that haemocyanin is the only protein in the blood. The spontaneous reduction of the blood on standing he correctly interpreted as due to bacterial action, but in spite of this Phisalix (1900) at a later date considered it a spontaneous chemical reduction in which micro-organisms played no part. Cuénot demonstrated the presence of copper in the haemocyanin molecule, and considered the pigment a rather useless analogue of haemoglobin, as the

oxygen it would take up was little more than could be carried by an equivalent amount of sea water. In working on the decapod Crustacea, he was able to demonstrate in the blood, not only haemocyanin and the lipochrome tetronerythrin, but a second protein, a fibrinogen, confirming Heim's contention(1892) that haemocyanin is not the only protein in the blood of the Crustacea Cuénot also found that the copper was held in loose combination in the molecule, and could be split off by treatment with acid into what he considered to be compounds analagous to the haematin and globin of haemoglobin.

Two other papers, published in 1892, reopened the whole question as to the copper content of haemocyanin, and its value as a respiratory pigment. Griffiths estimated the copper and the oxygen uptake of a large number of haemocyanin-containing bloods, found copper in all (which has since been confirmed by many workers) and also that the bloods could transport large quantities of oxygen. Heim failed to find copper in the blood of Palinurus, various crabs, or the crayfish, but found small quantities in Homarus, Maia and tha Cephalopods. No more oxygen was found in the blood than in sea water, with the exception of Palinurus blood, which contained no copper. Therefore Heim concluded that haemocyanin not only contained no copper, but was valueless as an oxygen carrier. Later workers proved Griffiths' analyses of oxygen high, and were able to demonstrate the presence of copper in all haemocyanin containing bloods.

As much valuable information as to the compounds of

haemoglobin with various gases and reagents had been obtained by the study of the position of the absorption bands of the compounds with the spectroscope, attempts were early made to study haemocyanin by the same method, but up to the present these have been comparatively unsuccessful in showing any changes in the pigment other than that from the oxygenated to the reduced form. By careful technique it is possible to demonstrate a band in the yellow in the neighbourhood of the D line, and the usual bands shown by proteins in the ultra-violet. Frédéricq was the first to attempt the spectroscopic investigation of the pigment, but found only absorption at the blue end of the visible spectrum. Rabuteau and Papillon(1873) obtained the same negative result. Krukenberg (1880) detected a band in the yellow, and absorption at both ends of the visible spectrum, which was less in the reduced form. Halliburton(1885) found no trace of Krukenberg's band in the yellow, nor could MacMunn(1885) find any absorptions in the oxyhaemocyanins of Helix or the Crustacea. More recently Dhéré (Compt. Rend. Acad. Sci. T 146) proved the presence of a band in the yellow in the chrystallised oxyhaemocyanin of the snail, which disappeared when the haemocyanin was reduced; and two protein bands in the ultra-violet. The presence of this band in the yellow has since been demonstrated in various animals by Dhéré(1914-1922) and by Quaglierello (1922), who found a band in the Cephalopods at 5790 A^{o}, and in Limulus at 5630 A^{o}. From the difference in position of the centres of these bands, and of Dhéré's band in Helix at from 3940-3280 A^{o} Quaglierello concludes that

the haemocyanins of different animals are of different chemical structures. More recent determinations of the position of the bands by Svedborg and Heyroth (1929) give the position of the yellow band in Helix at 3400 A^{o} and in Limulus at 3500 A^{o}. No compound analagous to methaemoglobin has as yet been demonstrated.

No information as to the chemical nature of the haemocyanin molecule could be obtained accurately till the protein was separated from the other blood constituents in pure form. In 1901 Henze prepared chrystalline haemocyanin from the blood of Octopus by precipitation with ammonium sulphate, and found the copper content to be 0.377% (average). The copper was in loose combination with the protein part of the molecule, and the amount of oxygen bound by the haemocyanin was 0.4 cc. for 1 gm. haemocyanin, showing that the blood has a real respiratory function. Henze later did further experiments (1904-5), finding that it was impossible to split haemocyanin into protein and protein-free bodies, as the haematin and globin moities of haemoglobin. Kobert (1903) chrystallised the haemocyanin from the blood of Eledone, obtaining chrystals very different from those of the Octopus. Alsberg and Clark(1910) prepared pure haemocyanin from Limulus by precipitation with half-saturated ammonium sulphate, thereby indicating the globulin nature of the protein, but could obtain no chrystals. Nor could they demon-

strate any haematin-like compound.

Later Dhéré, Burdel and Schneider, in a series of papers (1914-1922) published data on the chrystals in many different species, finding them of very different form, but as all the haemocyanins will not precipitate with the same treatment, it is fortunate that this evidence of the difference of the haemocyanin in the various species is confirmed by the differences in the copper content, and by the differences in the oxygen capacities, to be discussed later Quaglierello (1920 et seq) has also published data on haemocyanin chrystallisations.

From these various pure preparations the copper in the molecule has been estimated more accurately than was possible earlier when the estimations were done on whole blood (Griffiths, 1892) (Cuénot, 1891) (Dhéré, 1900, 1903). Kobert (1903), Dhéré, Alsberg and Clark (1910) Quaglierello(1916) determined the percentages of copper in preparations of pure haemocyanin in various bloods. Finally Redfield, Coolidge and Montgomery (1928) worked on the copper and oxygen combining ratio of the blood of Limulus, Loligo, Busycon and several Crustacea, and found that , as Frédéricq had suggested in 1878, one molecule of oxygen combined with haemocyanin per 1 molecule of copper, and Redfield, Coolidge and Shotts (1928) from the determination of the copper content of Limulus haemocyanin worked out the minimal molecular weight of this protein as 36,700. But Cohn (1925) found by ultrafiltration methods that the molecule

of this haemocyanin was intermediate in size between haemoglobin and pseudoglobin, that is, its molecular weight was between 66,800 and 81,000. If there were two atoms of copper in the molecule the weight would be about 73,000, and fit in with Cohn's experiments. Mention should , however, be made of experiments by Svedborg and Chirnoaga (1929) using the sedimentation velocity method which indicate a molecular weight in the region of 5,000,000. The significance of this determination is doubtful, but seems to indicate, at least, that the molecule of Limulus haemocyanin is much larger than the ultrafiltration experiments show, or possibly that this haemocyanin in solution exists as an aggregate of many molecules.

Philippi(1919) has claimed to have identified a pyrrol group in the molecule, but this observation has never been confirmed.

Haemocyanin is an amphoteric substance, and so a buffer of importance in the blood of the animals containing it (Parsons and Parsons, 1923)(Quaglierello, 1920)(Kerridge, 1926). Investigations of the isoelectric point were first undertaken by Quaglierello using the cataphoretic method, and later by Stedman and Stedman(1927) who, using the same method, determined the isoelectric point of Cancer and Homarus at pH 4.7, of Helix at 5.3 (the isoelectric point of Limulus haemocyanin has not been accurately determined, but is at about pH 6.3 (Stedman and Stedman, 1926)).

The haemocyanins are thus a group of chromoproteins ~~containing copper~~

containing copper in the molecule, in a loose combination which is easily broken by treatment with acid, but the split does not result in a compound of a haematin nature The protein combines with oxygen in a 1:1 ratio of copper to oxygen. The evidence of the diversity within the group rests on the difference of position of the absorption band in the yellow part of the spectrum, the differences in facility of chrystallisation, with the variety in the chrystals obtained, the varying amounts of copper in the molecules of haemocyanin from different species, and on differences in oxygen capacity and behaviour towards CO_2, and in the effect on the pigments of temperature, hydrogen ion concentration changes, and changes in salt concentration in the medium in which the haemocyanin is dissolved. For all these reasons it is legitimate to consider the haemocyanins from different animals as different chemical entities.

Since the observations of Jolyet and Regnard on the oxygen uptake of haemocyanin-containing blood, and the contradictory results of Heim, Cuénot and Griffiths, this question has been extensively investigated both in blood and in solutions of the pure protein. Cuénot obtained for Helix the value of 1.15-1.28 cc. oxygen per 100cc. blood, as compared with o.42 cc. for water (fresh, not sea water) Griffith reported oxygen capacities as high as 20 cc. per 100. The worthlessness of Griffiths' analyses were soon

proved by Dhéré (1903) and later by Winterstein(1909). Dhéré's analyses for oxygen and copper are given below, as well as those of Winterstein for oxygen. The results are in good agreement with each other, and Dhéré's prove Cuénot's hypothesis (1892) that the oxygen uptake is in direct proportion to the copper content of the blood.

	Dhéré.		Winterstein.
	% oxygen	Cu.%	% oxygen.
Octopus -------	4.2	23-28.5	4.2-5
Maia ----------	1.6	3.5-4.5	0.5-1
Palinurus ----------			1.4
Carcinus --------	3.9	8.5-10.5	--------
Cancer ----------	1.6	5.5-7.5	--------

Winterstein notes that in the Octopus the blood is almost completely saturated as it leaves the gills. The low fig - ure of γ 0.5 cc. for Maia was the oxygen content of the pericardial blood, but the blood could take up double that amount.

With Limulus, which has a haemocyanin with a great affinity for oxygen, Alsberg and Clark (1914) were unable to demonstrate complete reduction with the vacuum which they obtained. They were unable to find more oxygen in the serum than would be carried by an equivalent quantity of sea water. A solution of pure haemocyanin, containing per volume three times the amount of haemocyanin in the serum, took up 20% more oxygen than sea water, but they consider this amount

too small to justify the opinion that haemocyanin in Limulus functions as a respiratory pigment in the way in which haemoglobin, which gives up its oxygen at low partial pressures, does. However, as the pigment is obviously completely decolorised in the animal, it possibly acted by giving up its oxygen by chemical reaction to substances which were themselves incapable of combining with molecular oxygen.

It has since been shown that both Limulus (Redfield, Redfield and Hurd, 1925) and Helix (Pantin and Hogben, 1925) have a much greater affinity for oxygen at low partial pressures than have the Cephalopods or the Crustacea, which explains the difficulty experienced by Alsberg and Clark in reducing the pigment. To quote the figures of Stedman and Stedman: Limulus haemocyanin is 80% saturated at oxygen tensions of 2 mm.(1925), while the Crustacean protein is 85% saturated at 20 mm.(1926)

Parsons and Parsons (1923) investigated the CO_2 carrying power of the bloods of Maia, Palinurus and Octopus, and found that two to three times as much CO_2 was transported as could be carried in solution in sea water, whereas those bloods which had no haemocyanin (Aplysia, Phallusia) showed no adaptation to CO_2 carriage. The fact that haemocyanin in the blood is on the alkaline side of its isoelectric point and therefore behaves as an acid would assist in the expulsion of the CO_2.

Carbon-dioxide depresses the affinity of most of the haemocyanins for oxygen, their behaviour in this respect being the same as that of haemoglobin (Pantin and Hogben, 1925, Kerridge, 1926, Redfield and Hurd, 1925). But the Bohr effect is apparently reversed in the haemocyanins of Limulus and Busycon, where CO_2 increases the affinity of these proteins for oxygen. But as with increasing hydrogen ion concentration the affinity of haemocyanin for oxygen decreases till a certain "critical pH" is reached, and then increases again with a further increase in hydrogen ion concentration (Hogben, 1926, Hogben and Pinhey, 1926 and 1927) the nature of the first effect of increasing the hydrogen ion concentration depends on whether the haemocyanin in the blood is on the acid or alkaline side of this "critical pH". The work of Rona and Ÿllpo(1916) suggests that the same phenomenon occurs in haemoglobin, where the affinity for oxygen shows an increase at hydrogen ion concentrations greater than pH 6. The experiments of Redfield (1925) were carried out using a colorimetric technique for the estimation of the oxygen saturation of the pigment, and this same method was used by Pantin and Hogben in working out the oxygen dissociation curve of the haemocyanin of Palinurus and Helix, and the effect on the curve of different pHs and of a few salts. In 1925 and 1926 Stedman and Stedman repeated the experiments of Hogben and Pantin on the dissociation curve of some decapod Crustaceà, and the effect of pH on the oxygen uptake of Homarus haemocyanin, using an accurate gas analysis method. From this point the investigations recorded in

the present paper began.

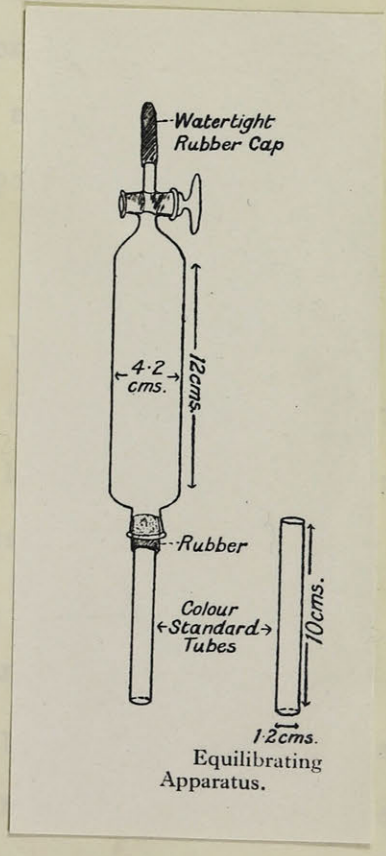
2. Experimental.

From the above survey, it will be clear that to decide definitely the usefulness of haemocyanin as a respiratory pigment in the animals in which it occurs it is essential to know the effect on the oxygen uptake by the the protein of such factors as hydrogen ion concentration, salt concentration, and temperature, as all these have been shown to influence the character of the oxygen dissociation curve. The investigation is based on the colorimetric method of estimating the extent of oxygen saturation of the pigment, taking advantage of the fact that haemocyanin is colorless in the reduced form and deep blue in the oxygenated. That no error is involved in such estimations has been shown directly by Redfield and Hurd (1926) in a series of experiments in which they compared the results obtained by the colorimetric and gasometric methods, and found them identical within the limits of experimental error; and indirectly by the identity of the curves obtained on the same haemocyanins by the colorimetric method of Hogben and Pinhey and the gasometric method of the Stedmans.

Methods.

The blood from the various experimental animals is shaken with chloroform to remove the lipochrome pigment when

present, and to get rid of excess protein in the blood. The precipitate is filtered off, and the clear blue filtrate containing the haemocyanin can be kept over an excess of chloroform on ice. Colour standards are made from suitable dilutions of the serum. As the reduced serum always has a slight opalescence, and in the case of Helix a yellow tint as well, (which cannot be removed, as can the lipochrome pigment of Crustacean blood, with chloroform extraction), the diluting fluid for the standards is matched with the reduced serum of the experimental animals by adding to distilled water slight quantities of egg-albumen and some inert pigment where necessary. In experiments where dialysed serum was used, the colorimeter is of course made from the same dialysed solution of haemocyanin, and where buffered haemocyanin is used in experiments on the salt effect, the control and the colorimeter are buffered to the same extent. The chloroform is removed before using the haemocyanin for an experiment by centrifuging and



Equilibrating Apparatus.

Fig. 1

by blowing through a current of CO_2-free air.

The samples are equilibrated with suitable partial pressures of oxygen in tonometers to which tubes similar to the colorimeter tubes are attached. The equilibrating vessel is shown in Fig. 1: a tube identical with the carefully selected colorimeter tubes is fitted to the neck of the tonometer. The other end of the tonometer has the usual tap, and can be connected by pressure tubing to the pump and the manometer. Since it is not necessary to remove the blood from the vessel in which it is equilibrated, it is unnecessary to prepare gas mixtures to submit the sample to the desired partial pressure of oxygen. The oxygen content of the air is constant, the appropriate correction for the vapour pressure of water at the temperature of the apparatus can be obtained from tables, so that the required partial pressure of oxygen can be calculated by the formula:

$$p = 0.21 (b - m - v),$$

where p is the partial pressure of oxygen required, b is the atmospheric pressure, m is the height of mercury in the manometer, and v is the vapour pressure of water. By connecting the equilibrating vessel with a vacuum pump connected to a manometer, the blood can be exposed to the various partial pressures desired in the experiment. When the pressure has been adjusted to the required value, the stopcock is closed and the tonometer placed in a constant temperature

bath in an arrangement for shaking it. This consisted of five sets of metal strips with spring clips to hold the five tonometers in place, the sets rotating simultaneously at high speed, driven by an electric motor. The complete apparatus is shown in Fig. 2 on the next page. This method enabled one to determine a five point curve with one equilibration. With the pressures in the five tonometers suitably adjusted, a preliminary mixing in the bath was given, after which the pressure in the tonometers was tested to make sure it remained constant. The fluid is then allowed to drain from the large space in the the tonometer into the small tube, air is instantaneously readmitted, and the original pressure of each tube restored. The object of this is to eliminate any error due to the possible giving off of oxygen by the blood, an error which in any case is practically removed by the relatively large air space in the manometer. A second mixing is then carried out.

