Thesis submitted to the Faculty of Science of the University of London, for the degree of Doctor of Philosophy.

August, 1953.

'A study of the haemochromogens found in the intestine of some invertebrates'

-by-

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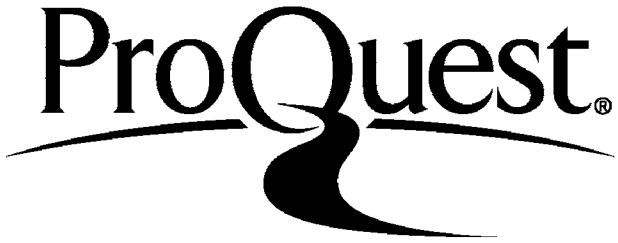

coast.

I am most grateful to my father and brothers, who collected most of the material used for the work described in Section 11.

I would also like to thank Professor J.Roche for kindly lending me a copy of Dr.J.Morena's thesis on helicorubin, and finally all my non-zoological friends who discussed the work with me so willingly.

T he work was financed by a maintenance grant from the Agricultural Research Council, to whom I offer my thanks for their generosity.

CONTENTS

INTRODUCTION

Metalo-porphyrin compounds have long been of fundamental importance to life on this planet. ~~Thus~~ At the present time, life is almost entirely dependent on the photosynthetic activity of green plants, in which chlorophyll, a magnesium porphyrin, plays a major rôle. During this process, light energy is transformed into chemical energy, and the carbohydrates, proteins, fats and vitamins necessary for the life of animals and plants alike are synthesised. At the same time, oxygen is formed, and this is a fundamental requirement of all aerobic cells. Photosynthesis has been of importance for many millions of years, probably long before the first plant fossils found in the Proterozoic and Cambrian (31), but it is of interest that chlorophyll porphyrins, and their derivatives have been extracted from petroleum, probably formed in the Silurian and Ordovician [redacted], and from oil shales, earth waxes, asphalts and coals (33).

Our present knowledge of the origin of life is largely of a speculative and deductive nature, but it seems probable that long before ~~this process~~ photosynthesis was evolved, iron porphyrins, or haems, were already of importance to life and since this thesis is devoted to these compounds it is of interest to discuss their possible evolution.

According to Oparin and Morgulis (27) the first living organisms were anaerobic heterotrophs, and at this period the earth's atmosphere contained little free oxygen, but a relatively high percentage of

hydrogen. Hydrogenase, an enzyme which activates molecular hydrogen, is widespread among micro-organisms to-day, and is found in the butyric acid fermenters which, because of their poor energy metabolism and apparently little integrated enzyme system, are regarded as very primitive (18). Hydrogenase is therefore probably a primitive enzyme. There can be no doubt that hydrogenase is an iron compound (37), and inhibition experiments show that it is either an iron porphyrin (11), or an iron bile pigment compound (10). It seems probable that it is the most primitive enzyme of this type present to-day, and was of great importance to life at this early period.

At the next stage in the earth's history, although still little oxygen was present in the atmosphere, abundant carbon dioxide and probably hydrogen-sulphide appeared. Possibly at this stage the shortage of easily assimilable organic compounds necessitated the first attempts of organisms to utilise sulphur energy, and hydrogenase, which catalyses, among other reactions, the reduction of carbon dioxide by hydrogen-sulphide, may well have been important to life at this period also. It seems likely that this process preceded photosynthesis, since Gaffron (10, 36) found that photo-reduction of carbon dioxide occurred alone in purple bacteria, but side by side with normal photosynthesis in some green algae; and the same has been found to be true of some Cyanophyceae and diatoms (24).

At the next period in the earth's history, according to Oparin, oxygen was produced by the introduction of water as the hydrogen donor

for photosynthesis, and as soon as this occurred, the need arose for the protection of the organism against the action of oxygen. At this period catalase may have been evolved, although this enzyme may have arisen at an earlier period, since it is found in purple bacteria (9).

Following the appearance of atmospheric oxygen, the evolution of all the other haematin enzymes became possible. These with the exception of the cytochromes a_1 and cytochrome oxidase, which are probably formyl porphyrins (20), probably contain the prosthetic group protohaem. The evolution of these compounds therefore, was dependant in the main, on the evolution of suitable protein conjugants, which would both increase the rudimentary catalytic activity resident in the haem, and at the same time increase its stability. In this connection many interesting adaptations, both of the protein components, and of the systems in which the enzymes act, are found in cells to-day (21).

Finally, as the organisms became more integrated and efficient, it seems probable that they increased in size, and that the need must have arisen for the development of special oxygen carriers and stores, and hence to the appearance of haemoglobin, myoglobin, and chlorocruorin. The two former are protohaem compounds, while the last is akin to the cytochromes a (4).

All this probably occurred long before the first fossil records, but it is interesting that haem porphyrin derivatives have been isolated from the fossil excrements (coprolites) of crocodiles of the early

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SECTION I

CHEMICAL INVESTIGATION OF THE NATURE OF HELICORUBIN

HISTORICAL INTRODUCTION

Had the spectroscope not been invented, helicorubin would probably be still undiscovered, and work on this pigment even to the present day has been almost entirely devoted to the study of its spectroscopic properties. But little by little information has been accumulated by this method, until now we have a fair idea of its chemical nature.

At first progress was slow, for discoveries in other branches of Science had not yet been made. For instance, until the work of Dilling in 1910 and that of Anson and Mirsky in 1925 (2) the true nature of haemochromogens was unknown, and it was generally supposed that these compounds gave rise to haematin on oxidation. This led to much confusion and little progress in the earlier literature on helicorubin. Again, much of the earlier literature, such as some of the work of Dastre and Floresco, mentioned below, is inexact and is useful only in indicating profitable fields for further investigation.

In 1876 Sorby[x] examined the crop liquid of a number of Pulmonate Molluscs with a hand spectroscope and saw in them a spectrum similar to that of a haemochromogen prepared from mammalian blood, but displaced to the red end of the spectrum (47)[x]. His careful measurements of the

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[x]It is interesting that the first observation of the spectrum of cytochrome was described in this paper although Sorby thought that it was the spectrum of helicorubin (cf. 33).

oxidised and reduced forms of the pigment and thus to establish a relationship between the pH, and the amount of each of these in his preparation. He found that as the pH was increased the amount of the reduced form increased, until at pH 9.2 the pigment was fully reduced.

Thus it seems that the pigment first discovered by Sorby, and named 'Helicorubin' by Krukenberg, is a protohaemochromogen. The absorption maxima of its various forms, as measured by Vegezzi, and later confirmed by Morena, are given in Table I.

TABLE I

Compound	α band in mμ	β band in mμ	γ band in mμ
Haemochromogen	558.75	526.5	423.7
Helicorubin (alkaline)	562.5	529.7	427.5
Helicorubin (acid)	563.5	529.3	428.0
Oxyhelicorubin	571.7	533.6	415.0

Purification of Helicorubin.

Although Sorby (47) had predicted the presence of at least one other substance besides helicorubin in the crop in 1876, it was not until 1917 that any attempt was made to purify the pigment. In that year Vegezzi and Dhéré showed that helicorubin could be separated from a brown impurity by dialysis (11), and shortly afterwards Dhéré and Baumeler also achieved purification by ultrafiltration (9). Vegezzi

showed further that the brown impurity was a mixture of xanthophylls carotenoids and chlorophyllanes (53) and he called it 'Helicofuschine'.

Morena (38) investigated many methods of purifying helicorubin, including precipitation by ammonium sulphate and acetone, and adsorption onto koalin, finally finding two satisfactory methods. In the first method crop fluid, diluted by half, was run through a 20cm. high column of calcium carbonate, and he claimed that this removed all the carotenoids. The second method which he used was a modification of the ultrafiltration method of Dhéré and Baumeler. Morena states that both methods were good, but his criteria of purity were not satisfactory, and he admits that albuminoids were, and other impurities may have still been present in his preparations.

Thus no satisfactory method has yet been found either for purifying or for measuring the purification of helicorubin.

Chemical Investigations.

1. Oxidation of Helicorubin.

Confirming the observation of Sorby on crude gut fluid, Vegezzi (11, 53) showed that dialysed helicorubin was oxidisable by air only in acid media, and he showed that although no spectrum was then observable in the crude gut fluid, in the dialysate the reduced spectrum gave place in air to a weak, but definite new spectrum. He called this the spectrum of 'Oxyhelicorubin' for he thought that it belonged to the oxygenated form of the pigment. The oxidised form of the pigment, according to him, had a band at 587 mμ.

and could be formed by treating the reduced form with an excess of potassium permanganate. Sorby had also come to this conclusion.

Anson and Mirsky (3) also thought that oxyhelicorubin was the oxygenated form of the pigment because in their experiments, after exposure to air, the reduced spectrum returned in vacuo.

However, conclusive evidence that oxyhelicorubin was the oxidised form of the pigment was finally provided by Morena (38), who showed that the spectrum of a helicorubin dialysate was the same, whether shaken with air in an acid medium or treated at any pH with potassium ferricyanide. Moreover, he found that in acid media, air was not necessary for the conversion of helicorubin to oxyhelicorubin. Like Sorby and Krukenberg, Morena found that helicorubin could not be oxidised by air in alkaline media. Thus previous work has shown that crude or dialysed helicorubin is autoxidisable only in acid solutions. From the work of Morena it seems that oxyhelicorubin is more comparable with methaemoglobin than with oxyhaemoglobin (6) and it thus is more appropriate to call it Methelicorubin. This name will therefore be adopted here.

2. Reduction of Helicorubin.

Like Sorby, Krukenberg (26) thought that the spectrum of helicorubin was only seen in alkaline media, and he thought that ammonium sulphide caused this spectrum to appear because it increased the pH of the solution. However, MacMunn (33) showed that acid

hydrogen sulphide and neutral reducing agents also caused its appearance, and he thus wrote: "... caustic alkalies, no doubt also cause the bands to appear, but that is no argument against reduction and oxidation."

Vegezzi (53), working with a dialysate, described the spectrum produced by alkali. In acid media he thought that reducing agents caused the appearance of a spectrum which differed from the alkaline reduced spectrum in having a twinned α band. He named this latter the spectrum of acid helicorubin.

Morena (38) established a relationship between the pH of his dialysate, and the amount of the reduced form present. He found that at pH 9.2 the pigment was fully reduced and suggested two possible explanations for this fact: either alkali might liberate reducing groups on the pigment itself, or it might liberate or activate reducing substances from impurities in the dialysate. Since his helicorubin solutions were impure, he was unable to decide which alternative was the correct one.

Morena's second important contribution to this subject was in connection with Vegezzi's acid helicorubin. Morena found that not only the α band, but also the β band could be twinned. Further he found that fresh solutions did not show this effect in acid media, but that old solutions often showed it even at pH 7. Moreover it was frequently seen in solutions at pH 10, or even higher. Morena

of hydrazine. No consistent increase in the density of one maximum relative to the other was observed as the pH was lowered. Moreover, twinning was less marked when hydrazine was used than when hydrosulphite was used. The latter compound becomes decomposed in acid media, liberating sulphur and producing a turbid solution. Confirming the work of Morena (38) it was also found that twinning persisted until pH 10 or more in some solutions.

In addition to the twinned α band (53) and the twinned β band (8), it was observed that the Soret band was sometimes twinned and that the maxima were then separated by 3 to 5 mμ.

It may be mentioned here too, that twinning was seen even in the purified solution. This means that if the phenomena were caused by two individual haemochromogens, these must be very similar in nature to have remained in constant proportions throughout the purification treatment.

Oxidised Spectrum.

It has already been said that Vegezzi and others could see no absorption bands in acid crude gut fluid in equilibrium with air. Figure 2 confirms this.

Ultra-violet Spectrum.

In the ultra-violet region of the spectrum the crude liquid absorbs intensely.[x] This is partly due to the presence of

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[x]All measurements of absorption spectra were made with a Beckmann Spectrophotometer with ultra-violet attachment.

helicofuscine (53) and partly to the presence of proteins such as those from the food, digestive enzymes and foreign proteins such as albumins (38) added during extraction of the gut liquid. Figure 1 shows the spectrum of oxidised crop fluid in this region. The maximum at 410 mμ is the Soret peak (the γ band of Vegezzi) while that at 280 mμ is probably caused mainly by proteins (46), but also possibly by carotenoids (2) and other substances such as xanthine and uric acid (19). Since there is no sign of a maximum at 260 mμ nucleic acids are probably not present (4). In the red end of the spectrum there is also a maximum at 670 mμ (which is not shown in the figure). This may be caused by chlorophyll products, and if so it is of interest in connection with MacMunn's extensive research on enterochlorophyll (31, 32, 34).

In the presence of so many impurities it was impossible to learn much of the nature of helicorubin. Investigations were therefore made of means to purify the pigment. Only Morena had done any work on this previously, but as his thesis was not brought to the notice of the author until the work was nearly completed, methods were evolved independently.

Measurement of purification.

1. Estimation of helicorubin concentration.

Dilute sodium hydroxide was added to the various samples until they were just pink to phenol phthalein (pH 9.3). After the addition

of a few crystals of sodium hydrosulphite the density at 563 mμ was measured. This measurement was made as rapidly as possible since autoxidation (1, 29) of the pigment caused the ∝ band to fade rapidly. This density was used as an index of the helicorubin concentration.

2. Estimation of the purity of the solution.

Purification of helicorubin samples was followed by comparing the following ratios in the crude (non-dialysed) gut fluid with those of the solutions (after dialysis).

A. $\frac{\text{Dry weight per ml. of sample}}{\text{Concentration of helicorubin}}$ (i.e. density at 563 mμ)

B. $\frac{\text{Density of liquid at 278 mμ}}{\text{Density of liquid at 563 mμ}}$

The Purification Factors given below are the resultants of the ratios of these quantities in the crude liquid to those in the purified fractions. For the sake of brevity only those calculated by method B will be given in the introductory section.

After treatment both the ultraviolet and visual spectra were examined and if these appeared in any way abnormal, suggesting denaturation, the fraction was discarded.

Preliminary investigations.

1. Ammonium Sulphate Precipitation.

This method has been used satisfactorily in the purification of many substances including cytochrome c (23), whale myoglobin (24) and fumarase (36).

At 20.5°C no precipitate formed below 30% saturation, but helicorubin was precipitated gradually between 30 and 70% saturation. Its solubility was not affected by pH variation between pH 6 and 11.

The purification factors (method B) obtained from two successive precipitation treatments at 20.5°C and pH 6.0 at 70% saturation were 2.1 and 3.3. These were accompanied by about 20 and 40% loss of the pigment.

Thus purification was not great by this method, probably because precipitation occurred over too wide a range, and because it was too unspecific (cf. 38).

2. Adsorption on Tri-Calcium Phosphate Gel.

This method has been used to purify catalase (22) and various other substances.

It was found that much protein impurity, but little pigment was adsorbed between pH 7 and 9 and that two successive treatments of 5 ml. of crude liquid (diluted by five) with 2 ml. of gel (dry weight 0.047 g. per ml.) at pH 7.5 gave purification factors of 2.6 and 2.9 with 55 and 71% loss respectively of helicorubin.