The sampling tubes are then removed, the fluid allowed to drain into the small tube, and the oxygen estimated by comparison with the colour standards. Using this technique, the determination of a five-point dissociation curve can be achieved in about half an hour.

The previous preparation of a large stock of blood by the chloroform method described above admits of a large number of experiments being done on the same sample of blood, so that several series of experiments can be carried out on a solution of haemocyanin containing the same concentration

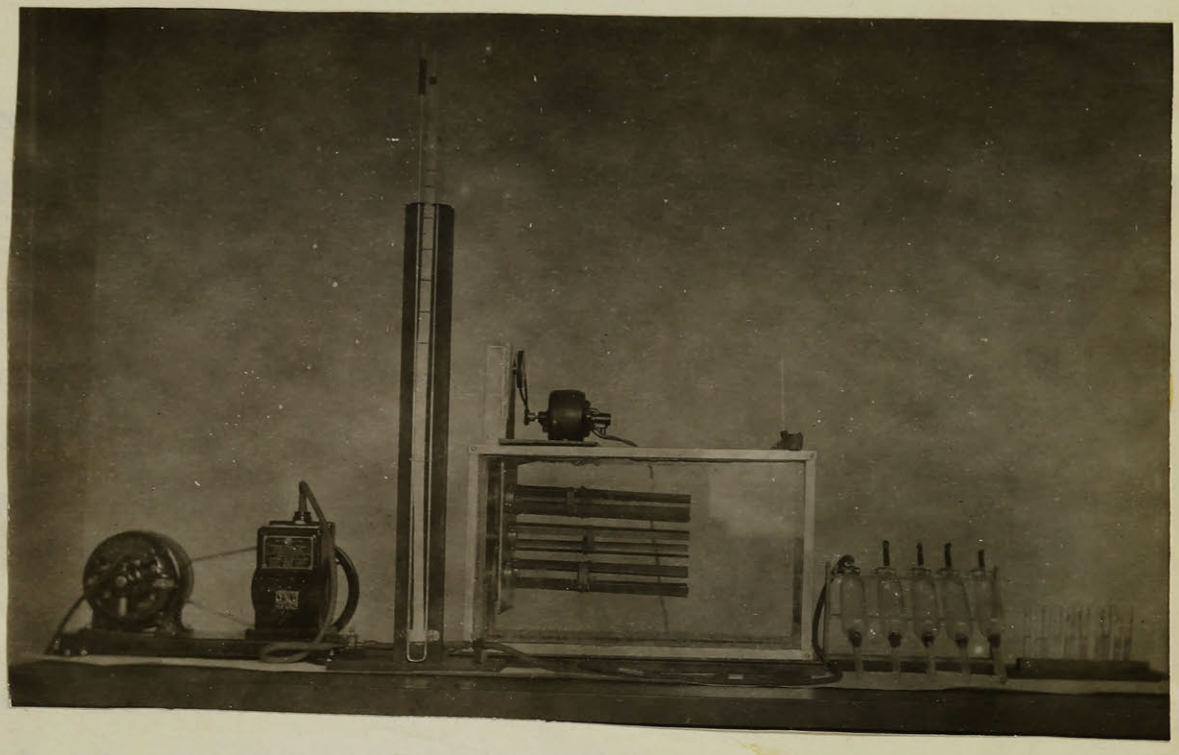


Fig. 2

of protein.

The Effect of Temperature.

Hogben has shown that in the Crustacea, rise of temperature markedly depresses the affinity of the haemocyanins for oxygen. The same will be shown to be true for the haemocyanins of Helix and Limulus, though in the case of Limulus the effect is much less than in the other haemocyanins. In discussing the experiments, the reasoning of Hogben(1926) is followed, in assuming a simple general relation of a stoichiometrical nature between reduced and oxygenated haemocyanin:

$$lHcy_o = mHcy_r + nO_2$$

where l and m are the numbers of molecules of oxygenated (Hcy_o) and reduced (Hcy_r) haemocyanin respectively, n is the least possible number of molecules of oxygen entering into the reaction. If the law of mass action applies we may write :

$$\frac{(Hcy_r)^m . (O_2)^n}{(Hcy_o)^l} = K$$

and for the condition when $lHcy_o$ is equal to $mHcy_r$, that is, when the haemocyanin is 50% saturated with oxygen,

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

$$\text{then } O_2)_{50}^{n} \propto x_{50} \propto K$$

where x_{50} is the partial pressure of oxygen corresponding to 50% saturation of the protein.

Expressing the above in logarithms and in the notation of the calculus, we have

$$n(d\log x_{50})_t = d\log K$$

where t is the temperature.

But the factor for the varying solubility of oxygen at different temperatures must be included, so that

$$n.d\log a x_{50} = d\log K$$

a being the factor for oxygen solubility.

Now, according to the van't Hoff isochore,

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

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

so 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 (T).

For simplicity in plotting, $\log ax_{50}$ is reduced to natural logs

$$\frac{Q}{n} = 2 \times 2.303 \tan \theta$$

where tan θ is the slope of the line.

Q so calculated gives the heat of reaction of 1 gram molecule of oxygen with haemocyanin in the water phase. It should be noted, as pointed out by Hogben, that the heat of solution of oxygen is not taken into account in the calculation.

If the reasoning given above is adequate, the values of Q for the reaction of oxygen at different saturations with the gas should be constant.

In all experiments on the effect of temperature on the oxygen dissociation curve, the haemocyanin sample was kept for one hour at the highest temperature to be used in the experiment, to eliminate any errors in the interpretation of the results which might be due to possible coagulative changes in the protein. The solution so treated was used in making up the colorimeter and for the series of experiments. All the solutions were strongly buffered.

Experiments on Helix.

A typical experiment on Helix is given in Fig. 3.

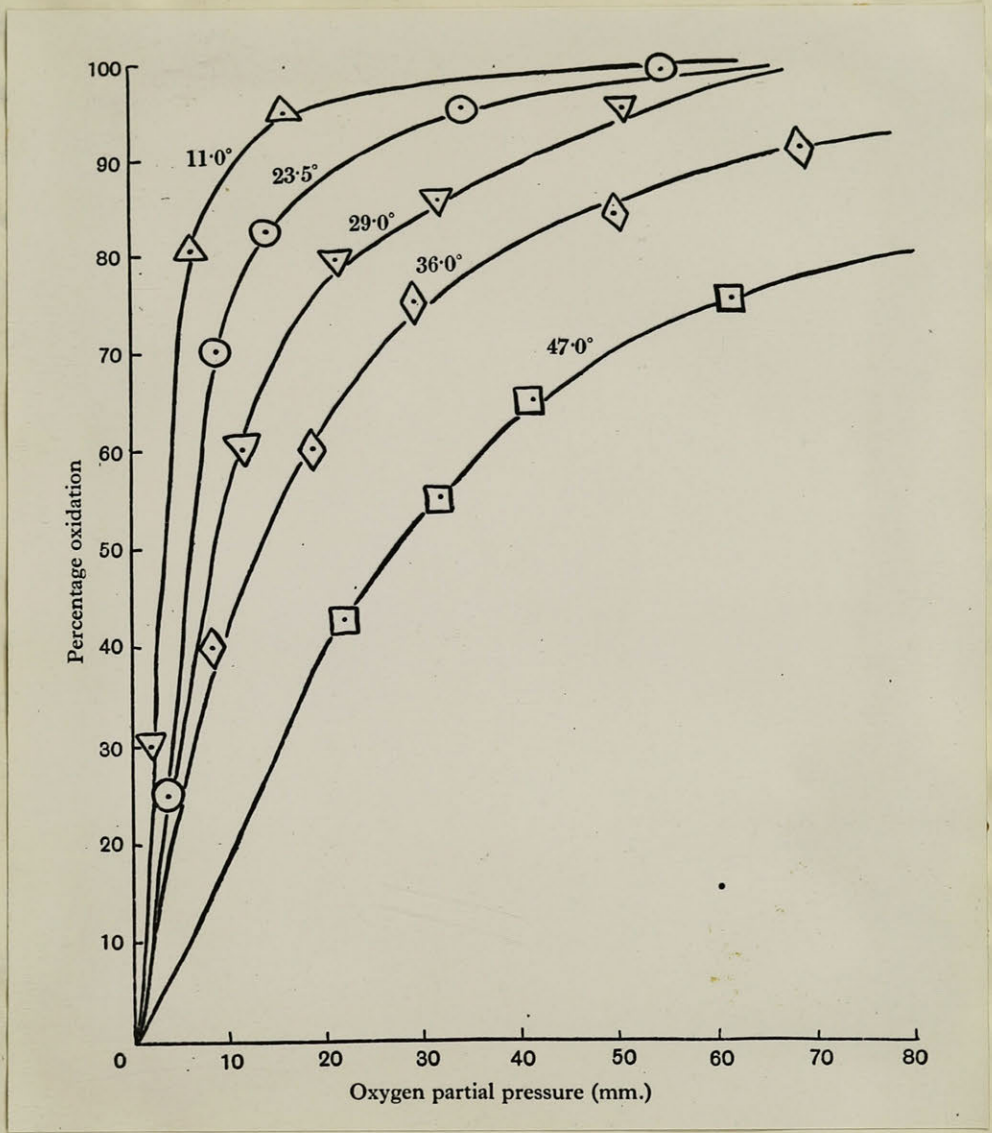

Percentage oxidation
100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Oxygen partial pressure (mm.)
11·0°
23·5°
29·0°
36·0°
47·0°

Fig. 3

The depression of the affinity of the protein for oxygen with increasing temperature is not as great as in the experiments of Hogben on the Crustacea, but is nevertheless considerable. If the results are plotted graphically to determine Q, a value of 8,050 calories is obtained, and as will be seen from the two curves in Fig. 4, the values for the 50 and 75% saturation findings are in good agreement.

Experiments on Maia.

A recalculation of Hogben's experiments on Maia, where the wrong solubility coefficient of oxygen had been used, thus indicating a Q of 9,500, gave Q equal to 9,100 calories, for the reaction with oxygen at 70% saturation. Whether the difference between these figures for Helix and Maia is significant is doubtful, especially as later work on a dialysed solution of Maia gave a much lower value. The result of the experiment on dialysed Maia serum is given in Fig. 5. If Q is calculated in the usual way (Fig. 6) the value is 5,200 calories, between the temperature limits of 13.8° and 36° C. at a pH of 7.7. The temperature coefficient increases suddenly at temperatures above this, but this is probably due to coagulative changes due to heat precipitation of part of the protein (note the difference in the character of the curve at 42° in Fig. 5), so it is not legitimate to include this curve in the calculations. This difference in the value of Q for Maia haemocyanin in the presence and absence of salts

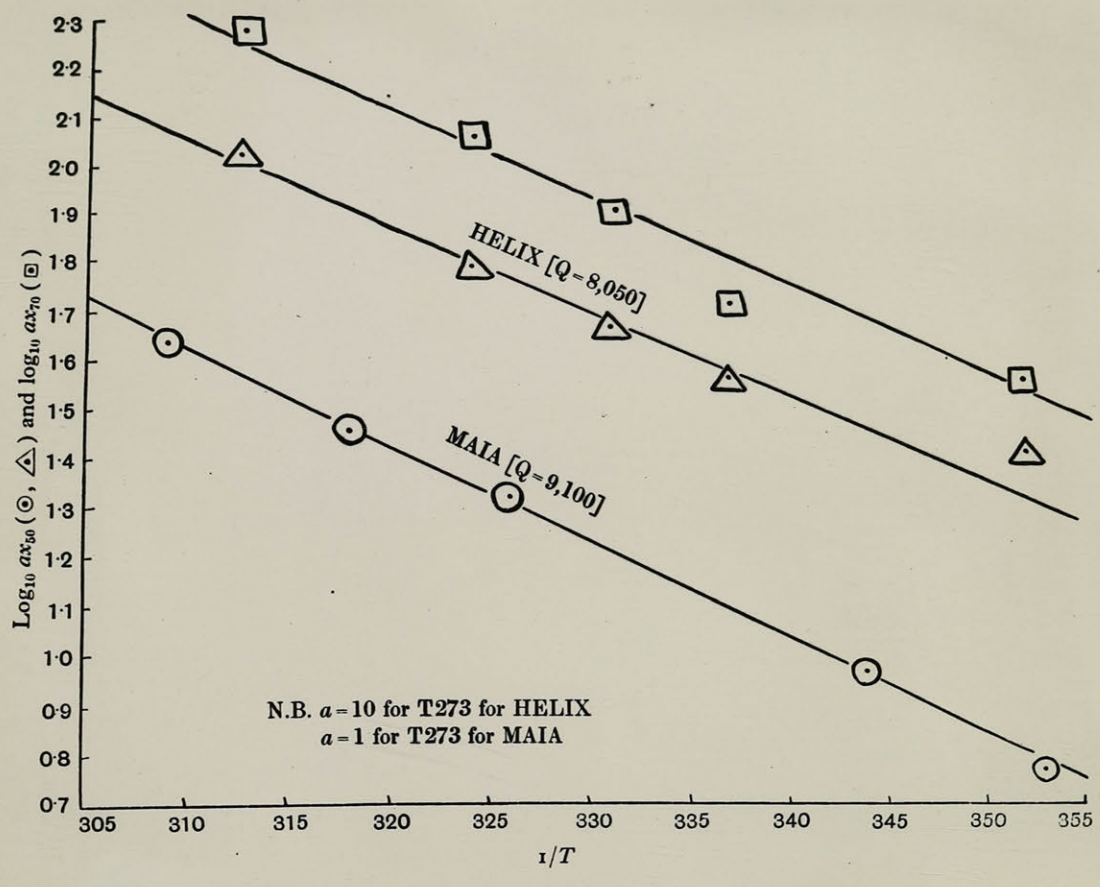

2·3
2·2
2·1
2·0
1·9
1·8
1·7
1·6
1·5
1·4
1·3
1·2
1·1
1·0
0·9
0·8
0·7
Log10 ax50 (⊙, △) and log10 ax70 (⊡)
HELIX [Q = 8,050]
MAIA [Q = 9,100]
N.B. a = 10 for T273 for HELIX
a = 1 for T273 for MAIA
305
310
315
320
325
330
335
340
345
350
355
1/T

Fig. 4.

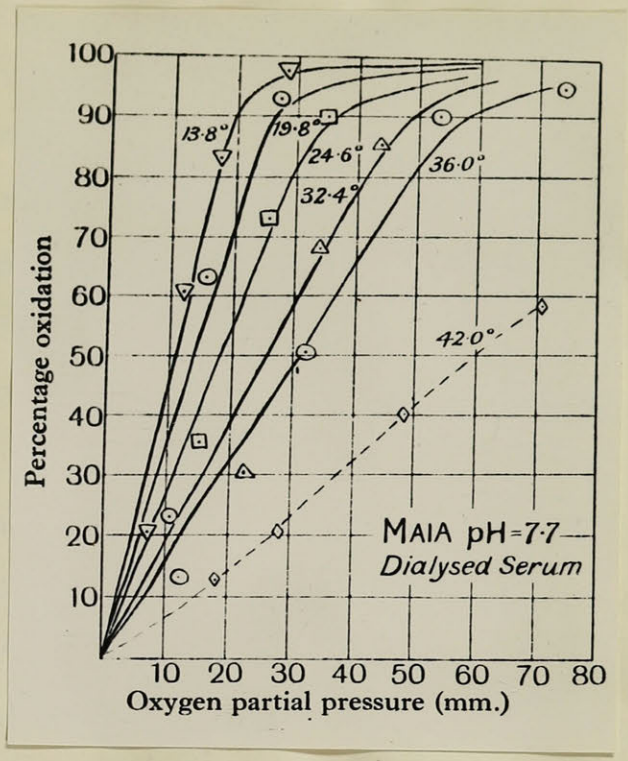

Percentage oxidation
100
90
80
70
60
50
40
30
20
10
13·8°
19·8°
24·6°
32·4°
36·0°
42·0°
MAIA pH=7·7
Dialysed Serum
10 20 30 40 50 60 70 80
Oxygen partial pressure (mm.)