Since this loss was great relative to the purification achieved the method was not considered satisfactory.

3. Treatment with n-butyl Alcohol.

Preliminary experiments using the method of Morton (39) showed that least pigment loss and optimum purification was obtained by

Filter paper Electrophoresis.

A paper electrophoresis apparatus similar to that described by Flynn and de Mayo (17) was used. Phosphate, acetate, barbitone and borate buffers were used at pH values between 9.9 and 4.2, and of ionic concentrations between 0.5 and 0.05. 6 by 30 cms. strips of Whatman Number 4 filter paper were placed over the bridge which was 9 cms. above the liquid in the electrode troughs, and after equilibration at room temperature, a current of 1 to 8 amps at voltages between 80 and 240 was run between the electrodes for 15 hours. The paper strips were then dried in an oven and were stained with azo-carmine B (40). No evidence was obtained from these experiments for the presence of more than one component in the purified solutions.

Estimation of the Minimal Molecular Weight of Helicorubin.

The dry weight of a sample of the solution was determined after drying it in an oven at 100°C until the weight was constant. A further sample was treated with sodium hydroxide and 20% pyridine, and after reduction with a few crystals of sodium hydrosulphite, the density of the solution at 555 mμ was measured. The millimolar extinction coefficient of pyridine haemochromogen was taken as 31 (11) and thus the molecular weight (assuming that there was one haem per molecule) was calculated as follows:

Dry weight of 1 litre of solution = 3.26 gms.

Density of pyridine haemochromogen sol. = 0.725 per ml.

Assuming ϵ^{555}_{mM} pyridine haemochromogen = 31

There are $\frac{0.725}{31}$ mM per litre = 2.34×10^{-5} M/L

= 3.26 gms.

$\therefore$ minimal molecular weight $\simeq$ 140,000.

Helicorubin after Purification Treatment.

The spectra of helicorubin solutions after purification treatment are shown in Figures 4 and 5. The density of the purified solution was measured at 563 mμ in acid and alkaline media after reduction. The haem in a sample was then converted to pyridine haemochromogen and the density at 555 mμ was measured. Taking ϵ^{555}_{mM} pyridine haemochromogen as 31 the 'millimolar extinction coefficients' of the α band of helicorubin was calculated (assuming again that there was one haem group per molecule). A similar method was used to calculate the extinction coefficients of the other bands. These values are given in the table below, together with the ratios of the intensities of the bands.

Discussion

Although no satisfactory purification of helicorubin had been achieved, previous work had suggested that the prosthetic group of this pigment was probably protohaem. The spectrum of crude and partially purified preparations had been examined, and it had been found that the pigment could be oxidised by chemical agents at all pH values, but was only autoxidised in acid media. Experiments had shown that helicorubin did not combine with cyanide, and probably not with carbon monoxide either.

During the present investigation the spectrum of crude helicorubin has been re-examined, and the observations of previous workers, already discussed in the introduction to this section, have been confirmed. The presence of reducing substances in the crop are of interest in connection with the work of Rosén (44, 45), who found that the proteinases of herbivorous gastropods could be activated experimentally by reducing agents. These reducing substances may therefore be of importance to the animal in this connection, although they may, in part at least, be merely excretory products from the gut and liver cells, and from the intestinal flora.

Working with the crude pigment, further observations have been made on the phenomenon of twinning. This could conceivably have been due to the presence of two independent haemochromogens. However,

three observations suggest that this is not so. These are: that no consistent increase in the intensity of one maximum relative to the other could be observed as the pH of the solution was lowered, that twinning was more marked in the turbid solutions reduced with hydrosulphite than in those reduced with hydrazine at low pH values, and, confirming the observations of Morena, that twinning persisted above neutrality. Moreover it was found that twinning of the α band was still observable in solutions of helicorubin after considerable purification treatment. It is considered that these observations support the suggestion of Keilin (21) and Morena (38) that the phenomenon is caused by the presence of the pigment in two physical states.

Evidence given in this section supports the work of Morena, showing that the pigment is oxidised rather than oxygenated. In the introduction to this section it was pointed out that this oxidised form should be named 'methelicorubin' rather than the 'oxyhelicorubin' of previous workers. Sorby, and Vegezzi, as already mentioned, found that potassium permanganate caused a shift of the α band, after reduction, first to 555mμ, and then, when more oxidising agent was added, to 587 mμ. They considered that the latter spectrum was that of the oxidised form of the pigment. Morena's experiments, and those described here, show that potassium ferricyanide treatment does not give these results. Potassium permanganate is a much more powerful

oxidising agent than ferricyanide, and it may well be that the α band shift to 555 mμ (that is to the same position as globin haemochromogen under these conditions) was caused by oxidative liberation of the prosthetic group. The further shift to 587 mμ may have been caused by oxidation of the protohaem side-chains. Recent work by Falk (15, 28) has shown that double bands in conjugation with the porphyrin nucleus of porphyrin methyl esters, give rise to absorption spectra at longer wave lengths than when these are not present, and further that this effect is more marked with aldehyde and acrylic acid than with vinyl side-chains. It seems likely that the potassium permanganate may have oxidised the vinyl side-chains of the protohaem to -CHO (cf. 30).

The chief contribution made during the present work to the study of helicorubin has been in the purification of this pigment, and for the first time the degree of purification achieved has been measured. The estimate of purification of the final preparation based on the ratio of the dry weight to the density at 278 mμ, is greater than that based on the ratios of the densities at 563 mμ and 278 mμ, and this is partly due to the presence of salts in the crude liquid. Indeed from the figures given by Duval and Fischer (13) it seems that about 17% of the dry weight of the gut fluid is due to these.

As judged by the ammonium sulphate precipitation test the resulting pigment was however still impure. No evidence was obtained by paper electrophoresis for the presence of more than one component in the purified preparation, but unfortunately this technique had not been

fully mastered so that the results are considered unreliable. Even specialists in this field will agree, however, that the method is not satisfactory for estimating purity.

On the basis of the dry weight of the preparation, a minimal molecular weight (that is one assuming that there is only one haem group per molecule) of 140,000 has been calculated. This is large, being about twice that of mammalian haemoglobin. In view of the readiness with which the pigment passes through collodion membranes (10, 11), and through calcium carbonate columns which have a pore size of 1.2 μ (54), and from the great stability of the pigment at different pH values, at least as judged by the spectrum in the visible region, it would seem more comparable in size with cytochrome c, which has a molecular weight of 16,500 (29) and with ribonuclease with a molecular weight of 15,000 (29). A more reasonable molecular weight is therefore probably about 14,000. This again suggests that the preparation is still impure.

From the figures given in Table V it is apparent that the crude gut liquid contained about 3.5% by weight of this 140,000 molecular weight material. This figure represents about 3.5 mg. as the average weight in the gut liquid of one hibernating snail, but since much helicorubin is also present in the digestive gland, the total weight per snail is probably much more.

The spectra of my purified preparation agrees well with the observations of Vegezzi given in Table I. However, my preparation shows a plateau in the absorption curve in the region of 555 - 565 mμ, rather than the definite maximum observed by Morena. It seems probable that this discrepancy is due to the greater purity, since this spectrum, Figure 4, is more like that of most parahaematins, than is that plotted by Morena. The small shift in the position of the maxima with pH is unusual for these compounds (29), but its significance is not known.

It is well known that when a pigment is denatured its specific properties, including its absorption spectrum, are altered. For instance, when sodium hydroxide and a reducing agent are added to a solution of haemoglobin the spectrum of oxyhaemoglobin is replaced by that of globin-haemochromogen. Even with helicorubin this phenomenon has been observed, for Vegezzi found that heat treatment caused a shift of the α band from 563 to 558 mμ (53). The maxima of the purified pigment shown in Figures 4 and 5, agree with those recorded personally on the crude fluid, and by others on dialysed preparations. It seems fairly certain therefore that the pigment has not been denatured during purification.

There is considerable discrepancy between the figures given for the relative intensities of the maxima by Morena (36) and those found in this preparation. Thus, calculations based on Morena's figures give

ratios of 0.90 and 1.20 for the intensities of the α and β bands of helicorubin and methelicorubin ~~respectively~~, at pH 5 and 10 respectively, while in the present preparation these are 0.81 and 1.90, both at pH 10.5. The reason for this may again be that the estimates are made at different levels of purity.

As pointed out previously it is still uncertain whether the extinction coefficients of helicorubin vary with the pH.

Morena, working with dialysates of the pigment, found that helicorubin was fully reduced in alkaline media. Since his preparations were impure he was unable to decide whether alkali activated or liberated reducing substances in the media, or liberated reducing groups on the pigment. It seems that alkali must have liberated or activated reducing substances from impurities in Morena's preparation since my preparation was autoxidisable at all pH values.

Confirming Morena's work, it was found that helicorubin does not combine with carbon dioxide. In this respect it is similar to cytochrome c and some other haem compounds (29), but the significance of this fact is not known.

Little can be said about the amino acid content of the preparation since it probably contained protein impurities. It is curious that little histidine was found in the preparation, while in the majority of haemoproteins this is one of the more prominent amino acids (55). In this respect the preparation is similar to cytochrome c, but differs from this latter in having a low lysine content. A