Fig. 5

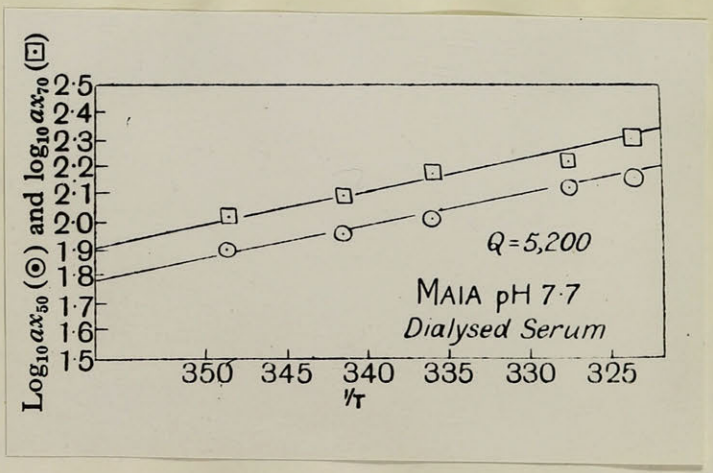

Log10 ax50 (⊙) and log10 ax70 (⊡)
2·5
2·4
2·3
2·2
2·1
2·0
1·9
1·8
1·7
1·6
1·5
Q=5,200
MAIA pH 7·7
Dialysed Serum
350 345 340 335 330 325
1/T

Fig. 6

reopens the whole question of the effect of temperature on the oxygen dissociation curve of the haemocyanins. It is obvious that comparisons can only be made in the complete absence of salts and, as the experiments on the effect of changing hydrogen ion concentration show, at the isoelectric point of the particular protein in question. This had not been determined at the time when these experiments were carried out. The same extraordinary variations in the value of Q are given by the different observers who worked on haemoglobin Adair (Journ. Biol. Chem.63, 1925) gives a table of some of these values, which vary from 10,000 to 27, 000, and which difference he interprets as due to differences in the hydrogen ion and salt concentrations in the haemoglobin solutions used by the various investigators.

Experiments on Limulus.

The effect of temperature on the oxygen uptake of Limulus haemocyanin is extraordinarily samll. Dialysed serum was used in the experiments, and this is less affected by changes in temperature than serum with its normal salt content, as shown by the experiments on Maia. But the highest value for Q for Limulus, from the curves in Fig. 7 where one at 47.5° is included, is 3,150 calories, (Fig. 8) a value significantly lower than the value for dialysed Maia serum. The temperature effect was explored on both sides of the "critical pH", that is, the pH at which the affinity of the haemocyanin

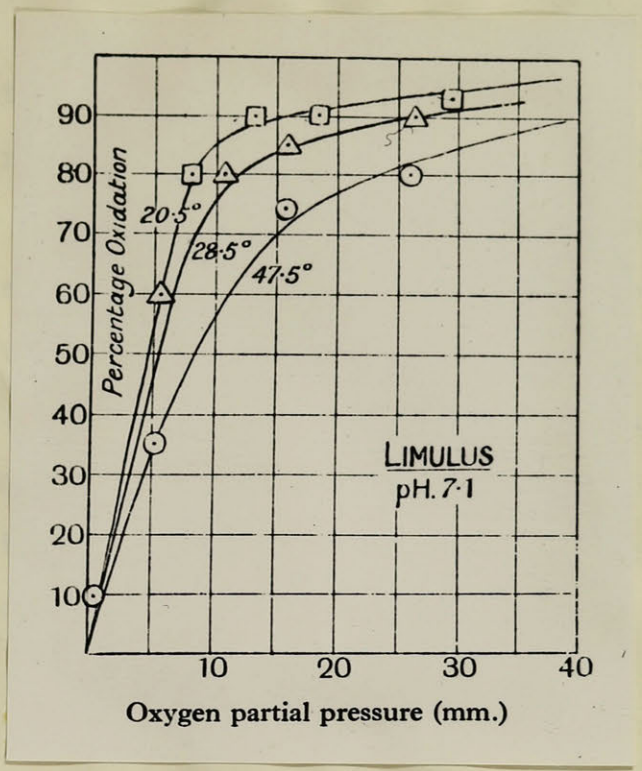

Percentage Oxidation
90
80
70
60
50
40
30
20
10
20·5°
28·5°
47·5°
LIMULUS
pH. 7·1
10
20
30
40
Oxygen partial pressure (mm.)

Fig. 7

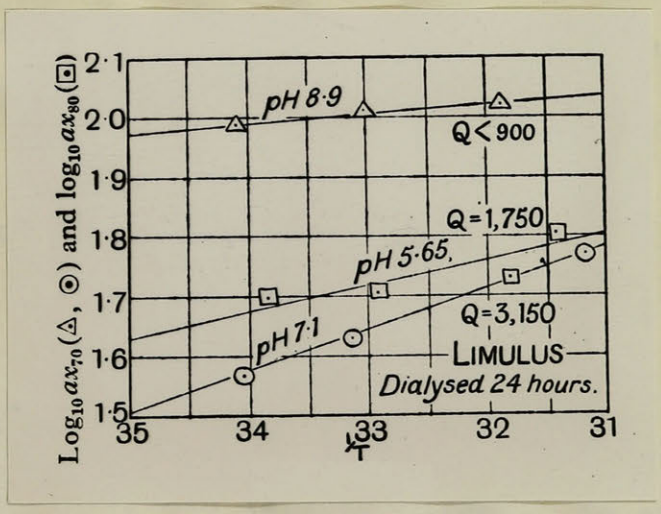

Log10 ax70 (△, ⊙) and log10 ax80 (⊡)
2·1
2·0
1·9
1·8
1·7
1·6
1·5
pH 8·9
Q < 900
Q = 1,750
pH 5·65
pH 7·1
Q = 3,150
LIMULUS
Dialysed 24 hours.
35
34
33
32
31
T

Fig. 8

for oxygen is at a minimum (Figs. 7, 8, 9 and 10). Low values for Q were obtained in every case, that for the serum at pH 8.9 being less than 900 calories. Comparison of these figures with the values for Helix is not possible, as the Helix values were estimated from experiments on undialysed serum, but it is probable that dialysed Helix serum would give a value in the region of the Limulus value of 3,150, since the removal of the salts from Maia haemocyanin reduced the value from 9,100 to 5,200.

The Effect of Hydrogen Ion Concentration.

The desired hydrogen ion concentrations were obtained by the addition of small quantities of phosphate buffers to the serum under investigation, and the values determined by the hydrogen electrode. In the case of snail blood, both the serum used in the series of experiments and that used in the colorimeter were from a stock solution diluted 3 to 7 with distilled water for convenience in colorimetry.

Experiments on Helix.

The effect of changes of pH on the oxygen dissociation curve of snail haemocyanin is very slight. In experiments on Helix aspersa Hogben and Pantin (1925) could find no difference in the curves at pH 8.7, 6.8 and 4.4. In the pres-

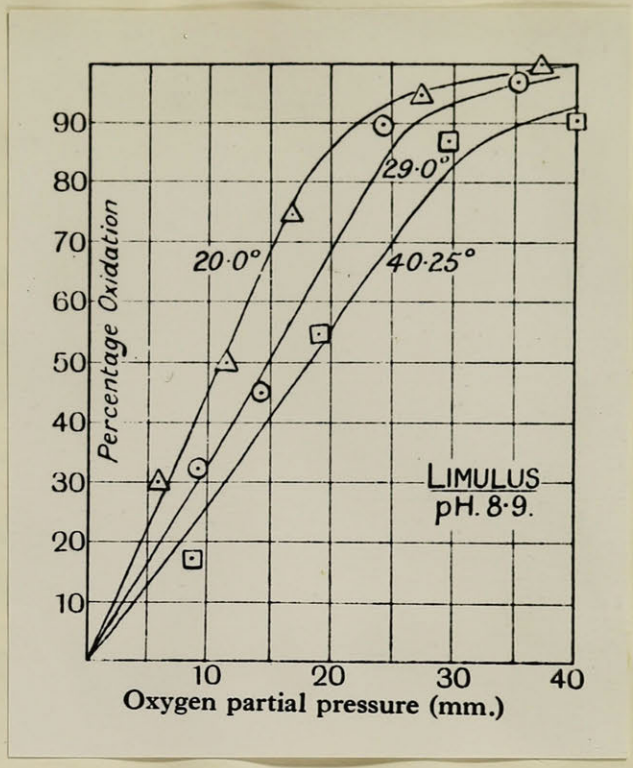

Percentage Oxidation
90
80
70
60
50
40
30
20
10
20·0°
29·0°
40·25°
LIMULUS
pH. 8·9.
10
20
30
40
Oxygen partial pressure (mm.)

Fig. 9

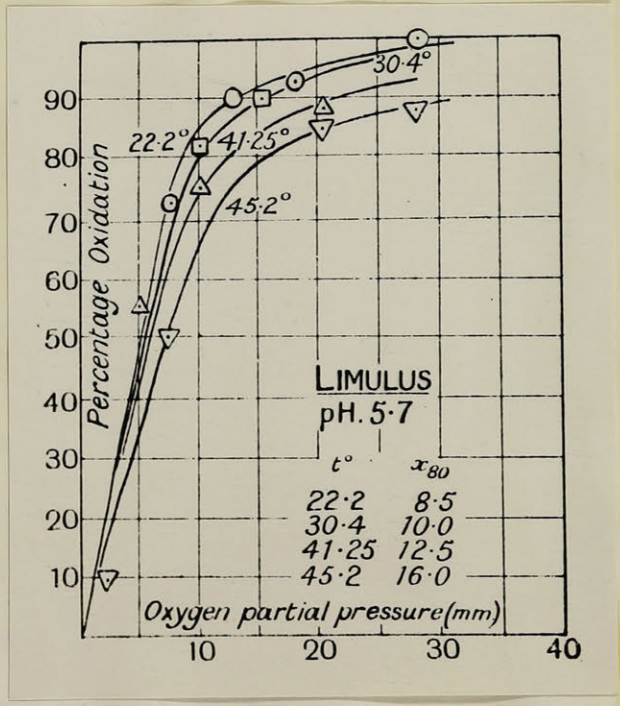

Percentage Oxidation
90
80
70
60
50
40
30
20
10
22·2°
30·4°
41·25°
45·2°
LIMULUS
pH. 5·7
t°
x80
22·2 8·5
30·4 10·0
41·25 12·5
45·2 16·0
Oxygen partial pressure (mm)
10
20
30
40

Fig. 10

ent investigation, Helix pomatia was used as the source of the snail haemocyanin, and the effect on the curve of smaller differences in pH was investigated in dialysed solutions of haemocyanin in serum. A typical experiment is given in Fig 11. It will be seen that the affinity for oxygen passes through a minimum at about pH 8 (calculated from the 50% saturation with oxygen points). There is only a very small differences in the partial pressures corresponding to 50 % saturation between pH 9 and pH 6.8, but that is a real difference is proved by the consistency of the result in four separate sets of experiments. The table gives the values from Fig. 11

pH	x_{50}
9.0	5.0
8.55	9.0
8.15	10.0
7.70	7.5
6.8	6.0

Thus the haemocyanin of Helix pomatia behaves in the same way with increasing hydrogen ion concentration as do the haemocyanins of the Crustacea, and, as will be shown below, the haemocyanin of Limulus; that is, 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

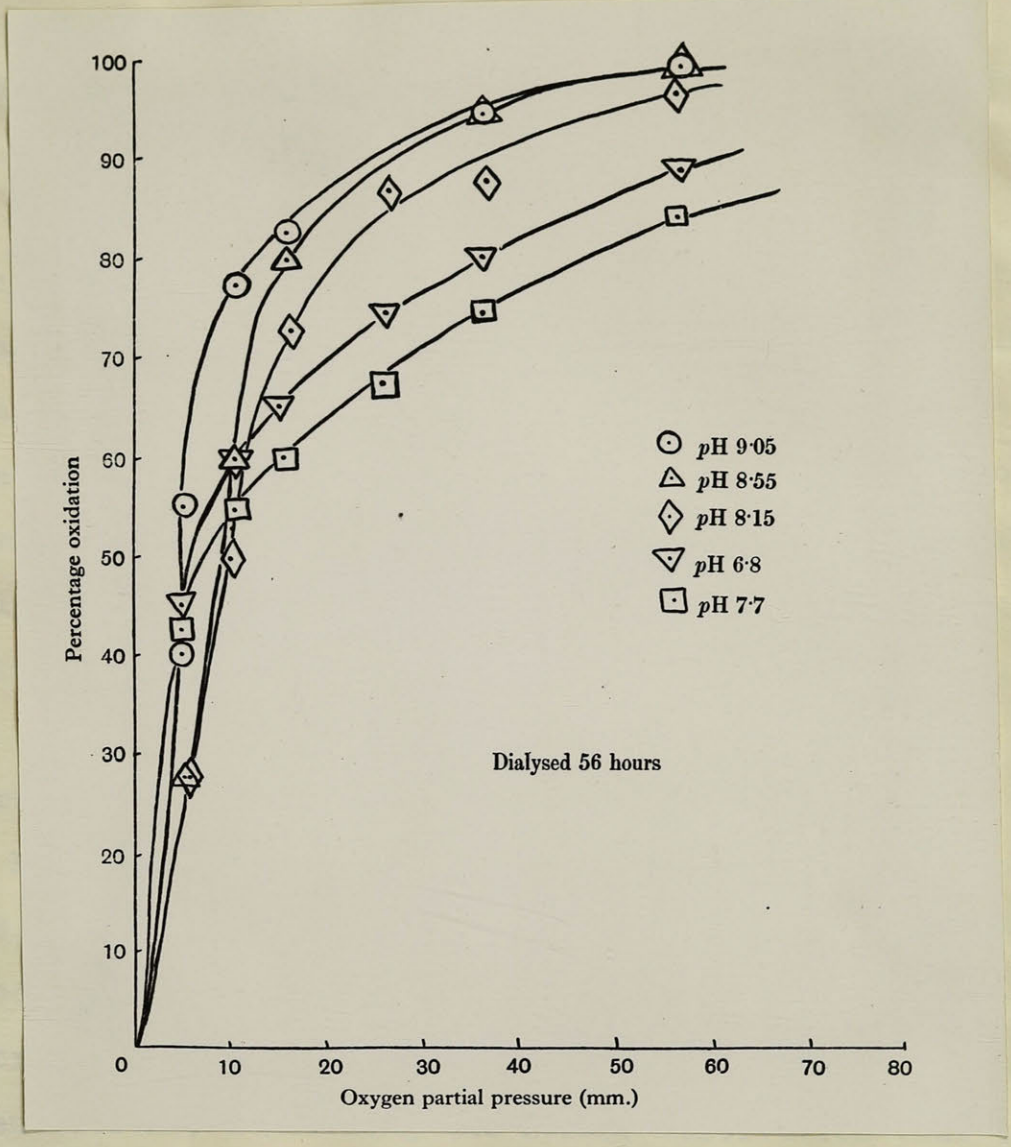

100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Percentage oxidation
Oxygen partial pressure (mm.)
pH 9·05
pH 8·55
pH 8·15
pH 6·8
pH 7·7
Dialysed 56 hours

Fig. 11

it. Though the effect is so slight it is interesting to note that the only appreciable shift in the curve comes in the region of the normal pH of the animal's blood, that is at a pH of 8.5.

More recently the Stedmans(1928), using a dialysed preparation of Helix pomatia serum, have been unable to demonstrate any change in the affinity of the haemocyanin for oxygen with change in pH But as they failed to explore the possibilities in the region of hydrogen ion concentration where the change in the oxygen affinity occurs, namely between pH 7.81 and 8.74, this omission may account for the apparent divergent behaviour of their solutions.

Experiments on Maia.

Hogben has shown that the "critical pH" of Maia blood is at 6.2 pH. As the pH was determined by the indicator method, the experiments have been repeated, using an electrometric method for the determination of the hydrogen ion concentrations, confirming the former observation that this point in Maia is in the neighbourhood of pH 6.2. This consideration is further reinforced by the work of Kerridge (1926) who finds that the buffering power of the blood of Maia is at a maximum for reduced blood at pH 6.39, and for oxygenated at 6.2. The CO_2 dissociation curves do in fact cross in the neighbourhood of 6.3. If, as Parsons and Parsons (1923) have shown, the main buffer action in the blood is due to

the respiratory protein, haemocyanin, itself, and the oxygen affinity is at a minimum at the above point, it follows that the CO_2 dissociation curves of reduced and oxygenated haemocyanin must cross at this point Thus the work of Kerridge is confirmatory of the determination of the oxygen minimal affinity point by investigation of the oxygen dissociation curves.

The Stedmans (1926) have confirmed Hogben's result on the blood of Cancer, that the point of minimal affinity for this species is in the region of pH 7.

Experiments on Limulus.

On the alkaline side of the normal pH of Limulus blood there is a critical pH , on the alkaline side of which increasing hydrogen ion concentration diminishes the affinity of the protein for oxygen, and on the acid side increases it. As the normal pH of Limulus serum is in the range where increasing hydrogen ion concentration increases the affinity of the haemocyanin for oxygen, the apparent reversal of the Bohr effect (Redfield, and Hurd, 1926) is explained. In all the haemocyanins a critical pH has been demonstrated on one side of which the Bohr effect will be reversed, and indications that the same phenomenon occurs in haemoglobin at pHs more acid than pH6 are found in the work of Rona and Yllpo (1916) who found that the affinity of haemoglobin is increased for oxygen at more acid pHs.

For Limulus, this critical pH is between 8.7 and 8.45, and, similar to the effect which can be seen in the curves for the effect of pH changes in Helix, the curves on the acid side are flatter as they approach complete saturation. The presence of this point of minimum affinity in Limulus haemocyanin has been confirmed by Redfield.

The phenomenon first noted by Hogben in the Crustacea is thus shown to be a general characteristic of the whole group of the haemocyanins, but what the nature of the change in the protein at this point may be remains uncertain. At the time of these experiments the possibility that the critical pH coincided with the isoelectric point of the protein could not be excluded, but it was suggested that possibly there existed two tautomeric forms of the pigment with different oxygen affinities. It was thought that the spectroscope might reveal some difference in the protein on the acid and alkaline sides of this point. But experiments carried out in collaboration with F. R. Terroux, in which the spectra obtained were carefully analysed with a spectrophotometer showed only the usual absorption band in the yellow, with no shift in its position on the acid or alkaline sides of the critical pH.

That there is no possibility of the isoelectric point coinciding with this critical pH is now known definitely from the recent experiments of the Stedmans (1927) who determined the isoelectric points of various haemocyanins by the

cataphoretic method, and also correlated the point of minimal affinity of the protein for oxygen with the pH of maximal viscosity. This maximal viscosity, according to Pauli, is due to maximal hydration of the protein, which occurs at the pH of maximal ionisation. The protein may be assumed to take up less oxygen in the ionised than in the comparatively un-ionised state. But this last statement is, as the Stedmans point out, merely an assumption, and whether the ionisation of the protein would affect its affinity for oxygen remains to be proved. To date, however, the hypothesis of the Stedmans is the only contribution to the elucidation of the problem.

The Effect Of Salts.

In Cancer, Maia, and Homarus, Hogben showed that concentrating the serum with neutral salts increased the affinity of the haemocyanins for oxygen at low partial pressures(but no control of the pH of the experiments was attempted) This is analagous to the effect of adding similar salts to a solution of haemoglobin. There are two effects involved here, the specific effect of the anion and kation of the salt, and the effect due to the valency of the kation. In proteins generally, the effect of adding a neutral salt with a common kation on the alkaline side of the isoelectric point is qual-

itatively similar in a general way to the effect of increasing the hydrogen ion concentration. It does not seem by any means certain, and the effects to be described in the case of Helix haemocyanin reinforce this consideration, that the well known effect of chlorides on respiratory pigments is a kation effect. This raises the question as to the possibility of the phenomenon described in Crustacean haemocyanin being an equilibrium in which the complex haemocyanin anion competes with other anions for the available kations: and if there is anything in this possibility, further light might be obtained by a comparison of the effects of such salts as sodium iodide and sodium chloride. On the other hand, as haemocyanin and haemoglobin are proteins, the common kation should have some effect. 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 by Hogben, and confirmed in this paper, to depress the affinity of Helix haemocyanin for oxygen.

Experiments on Helix.

Two series of experiments were carried out. In the first series, the effects of sodium, lithium and magnesium chlorides are recorded (Figs. 12 and 13). When the dissociation curve of a sample of dialysed serum at pH 8.55 diluted with an equal quantity of distilled water is compared with a sample of dialysed serum at the same pH to which an equal quantity of

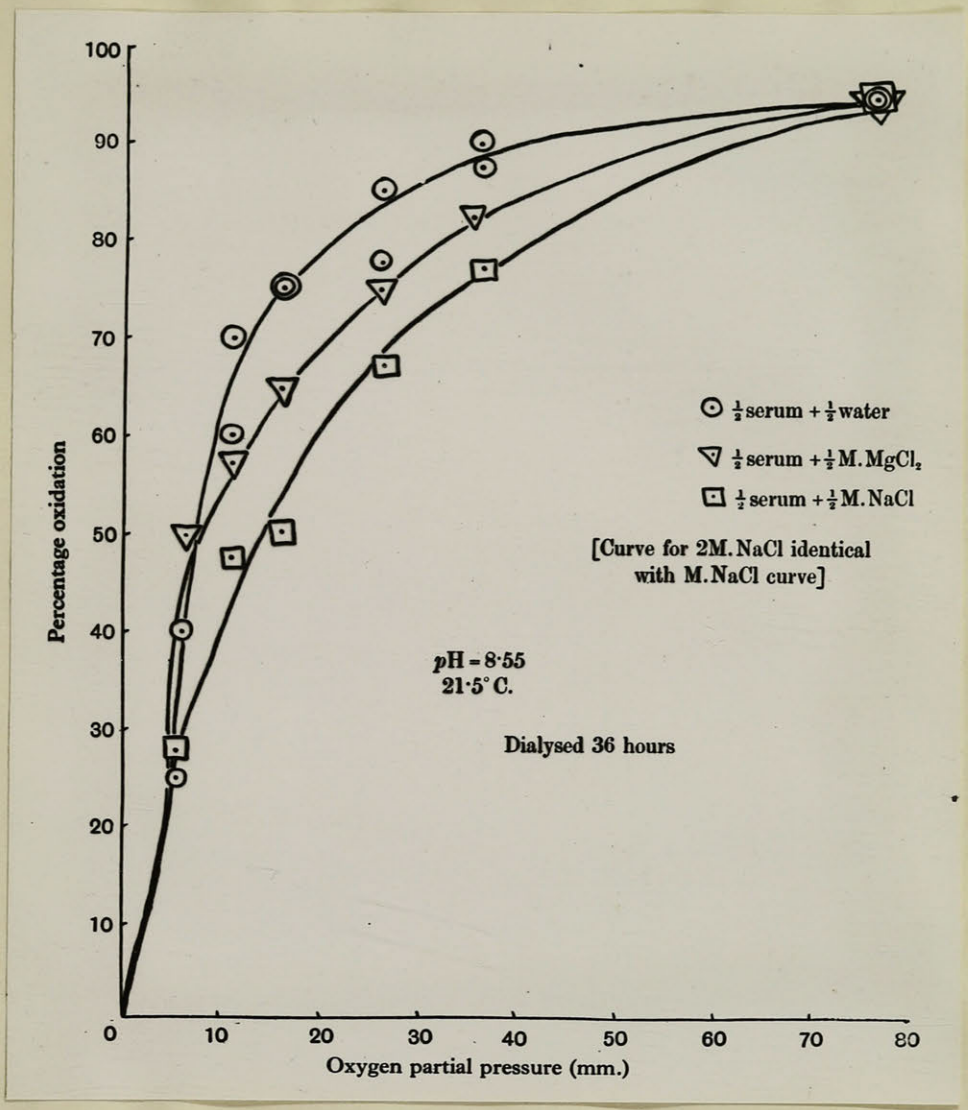

100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Percentage oxidation
Oxygen partial pressure (mm.)
½ serum + ½ water
½ serum + ½ M. MgCl₂
½ serum + ½ M. NaCl
[Curve for 2M. NaCl identical with M. NaCl curve]
pH = 8·55
21·5° C.
Dialysed 36 hours

Fig. 12.

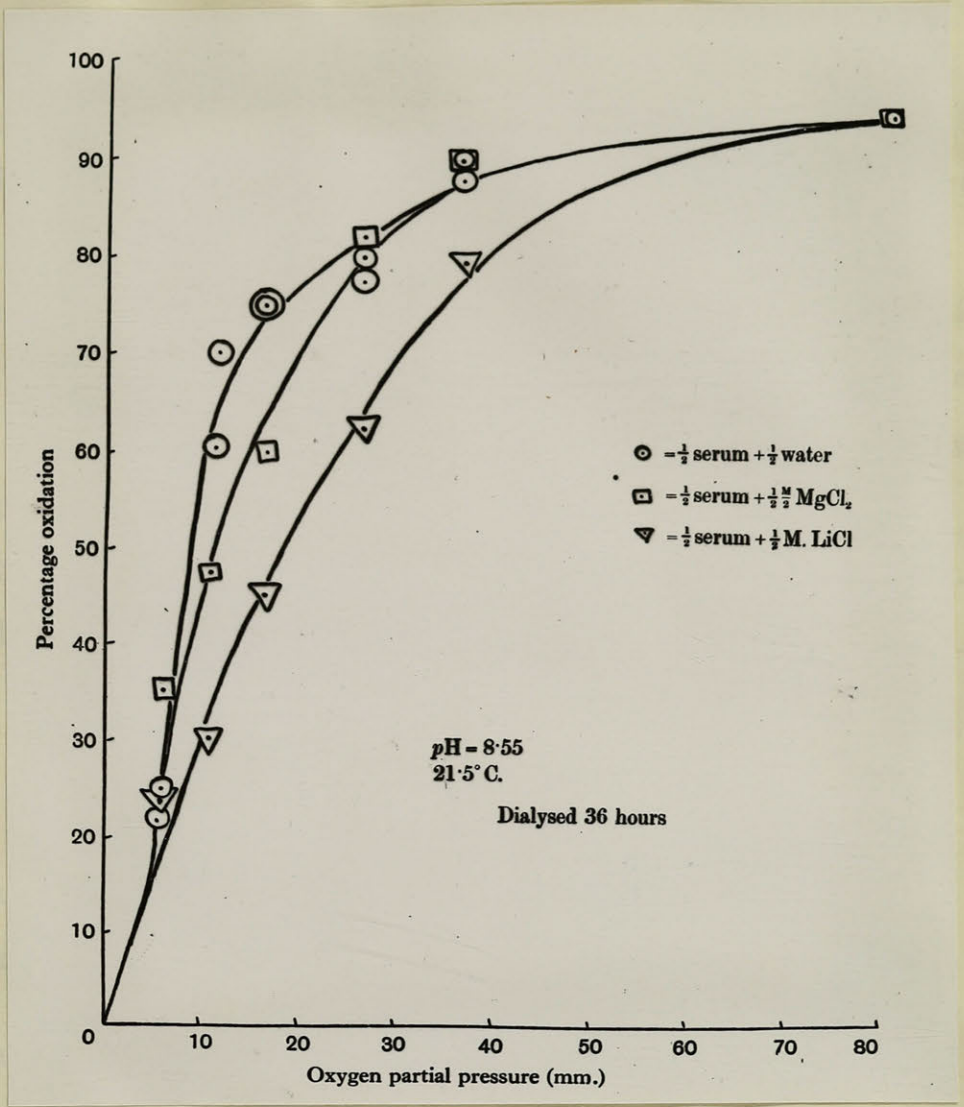

100
90
80
70
60
50
40
30
20
10
0
Percentage oxidation
10
20
30
40
50
60
70
80
Oxygen partial pressure (mm.)
⊙ = ½ serum + ½ water
⊡ = ½ serum + ½ M/2 MgCl₂
▽ = ½ serum + ½ M. LiCl
pH = 8·55
21·5° C.
Dialysed 36 hours

Fig. 13

molar NaCl is added, there is a striking depression of the extent of oxygenation at low tensions in the serum with NaCl. Double this quantity of NaCl (half serum, half 2-molar NaCl) does not further depress the oxygenation (Fig. 12)

The effect of a corresponding amount of LiCl (half serum, half molar LiCl) is the same(Fig. 13). But the addition of $MgCl_2$ in equi-valent quantity produces a significantly smaller effect. Thus, taking the 50 and 75% saturation pressures from the two series of curves, with serum at pH 8.5, the following table gives the results:

Sample.	x_{50}	x_{75}.
half serum, half water (1)	7.5	16.0
(2)	8.0	17.5
half serum, half M. NaCl or half 2M. NaCl	15.0	34.0
half serum, half M. LiCl	17.5	32.5
half serum, half M. $MgCl_2$	8.0	26.0
half serum, half $\frac{M}{2}$.$MgCl_2$	10.0	22.0

From the foregoing observations, it seemed desirable to investigate the effect of increasing the salt concentration at a higher hydrogen ion concentration of serum. By addition of buffer the serum was brought to pH 5.9. Identical dissoc-

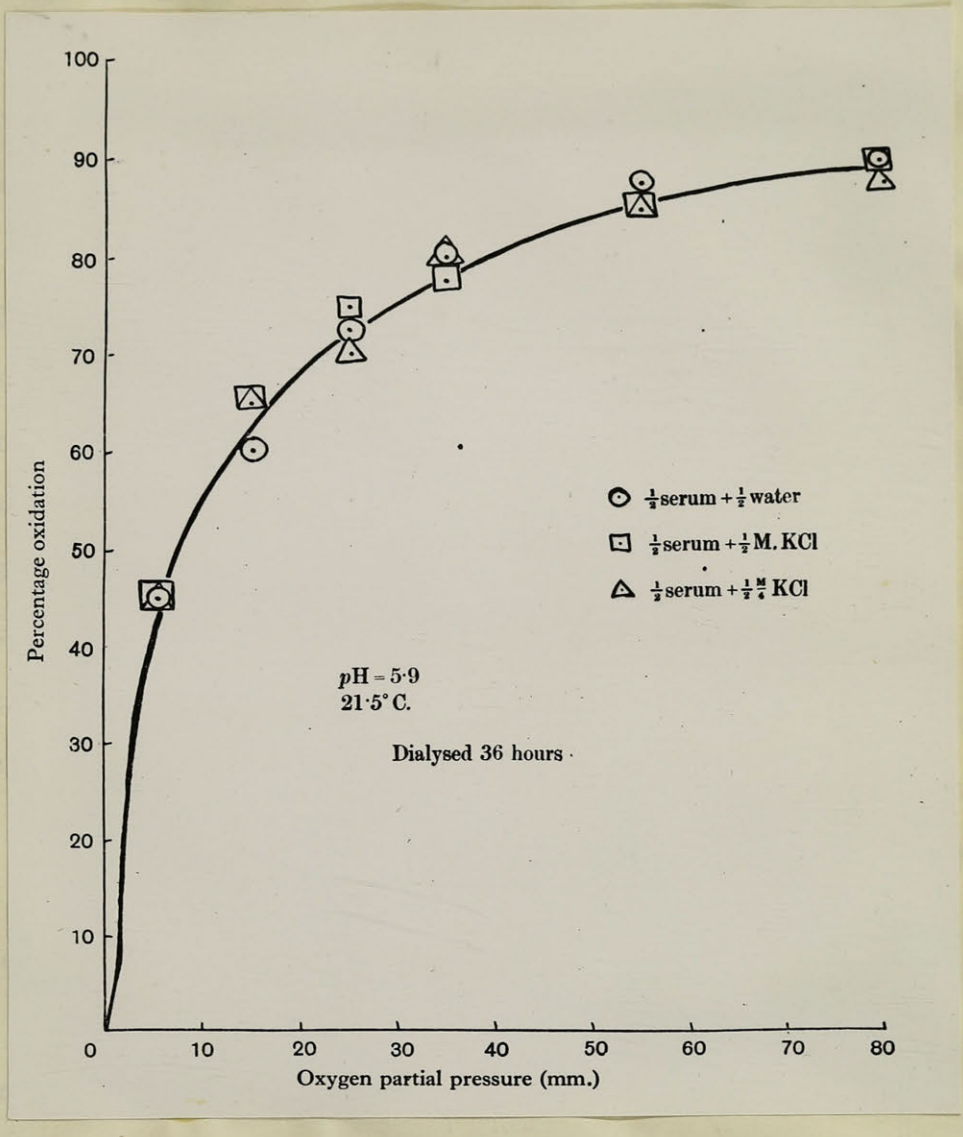

100
90
80
70
60
50
40
30
20
10
0
10
20
30
40
50
60
70
80
Percentage oxidation
Oxygen partial pressure (mm.)
½serum + ½water
½serum + ½M. KCl
½serum + ½ M/6 KCl
pH = 5·9
21·5° C.
Dialysed 36 hours

Fig. 14

iation curves were obtained from serum diluted with an equal quantity of water and serum diluted with equal quantities of NaCl or KCl.

In Fig. 14 the dissociation curve of dialysed serum at pH 5.9 is compared with the dissociation curves of serum roughly isotonic with the snail's own blood (half serum, half fourth molar KCl) and with Crustacean blood (half serum, half molar KCl); all the points can be referred to a single curve within the limits of accuracy of the method. The contrast betweem the behaviour of crustacean haemocyanin and the haemocyanin of Helix in relation to the effect on it of salts is even more striking than in relation to the effect on it of changes in pH. It is evident that a more searching investigation of the relation of ions other than the hydrogen ion to the oxygen affinity of these haemocyanins is required before this discrepancy can be profitable discussed.

Experiments on Maia.