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FIGURE IV

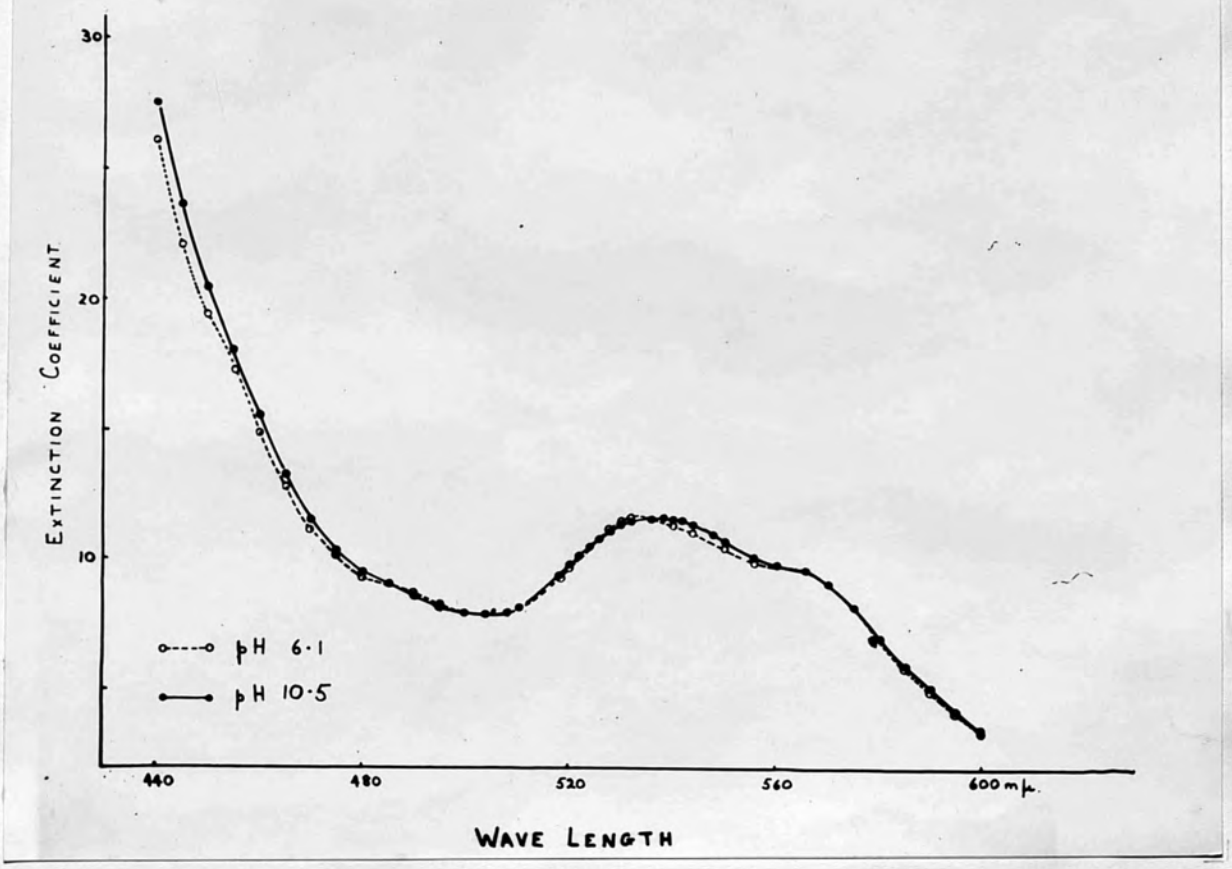


FIGURE V

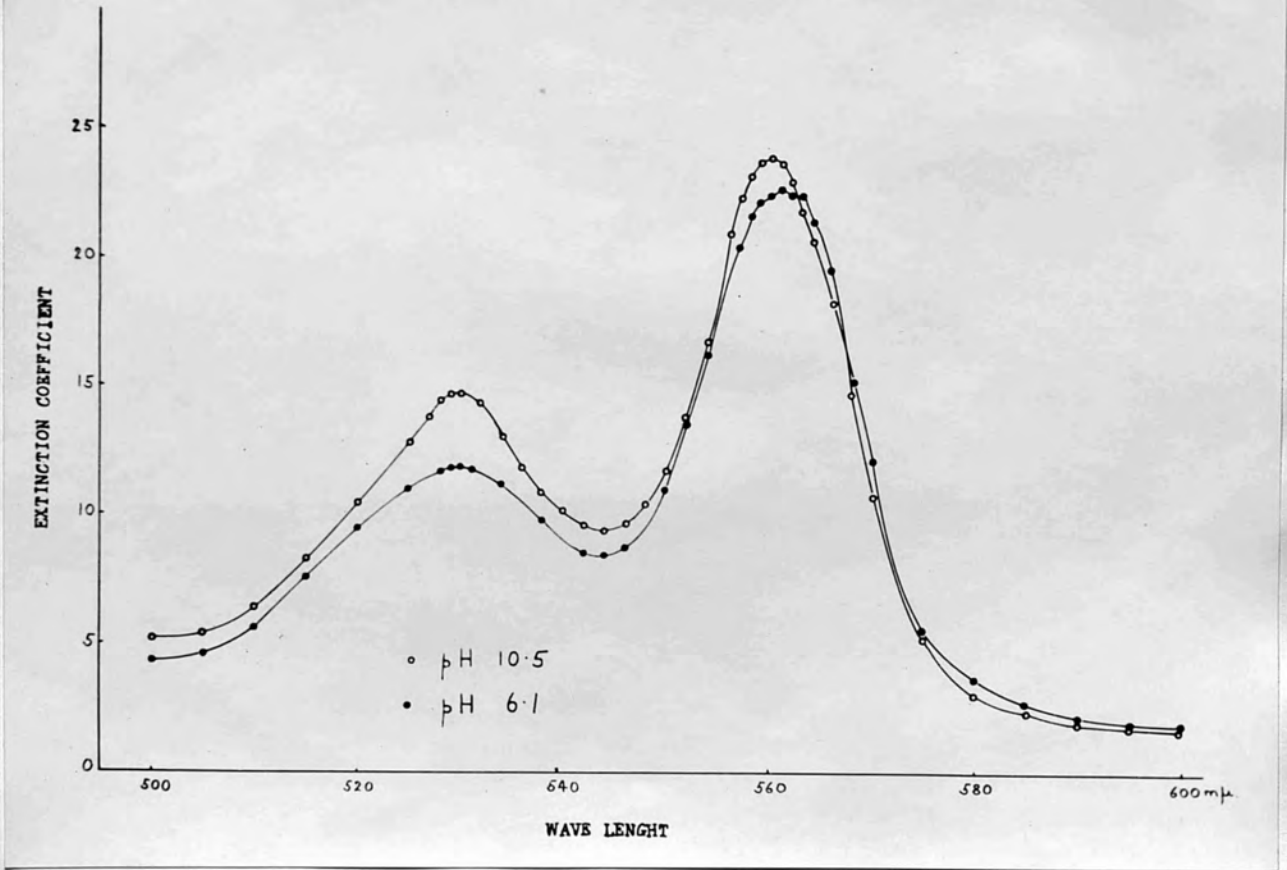
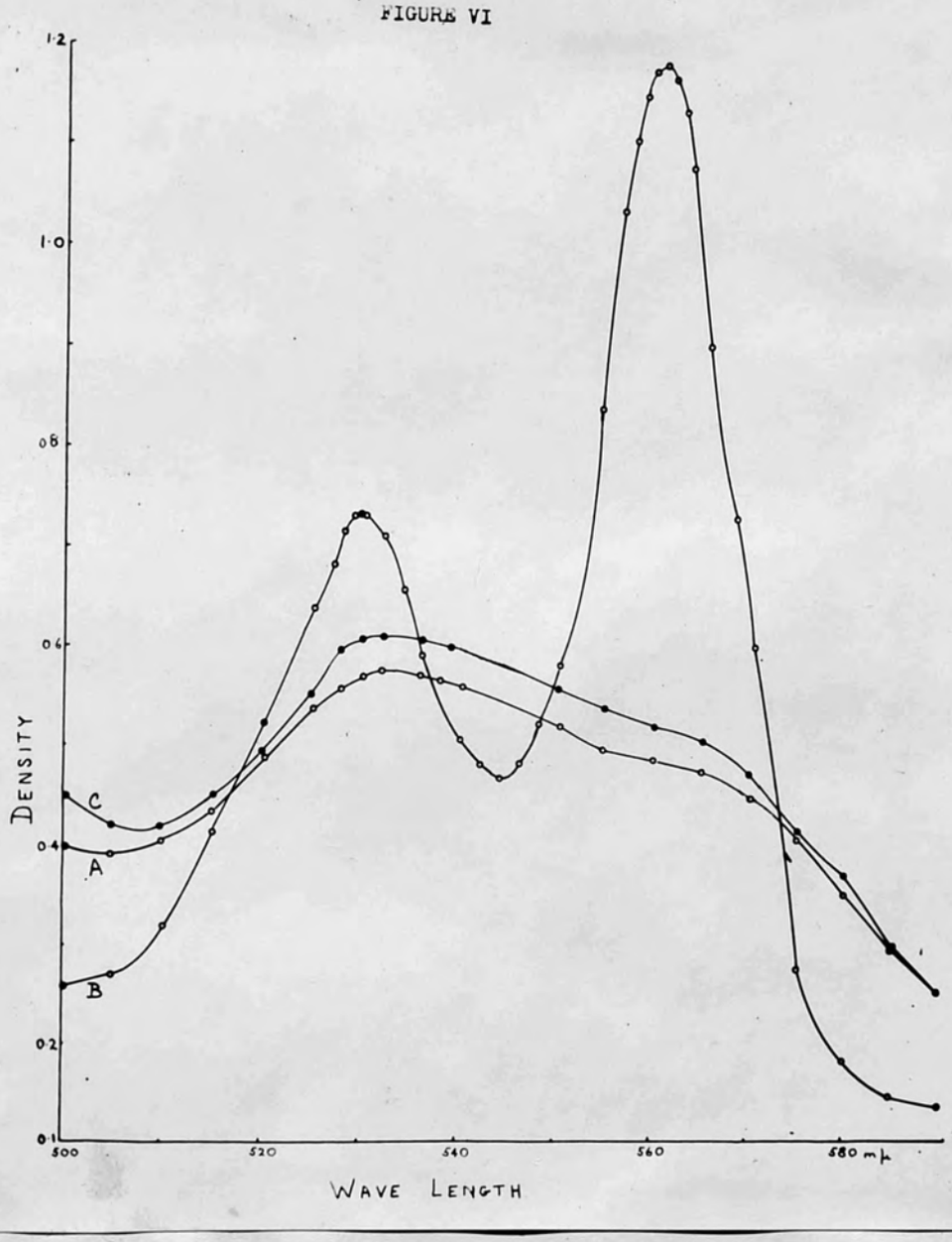


FIGURE VI


1·2
1·0
0·8
0·6
0·4
0·2
0·1
DENSITY
C
A
B
500
520
540
560
580 mμ
WAVE LENGTH

SECTION II

GUT HAEMOCHROMOGENS IN THE MOLLUSCA

Introduction.

Gut haemochromogens were found in the Mollusca about eighty years ago (36), but since that time work on them has been almost exclusively confined to the study of their chemical nature, which has already been discussed in Section I.

Observations of a somewhat random nature have, however, suggested that these pigments are widely distributed in the guts of members of this phylum, but that their concentration varies both between, and within species.

Thus Sorby (36) found that a haemochromogen-like compound was present in the crops of various Pulmonates, but that its concentration was not always high, for, although he found much of this compound in Helix, Zonites, Limax and Cyclostoma, he found less in Limnaea, and in Testicella, Pupa and Clausilia he was doubtful whether he could detect its presence at all. He observed a haemochromogen in the livers of all these animals, and also in that of Planorbis, but in this latter animal he found no gut haemochromogen.

Again Roche (30) detected a haemochromogen in the digestive gland liquid in the spiral stomach of Loligo vulgaris, thus showing that these compounds are not confined to the Gastropods.

Finally, Morena (26) noticed that the amount of helicorubin in the

Table II

Animal No.	Wt. in gms.	Stage of digestion of food.	Colour of gut liquid.	Helicorubin conc. in mg.haem/100 ml. x 10^{-1}	Vol.of liquid in ml.	Helicorubin x Volume x 10^{-2}
1	1.2			traces	0.02	-
2	1.4	Not	Yellow	0.027	0.11	0.03
3	3.3	digested	green	0.067	0.16	0.11
4	0.9			0.120	0.04	0.05
5	3.5			0.220	0.15	0.33
6	2.6	Partially	Brown	0.227	0.12	0.27
7	1.4	digested	yellow	0.233	0.06	0.14
8	1.9			0.367	0.09	0.33
9	1.8			0.433	0.07	0.30
10	2.8	Almost	Yellow	0.440	0.08	0.35
11	3.7	digested	brown	0.587	0.20	0.12
12	1.1			0.594	0.04	0.24
13	3.4			0.733	0.08	0.59
14	3.6	Completely	Red	0.760	0.08	0.56
15	3.2	digested	brown	1.101	0.06	0.66
16	0.6			1.148	0.04	0.46