As the haemocyanin of Maia is relatively less affected than that of the other Crustaceans studied by Hogben by changes in pH, this species was chosen for the investigation of the salt effect. As in the case of Homarus (Hogben, 1926) increased salt concentration increases the affinity of Maia haemocyanin for oxygen. In Homarus it was found that the

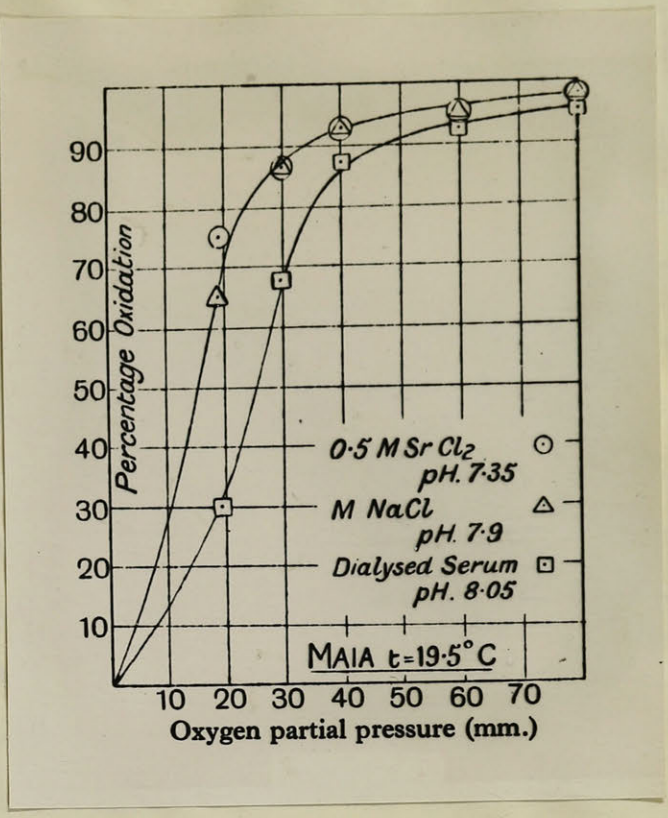

Percentage Oxidation
90
80
70
60
50
40
30
20
10
0·5 M Sr Cl2 pH. 7·35
M NaCl pH. 7·9
Dialysed Serum pH. 8·05
MAIA t=19·5°C
10 20 30 40 50 60 70
Oxygen partial pressure (mm.)

Fig. 15

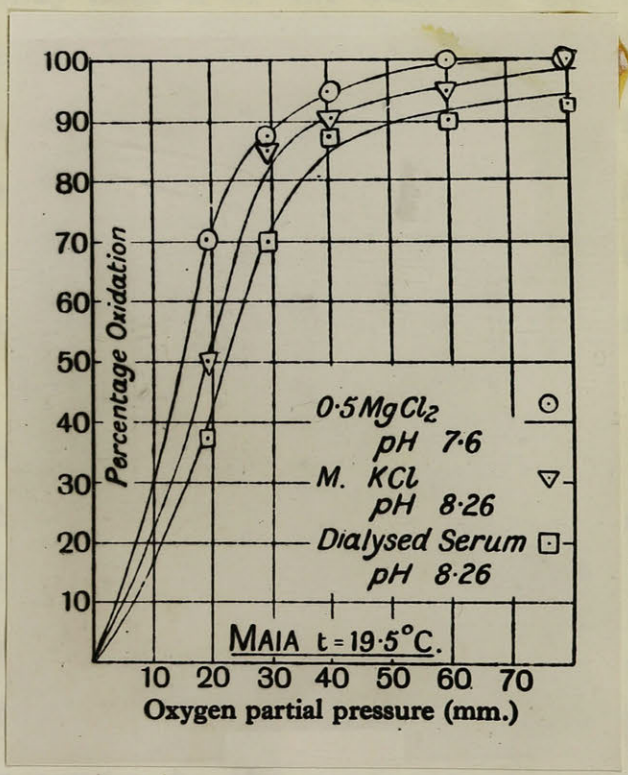

Percentage Oxidation
100
90
80
70
60
50
40
30
20
10
0·5 Mg Cl2 pH 7·6
M. KCl pH 8·26
Dialysed Serum pH 8·26
MAIA t=19·5°C.
10 20 30 40 50 60 70
Oxygen partial pressure (mm.)

Fig. 16

effect of molar concentrations of calcium, strontium and magnesium chlorides was in all cases at least as great as that of 2-molar solutions of the chlorides of sodium, potassium and lithium, when the serum was diluted fifty per cent with the reagent This suggests that it is not primarily the kation which affects the oxygen uptake. The series of experiments in Figs. 15 and 16 show the same general effects of the various salts on the oxygen affinity, but since in one series of experiments (several times repeated) the effect of $MgCl_2$ in half molar concentration was greater than that of KCl in molar concentration, it is evident that the kation effect is not the only action of the salt. (Fig. 16) Furthermore, a single experiment in which equivalent concentrations of KCl, KI and KBr were used revealed no significant difference in the effects of these three salts, though all increased the oxygen uptake at low partial pressures of oxygen, thus affording no evidence of a specific anion effect.

Experiments on Limulus.

As with experiments on the haemocyanin of Maia, dialysed serum was used. Both on the acid (Fig. 17) and on the alkaline sides of the critical pH addition of chlorides, iodides and bromides of potassium depress the affinity of the haemocyanin of Limulus for oxygen to the same extent , but in one

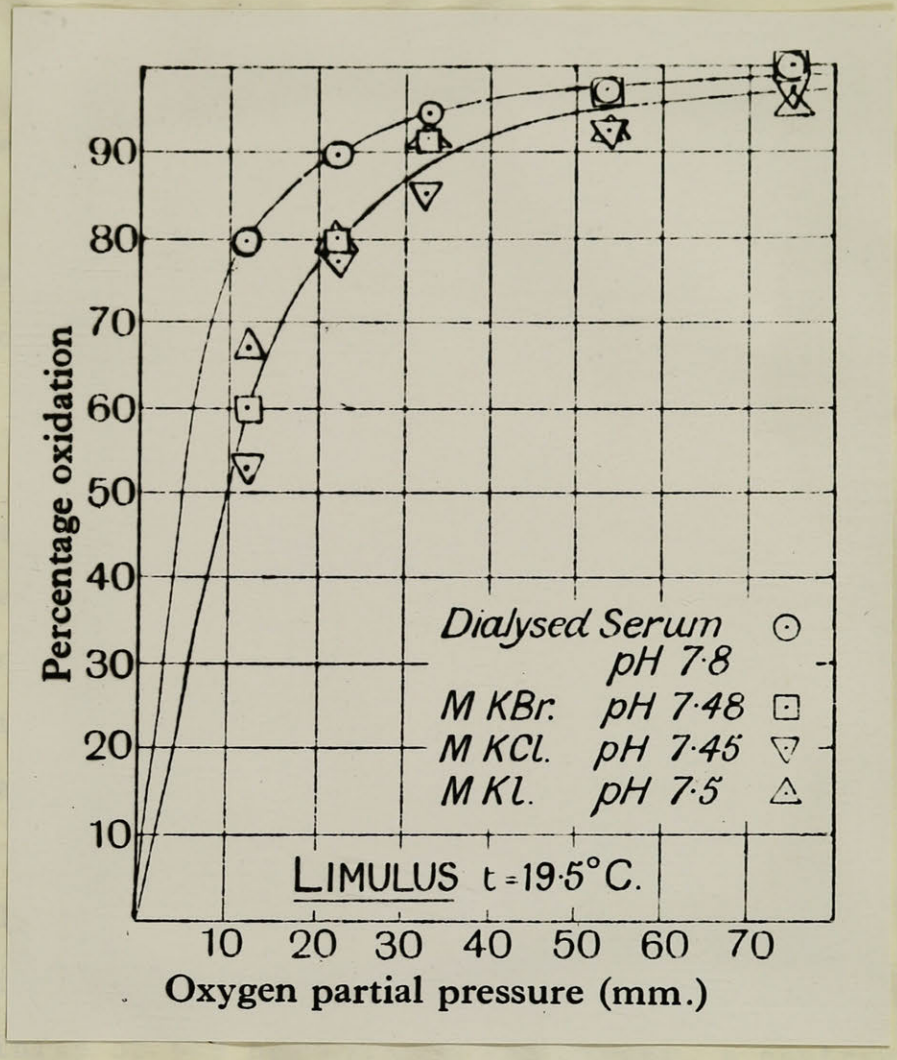

Percentage oxidation
90
80
70
60
50
40
30
20
10
Dialysed Serum pH 7·8
M KBr. pH 7·48
M KCl. pH 7·45
M Kl. pH 7·5
LIMULUS t = 19·5°C.
10 20 30 40 50 60 70
Oxygen partial pressure (mm.)

Fig. 17

experiment the effect of KBr was less marked than that of KCl.

As to the effects of the chlorides of the alkaline earths the experiments were not definite owing to the difficulty of keeping the pH constant unless large quantities of buffers, entailing large increases in the salt concentration, were added to the serum.

It is clear that the extent to which these various effects are due to different affinities for oxygen of the undissociated haemocyanin molecule and the dissociated salts of haemocyanin with different acid or alkaline radicals, cannot be profitably be discussed until the effects of a much greater series of salts are investigated. Indications of the complexity of the problem are given by two experiments in which the influence of a non-electrolyte was tested. Molar concentrations of urea added to serum at pH 7.3 greatly increased the affinity of the haemocyanin for oxygen, a contrary variation to any electrolyte so far investigated.

Summary.

The haemocyanins of Helix, Limulus and Maia are shown to behave very differently towards changes in the hydrogen

ion concentration, salt concentration or temperature of the medium in which they are in solution. The haemocyanins of Helix and Limulus resemble each other more than do either of these the haemocyanins of the Crustacea.

Maia haemocyanin subjected to changes of temperature shows greater depression of its oxygen affinity by increasing temperature than does Helix, and Helix is more affected than Limulus.

The affinity of all the haemocyanins for oxygen is diminished by increasing hydrogen ion concentration till a certain critical pH is reached, after which further increases in hydrogen ion concentration increase the affinity for oxygen. The apparent divergence in the behaviour of Limulus and Busycon (by analogy) with CO_2 may be attributed to the fact that in the blood these proteins are at a pH more acid than the critical pH, so that increase of the hydrogen ion concentration in any way increases the affinity for oxygen.

The effect of salts on the oxygen uptake is evidently a complex of kation and anion effects. In the Crustacea, increasing the salt concentration increases the affinity of the haemocyanin for oxygen. In Limulus, and in Helix on alkaline side of the critical pH, the addition of salts depresses the affinity. Increasing the salt concentration of solutions of Helix haemocyanin on the acid side of the critical pH (at a pH of 5.9) has no effect on the oxygen affintiy of the protein.

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PART 2.

Tyrosinase in Crustacean Blood

TYROSINASE IN CRUSTACEAN BLOOD.

1. Historical.

In the blood of certain crustaceans there is an enzyme system which brings about the formation of a dark pigment, melanin. Heim, in 1892, noticed in crustacean blood black granules, which were insoluble in dilute acids or alkalies, or the usual organic solvents, but were readily dissolved in hot strong mineral acids, producing a brown solution. He thought that they were produced through the action of a tryptic ferment on some protein body in the blood. Furth (1903) first showed that the enzyme concerned in the reaction was a tyrosinase.

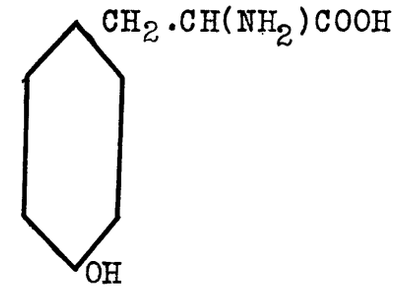
Tyrosinase is an enzyme, or complex of enzymes, which is capable of bringing about the oxidation of tyrosine in the presence of atmospheric oxygen, with the ultimate formation of melanin. It will also oxidise various phenols to coloured compounds, the chemical nature of which is not yet understood, and form oxidation products with pyrogallol and dihydroxyphenylalanine, producing from the latter a melanin identical with that obtained from tyrosine.

The means by which the enzyme brings about these oxidations is not yet understood, although Raper(1923 et seq.) and his collaborators have isolated and identified the series of compounds formed during the oxidation of tyrosine to melanin

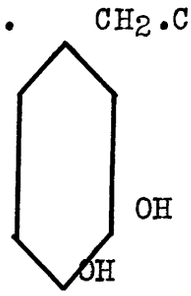
Chodat and Schweizer(1913) isolated ammonia from the products of a tyrosinase-amino acid-para-cresol system, which led Bach to put forward the theory that one of the components of tyrosinase was a de-aminase, which split off NH_3 from the tyrosine molecule, the p-hydroxy phenyl pyruvic acid formed being oxidised to melanin by the oxidase component of the enzyme. Haehn(1919) thought the system was composed of a tyrosinase proper plus a co-enzyme, which was a mixture of inorganic salts. Both these theses have been disproved by Raper and his co-workers. There is an organic substance in new potatoes, and sometimes in old, which will increase the activity of tyrosinase but this is not essential to the action of the enzyme, so the co-enzyme thesis is unnecessary.

When tyrosinase acts on tyrosine, neither p-hydroxy phenyl-pyruvic acid nor NH_3 can be detected at any stage of the reaction, nor does any change in the nitrogen content of the substances formed during the reaction support the theory of a de-amination process. The melanin from tyrosine contains slightly more nitrogen than tyrosine itself. A de-aminase, then, is not a necessary component of the enzyme. But when tyrosinase acts on an amino-acid in the presence of para-cresol or certain other phenols de-amination occurs and NH_3 is freed. This Happold and Raper(1925) consider a secondary reaction, the enzyme first forming an orthoquinone derivative from the phenol, which then attacks the amino-acid, freeing NH_3.

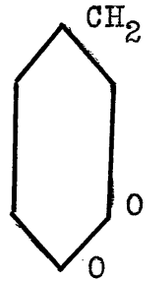
Further discussion of the nature of the enzyme system would not be profitable here. An excellent summary of the various theories is given be Raper in Physiological Reviews for 1928.
The course of the action of tyrosinase on tyrosine is given briefly below: Oxygen from the air is activated by the enzyme in some way not yet explained to bring about the oxidation of tyrosine(1) to di-hydroxyphenylalanine (2). The latter is readily acted on by tyrosinase, and gives rise to a red coloured substance, as tyrosine does, in the early stages of the reaction. The course of the reaction is the same whether the original substrate is tyrosine or di-hydroxyphenylalanine. Further oxidation produces an orthoquinone (3) which undergoes intramolecular change to an indole derivative (4). This is then oxidised to its quinone (5) which is the red substance appearing as the first visible product of the oxidation of tyrosine or dihydroxyphenylalanine by tyrosinase. For all these changes the presence of the enzyme is essential. The further processes involved in the formation of melanin proceed in the presence of atmospheric oxygen, without the action of the enzyme, though it is possible that the enzyme may accelerate them. On decolorising, the red substance gives rise to one of two indole derivatives, either by intramolecular change (6) or by splitting off CO_2 (7). Both these substances are readily oxidisable in alkaline solution by atmospheric air, and are the immediate precursors of melanin. Of the nature of the pigment little can be said-- but it must be a compound containing many of the indole molecules. Various workers give different values for the nitrogen

1. 

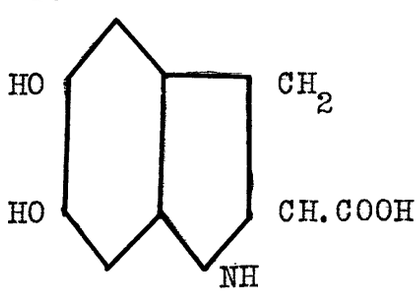
Tyrosine

2. 

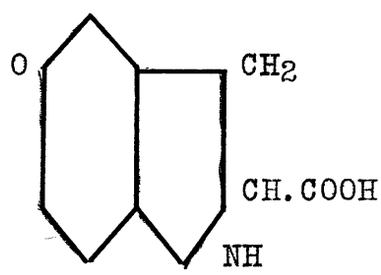
3-4 dihydroxyphenylalanine.

3. 

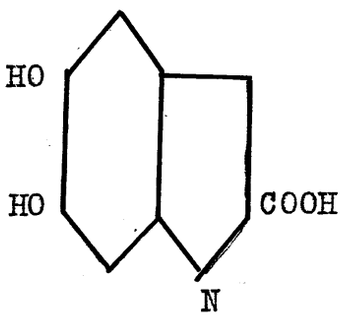
Orthoquinone of (2)

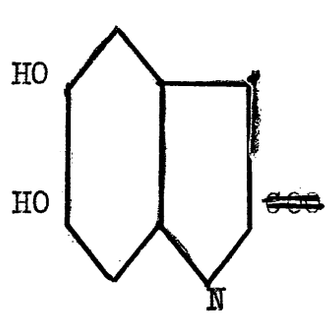
4. 

Indole derivative



Quinone of (4)--red compound.

6. 