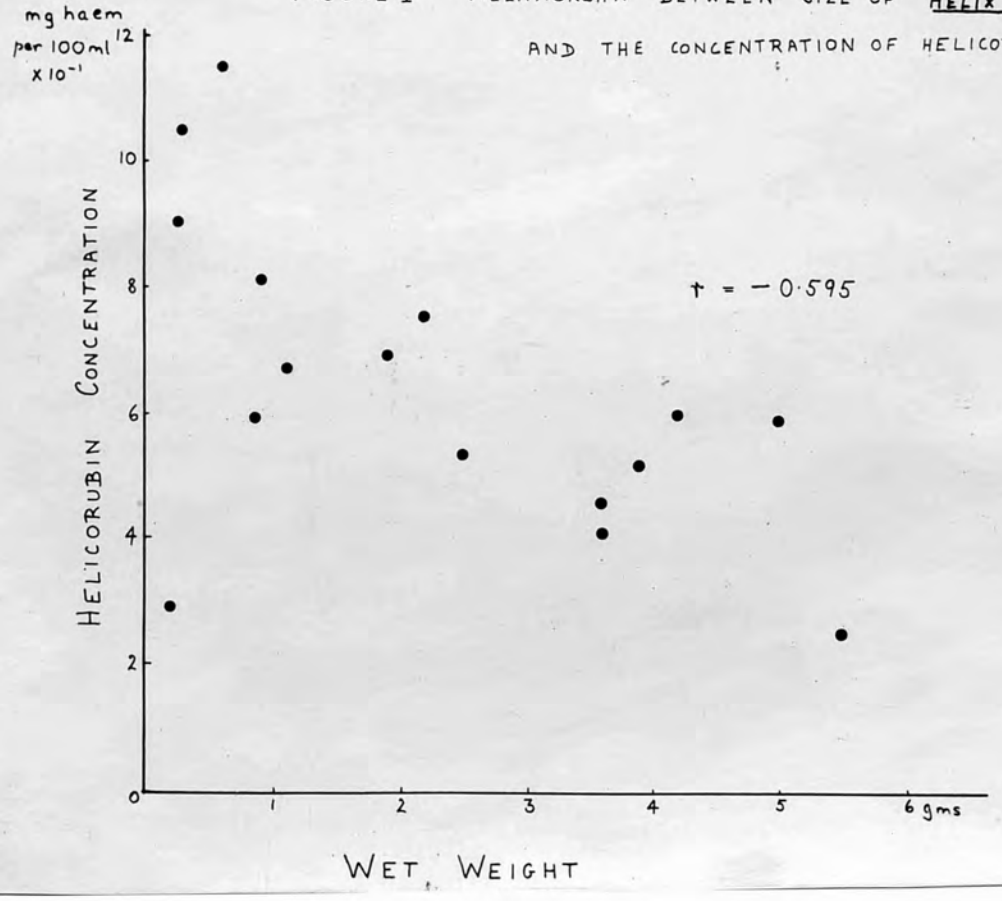
B. Variation with the size of the animal.

The size of the animals was estimated by their wet weights after the shell and crop liquid had been removed. Active animals, which had been starved for three weeks in the laboratory, were drowned, and it was found that the concentration, but not the total amount of helicorubin in the crop, was inversely correlated with the size of the animals. In Table III below the correlation coefficient between the weight of the snails and the helicorubin concentration is -0.59 which is significant at a level of P between 0.05 and 0.01. This correlation is shown in Figure I.

Table III.

Animal No.	Wt. in gms.	Helicorubin conc. in mg.haem/100 ml. x 10^{-1}	Volume of crop liquid in ml. x 10^{-1}	Hr. conc. x Volume x 10^{-2}
1	0.6	11.5	0.36	4.14
2	0.3	10.5	0.48	5.03
3	0.2	9.1	0.38	3.46
4	0.9	8.1	0.35	2.84
5	2.2	7.5	1.28	9.60
6	1.9	6.9	1.30	8.97
7	1.1	6.7	0.70	4.69
8	0.7	5.9	0.36	2.12
9	4.2	5.9	0.53	3.13
10	5.0	5.8	1.02	5.92
11	2.5	5.3	1.30	6.90
12	3.9	5.1	0.68	3.47
13	3.6	4.5	1.06	4.77
14	3.6	4.0	1.13	4.52
15	0.2	2.9	0.60	1.74
16	5.5	2.4	2.28	5.48

FIGURE I RELATIONSHIP BETWEEN SIZE OF *HELIX ASPERSA* AND THE CONCENTRATION OF HELICORUBIN.



However, this result may have been caused, at least in part, by water entering the guts of the different sized animals at varying rates during drowning, and thus may not be of any physiological significance.

C. Variation with hibernation and aestivation states.

It was found that both the concentration and the total amount of helicorubin was greater in hibernating than in active animals. This is shown in Table IV where nine animals in each state were examined.

Table IV.

A. Animals from a drainpipe near Box Hill, Surrey.

B. Animals from a churchyard near Cambridge.

	Average wt. in gms.	Average Helicorubin conc. in mg. haem/100 ml. x 10^{-1}	Average vol. of crop liquid in gms.	Hr. x Vol. x 10^{-2}	Hr. x Vol. A/B
A. Hibernating animals	2.6	10.4	0.1955	20.3	2.06
B. Active animals	2.8	7.5	0.1310	9.8	

In this experiment some of the variation may have been due to the animals being derived from different sources. However similar results were obtained with H. pomatia, all from one source, and Morena (24) had previously observed the same difference between animals in the two states, so that it is considered that these results indicate a real phenomenon.

b) Variation in the concentration of Helicorubin under experimental conditions.

Helix aspersa were kept in brevits and fed on different foods, a small dish of water being put into each brevit to keep the atmosphere humid and the animals active (15, 33). After two months they were starved for 4 days and then their gut liquid was examined. Table V shows a typical result in which ten animals from each food culture were examined.

Table V.

Food	Average wt. in gms.	Helicorubin conc. in mg. haem/100 ml. x 10^{-1}	Average volume of crop liquid in ml.	Average individual Helicorubin x 10^{-2} x volume.
Turnip	3.85	4.85	0.403	10.16
Carrot	3.30	2.61	0.267	7.00
Filter paper	3.52	8.93	0.091	5.42
Cabbage	3.52	2.15	0.237	4.64

The difference between the amount of helicorubin in the crops of the turnip and cabbage cultures corresponds to a t value of 2.09 which is just significant (at a level of P of 0.05). The difference between the amount in the other cultures corresponds to a P. value of more than this. Thus the nature of the food probably does not affect the amount of helicorubin in the crop.

After two months, no significant difference was found between the amount of helicorubin in the crop of animals which had been fed on turnip, carrot and filter paper, while the ~~difference in~~ concentration ~~between~~ in those fed on turnip was only just significantly different from that ~~of~~ in those fed on cabbage. Since filter paper contains no haem, this latter difference must be attributable to another cause than the haem content of the food. During the course of the experiment it was noticed that the snails fed more voraciously on cabbage than on turnip. Possibly therefore, the higher helicorubin in the animals fed on this latter food may have been caused by self-imposed periods of starvation, and thus be somewhat comparable with the high concentration of helicorubin found in hibernating and starving animals.

The filter paper cultures in this experiment are of interest in connection with the final point of this discussion, namely, whether the haemochromogen in the gut is excreted, or whether it is conserved by the animal. When animals were fed on filter paper their faeces were colourless (Frenzel, quoted by (25)) and indeed during the present work haem was only detected in the rectum or faeces of Helix immediately after hibernation. Further the amount of helicorubin in the gut, as mentioned above, was the same, whether the snails were fed on food containing haem and iron, or not. If the haemochromogen were being constantly lost from the animal, it ~~would see~~ is probable that its concentration in the crop would gradually decrease if the food did not contain these materials, because

of the gradual drain on the haem of the animal's body. Again if the haemochromogens were destroyed in the rectum, either by the micro-organisms found there (5, 13), or by enzymes or oxidation, its absence from the faeces could be accounted for, but not its high concentration in the crop of the animals fed on filter paper. It thus seems likely that the haemochromogen is not lost from the animal.