7. 

plus CO_2

The precursors of melanin.

content of melanin. The high value of 11.89% was obtained by Young(1914) from the skin of a negro For tumormelanin, Wolff gives 9.34% (1904), Brahm and Schmidtmann 11.03 (in Abderhalden). But Wolff obtained, in two preparations from the same melanosarcoma, 9.75 and 10.24%. Raper found values of 8.65% by the Dumas techmique, and 8.40% by the Kjeldahl. These are in good agreement with Brahm's preparation from melanotic urine. As the melanin prepared from tissues is always reported with a higher nitrogen content than that from liquid sources,it is probable that the the differences found are due to the inclusion in the preparation of varying amounts of tissue protein, rather than to a difference in the different melanins. But it would be premature to say whether melanin, a name loosely given to any naturally occurring black or dark brown pigment, is one substance or a group of related compounds.

2. Distribution

Tyrosinase is a widely distributed enzyme. First noticed in fungi by Bertrand and Bourquelot (1896) it is now known to be widely distributed in vertebrates, and is a component of the enzyme systems of all plants which turn brown on injury (Onslow,1920) It is very plentiful in the potato, which has been most generally used as a source of the enzyme.

In the arthropods, Roques (1903) found tyrosinase in a Trichopteran (Limnophilus flavicornis). The enzyme activity was at a maximum just before pigmentation began in the nymph, and he could get no reaction with tyrosine in the fully pigmented adult, nor did he identify the chromogen on which the enzyme acted.

Gortner (1910) working on the larvae of Tenebrio, the meal worm, established the oxidative nature of the enzyme , and also its thermolability. The larvae of this insect are colorless when shed, blackening in 10-12 hours. This change went on in decapitated larvae in air, but not in an atmosphere of nitrogen or CO_2. Larvae killed by boiling water still showed laccase activity, but no blackening ensued, even when tyrosine was added. The enzyme was therefore destroyed by heat, and was quite distinct from the laccase. In the pupae, darkening is slight, but the presence of an active enzyme could be demonstrated by adding tyrosine. In the adult, blackening again occurred without the addition of tyrosine. Gortner did not identify the chromogen, but states that it, like tyrosine, was not precipitated by phosphotungstic acid

Krukenberg(1908) noticed the same darkening in Hydrophilus but again found variations in the reaction. Some individuals (using the lymph for the experiments) darkened in fifteen minutes, some not in two days.

The activity of tyrosinase from the blood of Crustaceans varied enormously from individual to individual, and in the same individual at different times. This is similar to what Roques found in Tricoptera-- a variation in the enzyme content of the body fluids. In Tenebrio , the opposite occurred--

tyrosinase was always present, but the chromogen was produced intermittently.

In view of the recent work of Schmalfuss and Muller (1927) who identified dihydroxyphenylalanine in the cockchafer (Melolontha), and considered it to be the chromogen giving rise to the dark coloration in these animals, it seems probable that both Roques' and Gortner's chromogen was this substance. In the blood of Cancer and Maia, as will be shown later, the c chromogen is tyrosine itself The chromogen giving rise to the black pigmentation of the eyes and the tips of the walking legs in Maia, and of parts of the carapace in other Crustacea, has not been identified. Verne(1923) states that the melanin formation "est due a l'oxydation de tyrosine contenue dans le pigment amino-acide par une tyrosinase presénte dans les téguments exposés a la lumière"

Among Vertebrates, the presence of a true tyrosinase has not been established. But there are two systems which share something of the nature of a tyrosinase. One of these is Onslow's "tyrosinase" from the skin of rabbits, which oxidised tyrosine in the presence of hydrogen peroxide. The other is the "dopa" oxidase of Bloch and Schaaf (1925) which forms melanin form dihydroxyphenylalanine (dopa) but will not oxidise tyrosine. Neither of these is a true tyrosinase, as neither will oxidise tyrosine in the presence of atmospheric oxygen. But both produce melanins, and the similarity of their action tempts one to suggest that further investigation will show them to be components of the same system as that recognised as tyrosinase.

3. Experimental.

When the blood of certain Crustaceans clots at a wound, the clot ultimately becomes black. This same blackening sometimes becomes troublesome in solutions of haemocyanin(in its natural serum) which are kept for any length of time in the laboratory. This will be shown to be the result of the action of a tyrosinase system in the blood, producing a black pigment, to which the general name melanin can be applied. The present investigation started with the purpose of finding a satisfactory method of preparing a blood solution which would keep without discoloration, without adding to it reagents whose later removal would present difficulties. Other problems presented themselves: granted that the system in question was a tyrosinase, what was its substrate in the blood? No blackening of the blood appears in the body of the animals, but only in clots at wounds, and when the blood is shed. What keeps the enzyme and its substrate apart in the circulating blood? Why are some preparations entirely free from this discoloration, and why does this variation in blackening occur?

Preliminary Experiments.

Blood from Maia, the spider crab, was used for most of the experiments. The enzyme preparation was the clear serum solution with the lipochrome and some proteins removed, as prepared for investigations on haemocyanin. Some preparations were dialysed for three days against running water, others were used without dialysis. These solutions were made by bleeding the animals

at room temperature, extracting the lipochrome pigment by shaking with chloroform, and allowing the precipitate to settle. The clear blue solution is then poured off and kept over an excess of chloroform. This treatment frequently fails to prevent subsequent blackening. Befofe experiments on the tyrosinase activity of the preparation were commenced, the chloroform in the solutions was removed by aeration. When a small quantity of such a solution is added to a tyrosine solution, and air supplied, a red colour develops, turning violet and finally black as the reaction proceeds. These are the characteristic colour changes produced during the action of tyrosinase on tyrosine. The presence of this enzyme was further confirmed by the production of an orange colour with para-cresol Other oxidase systems do not produce this colour, but instead a milky cloudiness, nor will they oxidise tyrosine. Blood solutions which do not blacken on keeping will not give these reactions, and therefore cannot contain the complete enzyme system. But if to some of the blood solution which does not show the presence of the enzyme be added a small amount of blood from a solution which does show blackening, the blood which failed to give the tyrosinase reaction will now discolour, showing that it must contain the substrate, as the blackening is very much more intense than that which could be produced from the the action of the enzyme on the small amount of substrate necessarily added with it Thus the variations in discoloration of various bloods are due to differences in the amount of enzyme present, the substrate being present in both cases.

Methods.

At this stage arose the necessity of estimating the strength of the enzyme in the blood. Raper and Wormall(1923) used an accurate method of allowing the enzyme to act on tyrosine, and at intervals estimating the remaining tyrosine by bromination.

Chodat (1910) had used a colorimetric method, and estimated the complex colour produced by the mixture of red and black pigments occurring during the reaction, using standards of varying quantities of Bismarck brown and corallin.

If crustacean tyrosinase preparations are allowed to act on a solution of tyrosine at pH 8.8,(or any more alkaline pH) and a constant current of air is bubbled through, no trace of red colour appears, as the red compound is oxidised as fast as it is formed. This is due partly to the fact that the enzyme is less active at this low hydrogen ion concentration, and partly because oxidation of the red quinone to the later products of the reaction proceeds more rapidly in alkaline solutions Under these conditions, the first visible sign of the oxidation of the tyrosine is a grey colour, which becomes more intense as the reaction proceeds and more quinone is formed, till the solution becomes inky black As the red colour requires tyrosinase for its formation, and obviously the grey colour can be formed only subsequently to the preliminary formation of the red quinone, the activity of the enzyme becomes the limiting factor in the reaction, and the estimation of the degree of greyness of the solution gives a quantitative measure of the amount of tyrosine converted. There are objections to this method: the

amount of blackness might change with changes in the aggregation of the molecules of pigment in the solution, and this would not necessarily be influenced by the activity of the enzyme. A measure of the oxygen uptake would not necessarily give a more accurate idea of the degree of oxidation of the tyrosine, as this would include the autoxidation of the quinone as well as the the preceding oxidation for which the presence of the enzyme is essential.

The concentration of tyrosinase varies with every preparation. If 0.5 to 1.0 cc. of fluid obtained from grinding potatoes is used for an experiment (the amount of blood preparation used in my experiments) the activity of this preparation at pH 8.8 will not be depressed sufficiently to ensure the instantaneous oxidation of the red compound, which will be formed faster than it can be oxidised under the experimental conditions. With less concentration of enzyme the conditions will be fulfilled at greater hydrogen ion concentrations. The value of pH 8.8 was chosen to cover the most active blood preparations, and all reacting mixtures were strongly buffered to this pH.

A set of standards made from the black solution produced by the action on tyrosine of the enzyme was found to be impracticable, as the melanin tends to precipitate in the course of a few days. Accordingly a set of standards was made from a suitable dilution of india ink. Test tubes of equal bore were selected for the colorimeter. The colorimeter tubes were sealed to prevent evaporation, and the same colorimeter used throughout all the experiments.

The tyrosine-tyrosinase reactions were carried out in similar tubes. Air was supplied through a system of rubber tubing, glass

tipped, leading from the compressed air main. The air was passed through a soda lime tube and washed in water. The rate of bubbling through the reacting mixture was regulated by screw clips Frothing was prevented by a thick layer of medicinal paraffin. Bubbling of the paraffin was checked by a drop or two of capryl alcohol, which is prevented from precipitating the proteins in the blood by the separating paraffin layer.

The importance of carefully controlled pH in experiments with enzymes cannot be overestimated. Except in experiments on the limiting and optimal pH of the enzyme action, every reacting mixture has been buffered with half-saturated carbonate-bicarbonate buffer to pH 8.8, 1 cc. of buffer to 5 or 7 cc. of solution, according to the experiment.

Tyrosinase is a catalyst that disappears during the course of the reaction: if to a tyrosinase-tyrosine mixture which shows no further blackening more blood is added, the reaction will proceed again, showing that the cessation of oxidation was due to failure of the enzyme. The amount of tyrosine which can be oxidised by any blood sample will vary greatly with the varying enzyme content of the blood of different animals. In all experiments the amount of tyrosine present was in excess of that which could be oxidised by the tyrosinase, and in all comparative experiments the amount of tyrosine was the same in every case, so that the concentration of tyrosine had no effect on the rate or endpoint of the reaction, the

concentration of the enzyme being the limiting factor in all cases. It should be pointed out that when, in the curves that follow, 100% oxidation is shown, this does not mean that all the tyrosine present is oxidised, nor that all the tyrosine which the enzyme present could oxidise is oxidised, but that the amount corresponds to the colour of a certain point on the arbitrary scale of the colorimeter. All the values are purely relative.

The Effect of Hydrogen Ion Concentration.

The limits of pH at which the crustacean tyrosinase is active are not significantly different from those found by Raper for potato tyrosinase, or by Venn(1920) for a bacterial enzyme. These two latter Raper, from a study of the products of their oxidation when acting on tyrosine, considers identical systems, and the findings of this investigation suggest the identity of the crustacean tyrosinase with the other two.

To determine the optimum pH for the action of the enzyme, the following series was set up: 4 cc. of 0.05% tyrosine solution in water, 1cc. of Maia blood with the lipochrome removed by chloroform, and either HCl or NaOH to the desired pH The buffering power of such tyrosine-blood mixtures is sufficient to keep the pH constant during an experiment. The volumes in the various tubes were equalised by distilled water. The pH values, as determined by the Clark and Lubs series of indicators, were as follows: pH 4.4, 4.6, 5.5, 6.9, 7.6, 8.0,

8.4, 9.0, 9.6, 10.0, 10.6 As it was not intended to determine the optimum pH within narrower limits than the intervals between the above hydrogen ion concentrations, no correction was made for salt or protein errors. The solution at 5.5 showed slight precipitation, and those at 4.6 and 4.4 heavier precipitation, as these approached the isoelectric point of the blood proteins. Air was blown through as described, and the experiment carried out at room temperature (17° C.) The series showed the characteristic colour range of the tyrosine-tyrosinase reaction. At the end of four hours the oxidation was prodeeding in all the tubes between 5.5 and 10, rose at the acid end and grey at the alkaline end of the range, where more rapid oxidation of the red quinone to melanin takes place. The optimum pH is 8.0; thus the enzyme is most active at a pH close to that of the blood, which is 7.8 It is inhibited below 5.5 and above 10.0. Although the experiment was continued for two days, no reaction occurred in the solutions at 4.4, 4.6, or 10.6.

An experiment using Cancer pagurus instead of Maia squinado as the source of the enzyme gave identical results.

The Effect of Temperature, and the Meaning of the "Temperature Optimum".

The crustacean enzyme, like the tyrosinases from other sources, is thermolabile. Strong preparations will show activity for two hours at 48° C. At 52° C. the blood proteins in

the reacting mixture coagulate in the first five minutes of exposure to this temperature, but if the coagulated mixture is removed from the thermostat and aeration continued at room temperature, it will slowly darken, showing that exposure to the higher temperature has not entirely destroyed the enzyme. If the same proceedure is carried out at 60^{o}, no activity of the enzyme can be demonstrated

Each series of experiments was carried out simultaneously with the same sample of blood as enzyme. The reacting mixture was 4 cc. of 0.05% tyrosine in distilled water, buffered with 1 cc. buffer solution, and with 2 cc. of lipochrome free blood as enzyme. Air was supplied as described, and the tubes immersed in thermostats at the required temperatures.

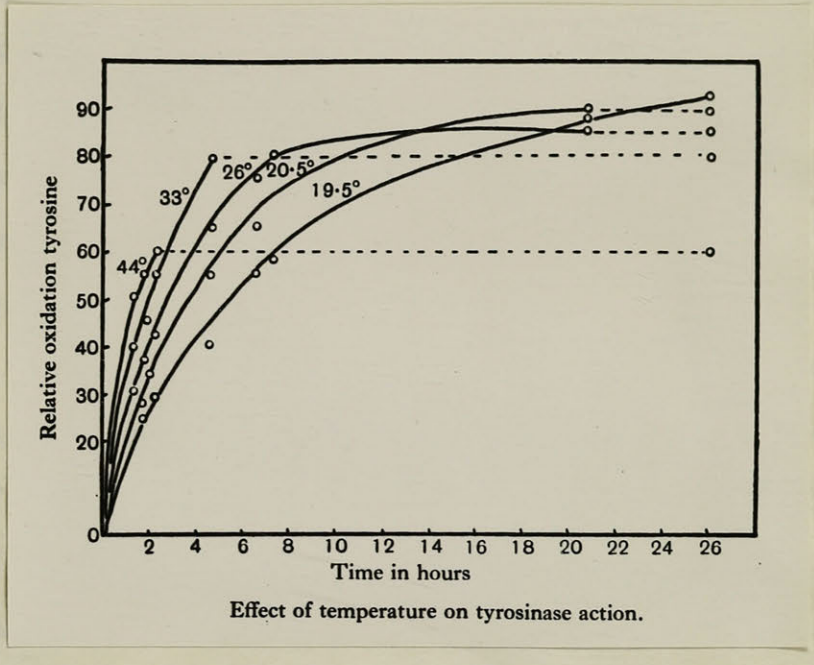
The curves illustrate an important point in the interpretation of the " temperature optimum". This is commonly understood to be the temperature at which the increase in velocity of the reaction due to heat more than compensates the decrease in velocity due to the destruction of the enzyme. Typical of the statements in this connection appearing in many textbooks is the following from Waksman and Davison's book on enzymes(1926): "The velocity of enzyme reactions is accelerated as the temperature is increased until a certain optimum is reached. On further increasing the temperature the reaction velocity begins to diminish until it ceases completely." These authors do point out that this optimum is influenced by the concentration of the enzyme and the nature and concentration of the substrate, and so cannot be

considered a constant for any particular enzyme, but merely for any given set of conditions. But they ignore the time factor. Instances of this could be multiplied from the literature; a common procedure is to allow the enzyme to act at a series of temperatures for some arbitrary time, plot the temperature against the amount of substrate transformed, and consider the highest point of the curve the optimum temperature. What such an "optimum temperature" really means is the temperature producing the greatest resultant velocity for a particular enzyme preparation acting on a particular substrate under certain given conditions for a given time.

Blackman, as long ago as 1905, emphasised the importance of this time factor. Working on the carbon assimilation of green leaves, he found that at high temperatures the rate of decrease in the velocity of the reaction was so great that it was practically impossible to measure the original rate, as the reaction had to proceed for a certain time before sufficient products could be obtained for quantitative estimation. By calculation and experiment he came to the conclusion that up to a certain hypothetical "extinction temperature", where destruction of the enzyme could be considered instantaneous, the initial velocity of the reaction increases the higher the temperature. Bayliss (1925), who quotes Blackman's results defines the "so-called optimum temperature" as "merely an expression of the fact that at a certain temperature the increased velocity due to this raised temperature is more than sufficient for a time only, to counteract the rapid destruction of the en-

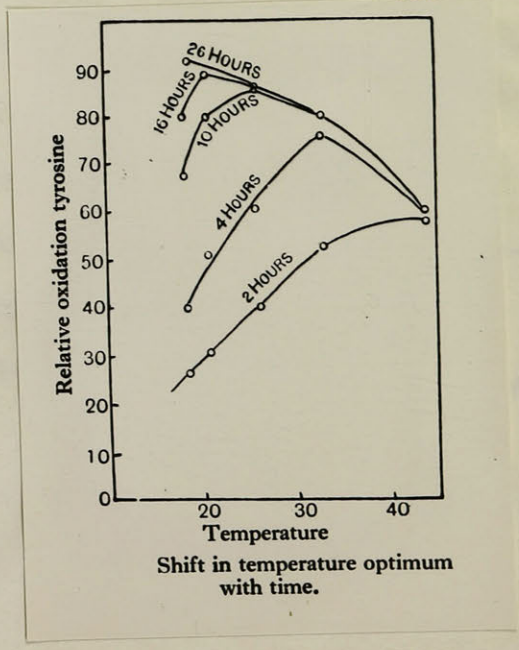
zyme," and rightly concludes that it is negligible, practically and theoretically.

The phenomenon is clearly illustrated below in the action of tyrosinase on tyrosine. At the end of two hours, the apparent optimum is 44° C., at the end of four, 33°, at the end of ten, 26°, and so on (Fig. 1)



Effect of temperature on tyrosinase action.

This same experiment is plotted in Fig. 2 in the usual way for the demonstration of "temperature optima". The shift in this optimum is obvious.



Shift in temperature optimum with time.

Though it is true that in all enzyme systems the initial velocity of the reaction is greater the nearer it approaches the temperature of unstantaneous extinction, the amount of shift of the optimum will vary greatly. In an enzyme system where destruction of the enzyme is not significantly affected by rise in temperature till a certain value is reached, and then is rapidly and increasingly destroyed by further rises in temperature, becoming totally destroyed within the limits of a few degrees, the temperature optimum can shift only within the limiting temperatures of that restricted range where destruction of the enzyme is going on. In tyrosinase, and other enzyme systems where destruction starts in at a relatively low temperature and increases steadily and slowly over a wide range of temperature, the effect is much greater, and the time factor must be taken into account in any discussion of the effect of temperature on the action of these enzymes.

It has been emphasised that the "optimum temperature" depends on the concentration of the enzyme and the conditions of the substrate, and is therefore variable. But this further variation, which may be very great, that the optimum is also dependent on the time factor, is too often ignored.

The rate of destruction of tyrosinase increases with rising temperature to such an extent that no exact estimation of the temperature coefficient can legitimately be made under the conditions of my experiments.

Variation in the Tyrosinase Activity of the Blood.

Blood collected from Cancer or Maia during the spring discolors less than that collected in the same way in the autumn and winter. It was thought that a seasonal variation in the tyrosinase content might be demonstrable, but experiments carried on over eight months led to no result more definite than the above general observation.

The experimental animals were allowed to walk about for about twenty minutes to get rid of the water in the gills, and then bled through a cut in the lower joint of one of the walking legs. A few cc. of blood were collected in a small test tube, then haemorrhage stopped by breaking off the leg at its natural shedding point, where there is a membrane which prevents further bleeding The blood was allowed to stand for half an hour at room temperature for the clot of leucocytes to settle, and 1 cc. of the clear serum used as enzyme. There was no chloroform extraction of the lipochrome. All the samples of blood were treated in the same way, and an arbitrary standard for comparison chosen. Even bloods rich in tyrosinase have ceased to show activity after four hours reaction with tyrosine at 31° C. The standard chosen was the amount of oxidation (measured by comparison of the colour against the standard colorimeter) of 4 cc. of 0.05% tyrosine, buffered with 1 cc. buffer, by 1 cc. serum, in four hours.

The enormous differences in different animals, and in the same animal at different times, are exemplified by the few records set forth in the table below. The blood from the

females showed slightly more discoloration than that from the males, but as only 75 animals were used in the experiments, the small difference found is not sufficient basis for a generalisation to this effect. The experiment was carried out from January to September on the same animals, but as, during a hot week in late August a large number of the animals died, and the survivors were probably in poor condition at the time of the estimations in September, only records from the first two series have been selected.

Animal.	% oxidation.	
	January	April
6	37.5	0.0
10	30.0	0.0
22	65.0	10.0
29	20.0	32.5
32	37.5	42.5
33	10.0	92.5
39	15.0	7.5
43	17.5	25.0
44	35.0	30.0
46	27.5	0.0
49	0.0	17.5
53	35.0	65.0
60	22.5	50.0
63	45.0	45.0
71	0.0	10.0

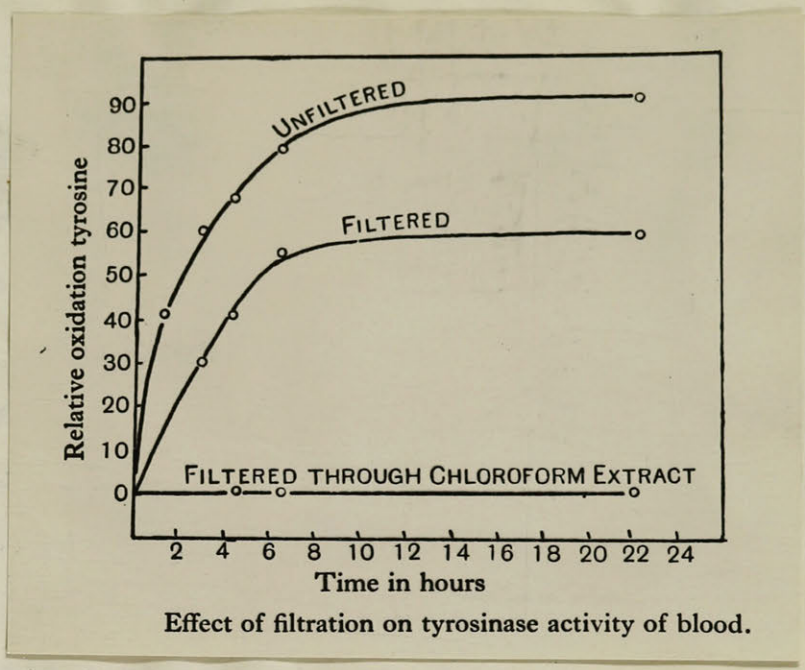
It is not improbable that there exist shorter cycles related to the variation in the number of the leucocytes, upon whose cytolysis, as will be shown later, the tyrosinase activity of the blood depends.

The Effect of Filtration.

Filtration suggested itself as a possible means of removing the enzyme. If the colloidal particles were of sufficient size, filtration through filter paper might be an effective means of checking the discoloration Ultrafiltration is too slow a process for the preparation of large quantities of blood.

The blood of a Maia was treated with chloroform as described and the clear solution poured off. The bulky chloroform precipitate was poured on to a filterpaper, and some of the fluid filtered through it, while a second portion was filtered through the paper alone. When 2 cc. of each of these solutions----the clear decanted solution, the portion of this filtered through the paper, and the portion filtered through the chloroform precipitate on the filter paper--- were tested for tyrosinase activity in the usual way, they showed striking differences.(Fig. 3)

A similar experiment on a different Maia, in which treatment of some of the original decanted solution by shaking with charcoal, and then filtering off the charcoal before using the blood for the experiment, was included, gave confirmatory results. (Fig. 4) Filtering the blood through the chloroform precipitate always depresses the enzyme activity, but does not always, as in the above experiments, remove it entirely. A sure method of keeping blood solutions from discoloration is discussed in the next section.



Effect of filtration on tyrosinase activity of blood.

Fig. 3

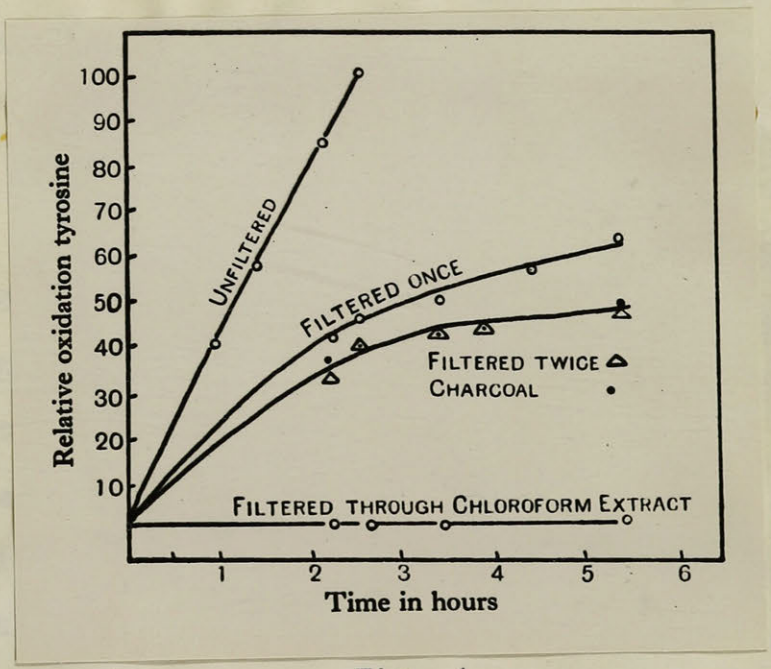


Fig. 4

The Tyrosinase System in the Animal.

The blood circulating in the animal does not discolor, therefore both the substrate and the enzyme cannot be free in the blood, unless one postulates the presence of an anti-enzyme, or the necessity for some activator which comes into action when the blood is shed. The main, or rather the first change in the shed blood is the bursting of the explosive corpuscles (Hardy, 1892). These explosive corpuscles are not present in the Arachnids and Mollusca, and the fact that the haemocyanin containing blood of Limulus, the king crab, and Helix, the snail, do not give a tyrosinase reaction with tyrosine supports the thesis developed below that the enzyme is freed from these corpuscles on their bursting when exposed to air. Qualitative experiments with the blood of Homarus vulgaris, Cancer pagurus(which was used in the pH experiment and in some early temperature experiments) Carcinus maenas and Portunus puber, proved the presence of a tyrosinase in these species as well.

The effect of hindering the cytolysis of the corpuscles is to inhibit the tyrosinase activity of the blood, as is shown by the following experiment. A Maia, packed in ice for three hours to lower the temperature of the blood before it was drawn, was partly bled at 0° C., and the corpuscles allowed to settle at 0° The same Maia was allowed to finish bleeding at room temperature --18.5°. In both cases most of the serum was poured off as soon as the clot had settled. A small quantity was left with the clot for about fifteen minutes at

room temperature.

A series of five test tubes was set up, in all 4 cc. of 0.05% tyrosine solution, 1 cc. buffer, and 2 cc. of the following portions of blood as enzyme:

A. From the blood drawn at 0^{o}

1. serum poured off the clot immediately at 0^{o}.
2. filtrate through filter paper of the above serum
3. serum left soaking with the clot.

B. From the blood drawn at 18.5^{o}.

4. serum poured off the clot immediately.
5. serum which had soaked with the clot.

All the separate portions were allowed to come to room temperature before adding them to the rest of the reacting mixture and commencing the experiment, the series was aerated, and the experiment carried out at room temperature.

The results, plotted in Fig.5, show clearly the increase in tyrosinase activity associated with cytolysis of the corpuscles. The amount of oxidation produced by the serum poured immediately off the cold clot is very slight, only about one-seventh that produced by the serum left soaking with the clots. The tyrosinase activity is obviously associated with the leucocytes, and the longer the serum is left with the clot, the more tyrosinase escapes into the serum. This has been proved by experiments on other Maia, where the amount of oxidation produced increased with the time the serum was left with the clot. The slight oxidation produced by the serum poured off

in the cold is due to the presence in it of a few leucocytes (this can be seen under the microscope), which cytolyse while the serum is being brought to room temperature for the experiment. If these are immediately filtered off while the serum is still near zero, the leucocytes are entirely removed, and with them the tyrosinase, so that no oxidation of tyrosine occurs, as in curve 2, Fig.5. Filtration after the serum has been associated with the corpuscles at room temperature is not successful in hindering the enzyme action, as in this case the enzyme has escaped by cytolysis.

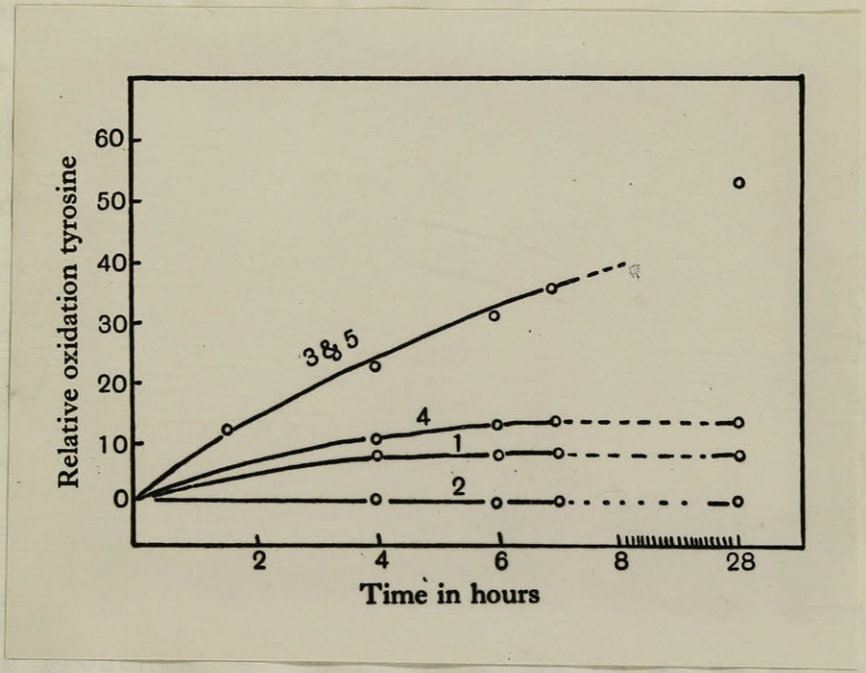


Fig. 5

The leucocytes are not discolored in the animal, so it seemed probable that the substrate was to be looked for in the serum. Early attempts to demonstrate the presence of tyrosine in filtrates from heat coagulums of whole blood, using Millon's or Morner's reagents, were unsuccessful. But free tyrosine may be demonstrated quantitatively in Maia blood by the use of the Folin-Denis (1912) phenol reagent(sodium tungstate, phosphomolybdic acid and phosphoric acid). If the animal is cooled to 0^{o} by packing in ice for three or four hours, and bled at this temperature, the blood proteins precipitated rapidly with Folin and Wu's tungstic acid reagent (1919) (sodium tungstate and sulphuric acid) there is little chance of any breakdown of the blood proteins. The filtrate tested with the phenol reagent gives a positive reaction, the colour can be estimated by a Dubosq colorimeter using a suitable tyrosine standard, and the percentage of tyrosine in the blood found. The average amount in the blood of the several animals tested is 0.004%. It is unlikely that other phenols are present in the blood stream to interfere with the estimation, and Folin and Denis(1912) have shown that tyrosine is the only amino-acid which will give a positive reaction. Further, its presence in the blood would be expected as a normal product of protein digestion. Folin and Denis got positive tests for the presence of tyrosine in the blood of a normal cat by this method, but did not estimate the small quantity present. 0.004% tyrosine is quite sufficient to account for

the degree of blackening in the blood. Later experiments with the blood of animals bled at room temperature, and with small quantities of serum left soaking on the leucocyte clot did not give a higher percentage of tyrosine, so it is concluded that that the tyrosine found in the cold-bled blood is not a product of the breakdown of the proteins under the conditions of the experiment.

The tyrosine content of the blood will vary with the usual increase in amino-acids after digestion of food; there are no available figures for the changes in amino-acid content of crustacean blood, but this change cannot account for the large differences in enzyme activity of the various preparations, as the amount of substrate added with the blood used as enzyme is negligible in the quantity of tyrosine solution supplied as substrate. The quantity of enzyme present per cc. of blood must vary. In the comparative experiments reported earlier, where the enzyme was allowed to escape from the corpuscles through their cytolysis, varying quantities of tyrosinase must have been present in the bloods used. As the enzyme is contained in the leucocytes, either the quantity of enzyme present per leucocyte must change, or the number of leucocytes, or both these vary. What the true facts are must be left for further experiments when the leucocyte count is done simultaneously with the test for tyrosinase activity. It may be mentioned here that experiments on the comparative tyrosinase activity of different bloods showed the same large variation even when the clot was allowed to cytolyse several days in the

serum which was later used for the experiment, indicating that the differences formerly obtained are real differences, and cannot be due merely to a possible unequal cytolysis of the samples in the short time during which the clot was allowed to settle out.

This explanation of the tyrosinase system explains why blackening of the blood occurs only at wounds or when the blood is shed, as it is only when the corpuscles burst or cytolyse that the tyrosinase can come into contact with its substrate, the tyrosine which is normally present in the blood serum.