The crop of Helix, and other gastropods, contains much liquid, while the faeces are usually passed in a semi-solid state. Thus water must either be squeezed out of them or be re-absorbed, before they are voided. Carricker (5) has found that in Limnaea both processes occur, and if the latter process occurred in Helix also, then the haemochromogen would tend to be conserved by the animal.

The observed decrease in the concentration of helicorubin immediately after feeding again suggests that the pigment is either being re-absorbed, or passed into the distal diverticula of the liver, since it was not found in the faeces. It is well known that the liver is capable of phagocytosis, and that it can absorb dyes substances from the gut (32, 33, 34). Moreover, in Section I it was shown that helicorubin is a small, easily diffusable molecule. Thus it could easily be absorbed by the liver cells.

Finally, although Cuenot (6) considered that the intestine had an excretory function, in Helix the normal excretory path is the nephridium (8, 27, 28). Thus, although uric acid is formed in the

SECTION III

A SURVEY OF THE DISTRIBUTION OF HAEM PIGMENTS IN THE GUT LIQUIDS OF POLYCHAETA.

INTRODUCTION

Fox (9) found protohaemochromogen in the guts of various members of the Serpulidae and the Terebellid Polycirrus calendrium Claparède, and in the oesophageal pouches of Arenicola marina (L) gut haemochromogen has also been found in Aphrodite*

At the time when this work was undertaken, it was suspected that the concentration of haemochromogen in the gut of the Cladoceran Daphnia magna Straus varied in relation to that of the haemoglobin in the blood. The Polychaeta show great diversity in the nature of their respiratory pigments (8, 15, 16, 17) for all the known respiratory pigments, with the exception of haemocyanin, are found in the class. The predominant pigment is haemoglobin, and this is found in solution in the blood plasma of a great many species. In some families, however, such as the Capetellidae and the Glyceridae, it is present, not in the blood, but in corpuscles in the coelom, while in Travisia Forbesii Johnson and Terebella lapidaria Kahler, it occurs in both these places. Again neuro-, and muscle haemoglobin are found in many species in the presence or absence of other respiratory pigments. It was hoped therefore, that a survey of the distribution of gut haemochromogens in this class might give indications of their relation with haemoglobin.

* Pantin C.F.A. (1931) J Linn Soc., 37, 705.

The Polychaeta are unique in the possession of chlorocruorin, and since Fox had found a gut haemochromogen in Serpulidae possessing this pigment, it was thought that during the survey some connection might be found between the gut haem, and this pigment.

Finally the Polychaeta live in widely different habitats, and have different foods and modes of life. If the gut haemochromogens had no connection with the respiratory pigment, then it was hoped that a survey of their distribution in this class, might reveal a connection with one or other of these factors.

Methods used for detecting and measuring the concentration of haem pigments.

1. Haemochromogens

When it was not possible to extract the gut liquid, the whole animal was introduced into a Graham type compressorium (10) in water containing a little sodium hydrosulphite and the compressorium was placed on the stage of a microscope. It was illuminated from below by a strong light fitted with a variable resistance, and the eye-piece of the microscope was replaced by a Zeiss comparator spectroscope. Haemochromogens in the gut fluid, or elsewhere in the body, were then detectable by their α band at about 560mμ.

Whenever possible the gut liquid was extracted, for then

the haemochromogen could not only be detected, but its concentration could be estimated. This estimation was made by the method of Elliott and Keilin (6) and although the method is identical with Method 2, Section 11, for completeness it will be redescribed here.

A standard solution of globin haemochromogen was prepared by diluting 0.1ml of human blood with 150ml of distilled water. 50ml of this solution containing a little hydrosulphite was then diluted further with 20ml water and 30ml N. sodium hydroxide. This standard solution was introduced into a wedge-shaped trough (10 cms long, and 4cms wide at the base of the wedge) on a calibrated side stage of the microscope. The spectrum of this solution was matched with that of the haemochromogen in the compressorium by sliding the wedge-trough along the stage. The stage was calibrated into 100 millimeter divisions, and when intensity of the α bands of the two spectra were identical, a reading was taken on this scale. This estimation was repeated 3 times, as the reading were inclined to be variable.

Calibration graphs were made by introducing known dilutions of globin haemochromogen, prepared as above, into the compressorium, and estimating their concentration as above, at 0.5, 1.0 and 1.5 turns of the head screw of the compressorium from its lower limit. The calibration graphs at each position of the head screw were then obtained by plotting the

concentration of haemochromogen against the corresponding scale reading. The haem content of the blood was measured with an M.R.C. Grey wedge photometer (11), so that from these graphs it was possible to estimate the concentration of a haemochromogen solution, and to express the result in grams of haem per 100 ml.

In order to estimate the concentration of haemochromogen therefore, liquid was sucked out of the gut and transferred to the compressorium. After the addition of a little hydrosulphite, the head screw was racked down to one of these three positions of the head screw, the position chosen depending on the concentration of the pigment. An estimate was then made of the concentration of the haemochromogen, and from the appropriate calibration graph, the concentration of haem in the sample estimated.

It has been found (5) that ϵ^{558}_{mM} globin haemochromogen = 30.6 - 30.9

and (Section 1) that ϵ^{563}_{mM} acid helicorubin = 22.6

In estimating the concentration of haem in the gut haemochromogen solutions, it was assumed that their extinction coefficients were identical to that of acid helicorubin, and a correction factor of $\frac{30.9}{22.6}$ was applied to the graph reading.

The table below shows the concentration range over which it was possible to estimate the haem in a solution of haemochromogen at each of the three positions of the head screw,

and the upper limit could of course be extended by diluting the solution with distilled water.

Table I

Position of head screw	Concentration of haemochromogen in mg/100ml X 10 measurable as	
	Globin haemochromogen	Gut haemochromogen
0.5	28 to 8	38 to 11
1.0	12 to 4	16 to 5
1.5	8 to 2	11 to 3

2. Haemoglobin and Chlorocruorin

These pigments were detected by their colour, and by their oxy-form spectra. Their concentration was estimated with the globin haemochromogen standard, as described above, although this introduced unavoidable error (see'Discussion of Method'below.)

The ratio of the intensity of the α band of oxyhaemoglobin to that of globin haemochromogen is approximately 1 to 2 (5, 13), so that from the calibration graphs, the concentration of haem in a solution of oxyhaemoglobin could be estimated, remembering that there are 4 haems per molecule.

Again Fox (9) showed that for equal concentrations of iron, the intensity of the α band of oxyhaemoglobin to that of oxychlorocruorin is 30 to 49. Thus from the graphs the haem in solutions of this pigment could also be estimated.

Family Aphroditidae

Aphrodite aculeata Claperède

One specimen was dredged from 35m. This animal was 6cms in length.

There was a large volume of dark brown gut fluid, and this contained methaemoglobin, but no haemochromogen. No haemoglobin was found in the blood, but neurohaemoglobin was present in the brain and nervous system, and cytochromes in the pharyngeal muscles.

Hermione hystrix Savigny

4 specimens were dredged from 6m. These varied in length from 0.8 to 1.6cms. Haemochromogen was present in the gut at an average concentration of 5.3×10^{-2} mg haem / 100ml. Myoglobin was present in the pharyngeal muscles.