The Effect of Narcotics.

Warburg(1928) has shown that the reagents inhibiting oxidative processes may be divided into two classes, those that depress the oxidation when present in very dilute concentrations, and those requiring relatively larger concentrations to be effective. In the first class are those reagents which are known to have an affinity for metals, such as the cyanides, sodium pyrophosphate and carbon monoxide; in the second those substances such as the alcohols, the urethanes, various ketones, vanillin and thymol, which have no special affinity for iron or copper. Using charcoal as a respiratory model, Warburg was able to establish an analogy between

the action of the charcoal as a catalyst and that of an enzyme. The surface of the charcoal particle is a mosaic of charcoal and iron, with the latter occupying a small proportion only of the surface area. If the reacting substances be considered as acting through cutting off the charcoal (or enzyme) from its surrounding substrate through becoming themselves adsorbed on the active surface, the following striking facts present themselves: the members of each series in the second class are effective in smaller molecular concentrations the greater their molecular weights. Thus amyl alcohol is more effective than propyl, propyl than butyl, and so on; phenyl urethane is more effective than methyl urethane, methyl than ethyl. By calculation the effective concentrations can be shown to be proportional to the area covered if the reagent be considered to be adsorbed in a mono-molecular layer on the surface of the charcoal, and by analogy the enzyme.

But the cyanides and sodium pyrophosphate are effective in concentrations too dilute to be considered as covering the whole surface of the particle, and so are to be considered, both for this reason and for their known affinity for the metals, as combining selectively with the metallic portion of the surface. And as these small concentrations can inhibit the oxidation of the substrate, Warburg comes to the conclusion that a metal is an essential component of any respiratory or oxidation process. This conclusion is rein-

forced by the fact that small traces of added iron will accelerate the oxidation, and indirectly by the known inclusion of metals in the molecules of respiratory pigments. There are, however, oxidation processes brought about by enzymes whixh are not inhibited by cyanides, such as the aldehyde oxidases (Bernheim, 1928) indicating that there may exist oxidative enzymes in which a metal is not necessary to the reactive part of the molecule.

Haldane(1927) has corroborated the inhibitory effect of CO on respiration in the wax moth, and Keilin(1927) has shown that a polyphenolase present in yeast cells is acted on in the same way by CO and cyanides as Warburg's respiratory model. This enzyme takes part in the respiration of the cell, and is probably the same enzyme as the indophenol oxidase long recognised in the tissues of higher animals, and lately demonstrated in plants This enzyme is extremely sensitive to cyanides.

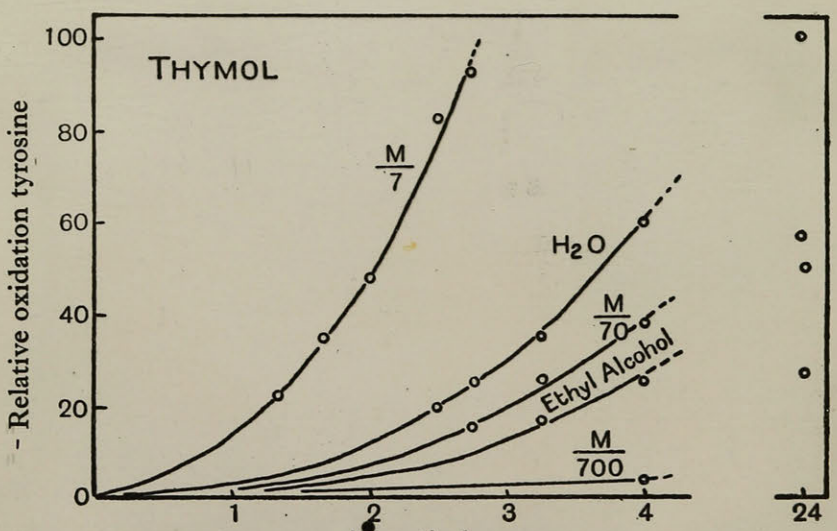
Crustacean blood will accelerate the oxidation of the "Nadi" reagent (dimethylphenylenediamine and a-napthol) to a blue indophenol derivative. But the indophenol reaction of the blood is inhibited only by much higher concentrations of NaCN than those required to inhibit the tyrosinase reaction of the same blood, and in fact at concentrations so high as to suggest that the NaCN is not combining selectively with what must be a very small portion of the molecule. In a typical experiment with blood from the same animal, the tyrosinase reaction was inhibited by $\frac{M}{3500}$ NaCN, and the indophenol reaction only at a concentration of $\frac{M}{70}$ NaCN. The

latter is an extremely high concentration. Recently Szent-Györgyi (1925) has shown that ortho-quinones will autoxidise in air, and will therefore give positive tests for a direct oxidase reaction with guiacum, guiacol, "Nadi" and other autoxidisable substances. In the shed blood traces of ortho-quinones will be present as a result of the action of tyrosinase on tyrosine, and may account for the indophenol reaction of the blood, so that no importance is attached to the presence of this reaction at this juncture.

The amount of any narcotic required to inhibit the reaction will of course depend on the strength of the particular enzyme preparation used, and so will vary from serum to serum, those showing great tyrosinase activity requiring higher concentrations of narcotics for any given effect than those of weaker enzyme activity.

The tyrosinase of the blood may be depressed and inhibited by narcotics of both Warburg's classes. Among the alcohols, ethyl alcohol is effective in lower molecular concentration than methyl, producing a distinct depression of the oxidation of tyrosine in 2.7 molar concentration, while methyl alcohol is ineffective in concentrations of 3.7 molar, as are the low concentrations of the higher alcohols which it is possible to get into watery solution, their insolubility preventing the investigation of a complete series; as tyrosine is almost insoluble in alcohol. The ethyl- methyl result is, however, the expected one from Warburg's thesis.

With thymol, an unexpected phenomenon occurred. This reagent depresses the oxidation of the tyrosine in small concentrations, the inhibition increasing with the concentration up to a certain point, while in greater concentrations it increases the oxidation to an astonishing extent, affecting not only the rate of oxidation but the actual amount of tyrosine oxidised. Phenyl urethane and methyl urethane also increase the oxidation in some concentrations and depress it in others, but the results have not been as regular as those obtained with thymol. In a reacting mixture $\frac{M}{7}$ for thymol, not only was the inhibition due to the ethyl alcohol in which the thymol was introduced removed, but the reaction was more powerful than in a watery solution of the same enzyme preparation. To eliminate the possibility that thymol was acting through a possible further cytolysis of the leucocytes in the serum used as enzyme, the experiments was repeated using filtered blood, with the same results.(Fig.6 below)



Effect of thymol on tyrosinase action.

In the above , and all other experiments with narcotics, the proceedure of the experiments was the same as in the earlier experiments, the reacting mixtures were strongly buffered as before, each series is on blood from the same animal at the same time, and controls are included in every case. In the experiments with narcotics filtered blood was used in all cases.

Thymol and phenyl urethane are necessarily introduced into the reacting mixture in alcoholic solution. The higher concentrations of the reagents, although the alcohol introduced with them is the same quantity as in the dilute concentrations and the alcohol control, produce an obvious emulsion It is suggested that in dilute solutions the thymol becomes adsorbed on the surface of the colloidal enzyme particles, separating them from their substrate; but when the reagent added produces an emulsion, the enzyme, and possibly the substrate, become adsorbed on the surface of the thymol globules, and their contact so assisted The velocity of the reaction of the tyrosinase on the tyrosine is changed, as estimated colorimetrically, and so is the ultimate end-point of the oxidation, as can be seen in Fig. 6 by comparing the twenty-four hour values for the various curves. There is no further change in the depth of colour of the solutions even when kept for several days. The enzyme in contact with its substrate tyrosine does not retain its activity for longer than 30 hours at room temperature, so that any means of hastening the association of the enzyme with its substrate

and thus changing the velocity of the reaction, would also alter the end-point. A word of caution is necessary in considering this theory of the increase in velocity of melanin formation; what is measured colorimetrically is the depth of colour, and not the oxygen uptake of the system. If this should prove to be altered in a different way than the melanin formation, the possibility that the thymol was causing some change in the aggregation of the colored particles, so the product of the reaction was more easily visible, would have to be considered. But the fact that the endpoint of the reaction is affected, as well as the velocity, supports the idea that this is a real alteration in the oxidative process.

If such a change in the physical properties of the solution as that suggested above were in fact taking place, a change might be expected in the viscosity of the reacting mixture. This possibility was tested, with negative results as recorded below. The determination of the viscosity was of course carried out using the same sample of filtered blood in the mixtures as that used in the oxidation experiment with thymol, -- in fact, portions of the same mixtures as those used in the oxidation experiment were used. The table follows on the next page:

Table of viscosities of reacting mixtures.

Experiment.	Solution.	Time of flow between two points for x cc. of solution used.
1.	Distilled water	10.15 sec.
2.	Reacting mixtures of Fig. 6: 3cc 0.06% tyrosine, 1 cc. buffer, 2 cc. filtered serum, plus 1 cc. of the following reagents: water	10.19 10.19
3.	Ethyl alcohol	10.23 10.24
4.	$\frac{M}{1000}$ thymol (in ethyl alc.)	10.30 10.30
5.	$\frac{M}{100}$ " "	10.29 10.30
6.	$\frac{M}{10}$ " "	10.31 10.30
7.	$\frac{M}{1}$ " "	10.29 10.30

There is here no confirmation of the adsorption on thymol globules hypothesis. In view of the crudity of the system used, the tyrosinase representing but one of the possible enzyme reactions of the blood, no further interpretation is possible till some method of preparing a purer tyros-

inase from the blood is found. Experiments on the effect of thymol on the oxidation of tyrosine by a crude preparation of tyrosinase from the potato showed the same phenomenon. It seems worth while to record the anomalous effect above, as if the interpretation be the the true explanation, it supports Warburg's thesis that this type of reagent acts by surface adsorption rather than by chemical combination with some constituent of the enzyme molecule.

Sodium fluoride in $\frac{M}{13}$ concentration depresses the oxidation of the enzyme by about one-half, but complete inhibition is not effected by stronger concentrations.

Sodium pyrophosphate inhibited the reaction of the serum sample used in a concentration of $\frac{M}{150}$.

The effects of NaCN and H_2S are plotted below, in Figs 7 and 8, and require no explanation. The effectiveness of cyanide in small concentrations ($\frac{M}{10,000}$ in one experiment) indicates the presence of a metal in the active group of the molecule. The reversibility of these inhibitions can be demonstrated, in the case of NaCN, by adding an acid buffer to the solution and blowing off theHCN formed; and in the case of H_2S, the aeration of the reacting mixture ultimately removes the H_2S, and the reaction then follows its normal course, as can be seen in the curve for $\frac{M}{3900}$ H_2S in Fig. 8 In the case of the latter reagent, the concentrations of NaSH given are of course the initial concent-

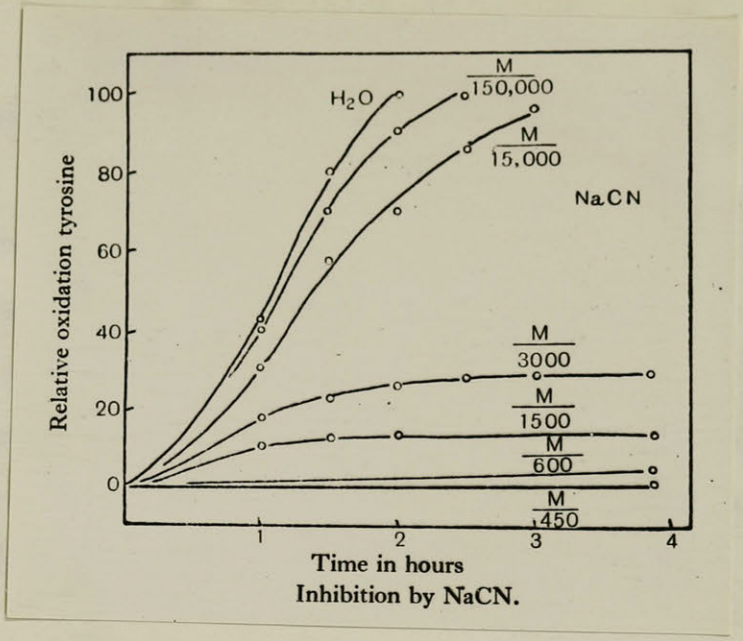

Relative oxidation tyrosine
100
80
60
40
20
0
H2O
M/150,000
M/15,000
NaCN
M/3000
M/1500
M/600
M/450
1
2
3
4
Time in hours
Inhibition by NaCN.

Fig.7

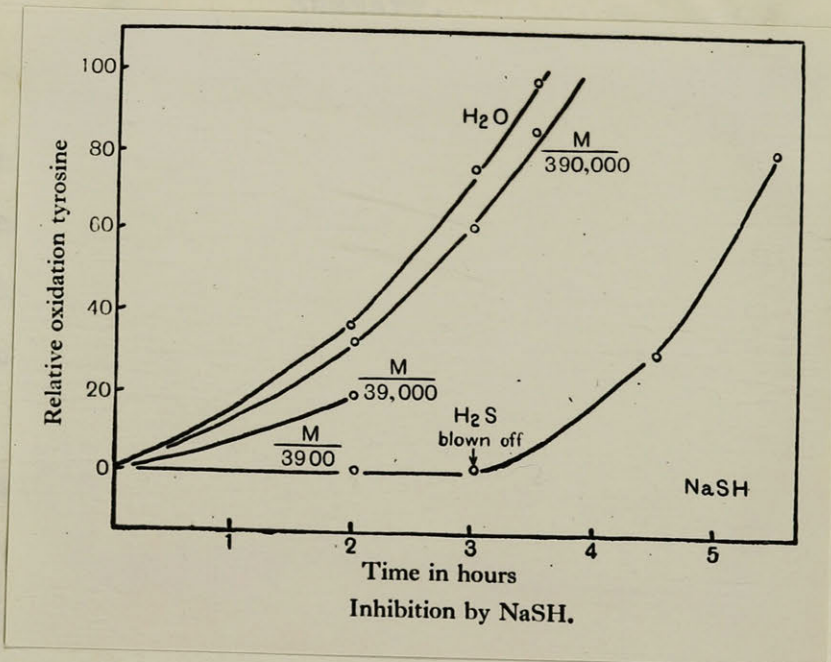

Relative oxidation tyrosine
100
80
60
40
20
0
H2O
M/390,000
M/39,000
M/3900
H2S
blown off
NaSH
1
2
3
4
5
Time in hours
Inhibition by NaSH.

Fig.8

rations in the reacting mixture, the blowing off of the reagent changing these as the experiment proceeds.

No acceleration of the oxidation could be demonstrated on adding small traces of iron, as $FeCl_3$ or copper, as $CuSO_4$. Both these reagents inhibit the oxidation in greater concentrations, $CuSO_4$ at a molar concentration of $\frac{1}{1400}$ (the rate was depressed to one-third by $\frac{M}{14,000}$; no intermediate values were used). $FeCl_3$ depresses the reaction velocity to one-half at $\frac{M}{700}$

Summary.

The blackening of crustacean blood when it is shed, or in clots at wounds, is caused by an enzyme similar to, and probably identical with, the tyrosinase systems previously described in various invertebrates, bacteria, fungi, and the higher plants The components of the system are an enzyme contained in the blood corpuscles, from which it is freed by cytolysis, and its substrate tyrosine, which is free in the blood stream. The enzyme is by definition a tyrosinase, since it will bring about the oxidation of tyrosine with the ultimate production of melanin, deriving the oxygen necessary for the reaction from the air.

The effects of temperature and of pH on the enzyme action are discussed.

The tyrosinase content of the blood is not constant, nor does it undergo a seasonal variation.

The blood will accelerate the oxidation of "Nadi" to the blue indophenol derivative; but as this reaction is comparatively insensitive to cyanides, it is unlikely that it is due to an indophenol oxidase, and is probably caused by the autoxidation of some substance in the blood such as an ortho-quinone.

The tyrosinase action is inhibited by low molecular concentrations of cyanide, indicating the presence of a metallic group as the active part of the enzyme molecule.

The activity of the enzyme can be depressed by H_2S, $CuSO_4$, $FeCl_3$, NaF, sodium pyrophosphate, and the alcohols. Of the last, ethyl is effective in depressing the oxidation in concentrations of 2.7 molar, while methyl tested on the same serum had no effect in concentrations of 3 5 molar. This is the expected result from Warburg's hypothesis, but as tyrosine is insoluble in alcohols, and the amounts of the higher alcohols which could be introduced into the watery solution were too small to have any effect, a series could not be investigated.

Thymol, phenyl and methyl urethane will depress the oxidation of tyrosine in some concentrations, and increase it in others. A possible theory to account for this phenomenon is advanced.

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