Harmothoe imbricata Malmgren

2 specimens of 1.5 and 4.5 cms. length were dredged from 45m.

The large volume of gut fluid contained an average of 4.3×10^{-2} mg haem / 100.ml.

Neurohaemoglobin was present in the nervous system, and myoglobin in the pharyngeal muscles.

Lepidonotus squamatus Saint-Joseph

11 specimens were obtained from 6 and 10m. These were from 1.5 to 3.0cms long.

The turbid brown gut liquid contained an average con-

Lepidonotus squamatus Saint-Joseph (continued)

centration of 11.2 X 10^{-2} mg haem / 100ml in the form of haematin. Protohaemochromogen was present in the brain, and neurohaemoglobin here, and in the rest of the nervous system. Myoglobin was present in the pharyngeal muscles.

Panthalis Oerstedi Kinberg

3 specimens were examined shortly after being dredged from 70m. They were of an average length of 10cms. Haematin was found in the gut, and its average concentration in these animals was 16.8 X 10^{-2} mg haem / 100ml.

3 specimens were examined after they had been kept for 2 days in a non-aerated aquarium. These animals had an average length of 6 cms, and an average haematin concentration in the gut of 61.6 10 mg haem/ 100ml.

Protohaemochromogen was found in the brain of all the specimens examined, together with neurohaemoglobin.

Family Eunicidae

Lumbriconereis fragilis (O.F.Müller)

Specimens collected from 70m

The gut contained a clear orange liquid in which no haemochromogen was detectable. Neither the spectrum, nor the colour of this liquid changed after the addition of hydrosulphite, but pyridine treatment revealed the presence of protohaem. This was therefore probably present in the gut as haematin. The concentration of this haem was greatest in the mid-gut.

There was a small volume, but high concentration, of plasma haemoglobin.

The results of the examination of 4 animals are shown below.

Table 11

Animal number	1	2	3	4	Average
Haem in mid gut in mg haem/ 100ml X 10^{-1}	4.5	4.7	6.2	6.7	5.5
Haemoglobin conc.in mg haem/ 100ml.	7.5	7.3	7.0	6.9	7.2

The correlation coefficient between the concentration of these two haem compounds is -0.97 ($P < 0.05$).

Family Glyceridae

Glycera convoluta Keferstein

One specimen was dredged from 14m. and was 4cms.long.

No haemochromogen was detectable in the gut, and the effect of adding pyridine was not investigated. Haemoglobin was present in corpuscles in the coelom.

Glycera alba Rathke

6 animals were dredged from 53m

The gut contained a turbid brown liquid, in which methaemoglobin, but no haemochromogen was detectable. Haemoglobin was present in the nervous system, and in corpuscles in the coelom. The results of the haem estimations of these animals are shown below.

Table III

Animal Number	1	2	3	4	5	6	Average
Length in cms	5	7	3	7	4	7	
Haem in gut, in mg / 100ml X 10^{-1}	2.16	2.32	2.88	2.96	3.24	5.64	3.20
Haemoglobin in coelom, in mg haem /100ml X 10^{-1}	4.0	5.6	5.6	4.7	5.3	4.1	4.9

The correlation coefficient between these two haem pigments is -0.29 ($P > 0.1$).

Family Hesionidae

Castalia punctata O.F.Müller

2 specimens were dredged from 6m. These were 0.9 and 1.3 cms in length.

The gut contained a brown liquid in which haemochromogen was detected, and its concentration estimated in the foregut respectively as 5.5 and 17.6 10 mg haem / 100ml. The plasma contained haemoglobin, but the concentration of this was not measured.

Family Nephthydidae

Nephthys caeca Fabricus

2 specimens in which the gut contained a dark brown liquid were examined. This liquid contained much haem, but no haemochromogen. The concentration of this haem was not measured but the haemoglobin concentration in these animals are shown below.

Table IV

Depth from which dredged in m.	Length in cms	Haemoglobin concentration in mg haem / 100ml
14	10	2.32
40	20	5.52

Platynereis Dumerilii (Audouin and M.Edwards)

One specimen was dredged from 80m. This specimen was 2cms.long.

A haemochromogen was detectable in the anterior region of the gut, but as the liquid could not be extracted, its concentration could not be measured, and the band may have been that of cytochrome. Haemoglobin was present in the blood.

Nereis diversicolor, O.F.Müller.

One specimen was dredged from 6m.

No haemochromogen was present in the gut. The presence of other haem was revealed by pyridine treatment of the extracted liquid, but its concentration was not measured. Haemoglobin was present in the blood.

Nereis pelagica Fauvel.

Animals were dug from muddy sand, inshore, at a depth of 1m.

The gut contained a clear brown fluid having no definite spectrum. No change in colour, or spectrum was observed after addition of hydrosulphite, but pyridine treatment revealed the presence of protohaem. This was probably present in the gut as haematin.

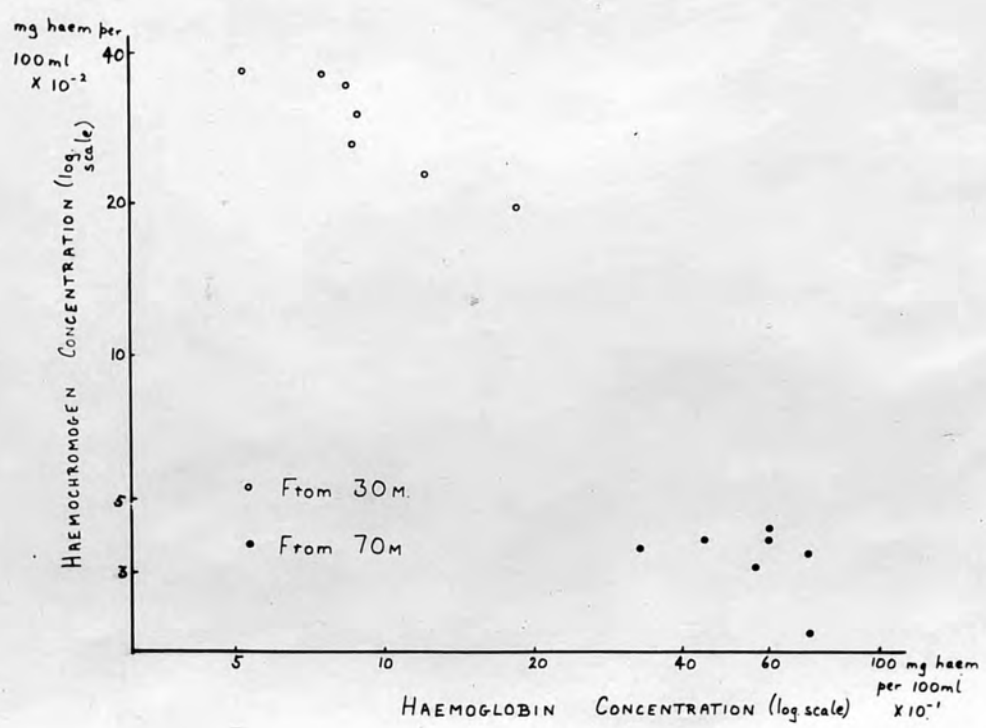
There was a small volume of plasma, but it was possible to extract this to measure the concentration of the haemoglobin

The average length of the 8 specimens from 70m was 4.9cms, their average haemochromogen concentration 3.9×10^{-2} mg haem / 100ml, and their average haemoglobin concentration 5.76mg haem / 100ml.

The correlation coefficient between the individual concentrations of these haems (shown in Figure 2) is -0.97 ($P < 0.001$) when calculated on all the specimens, -0.93 ($P < 0.01$) for the animals from 30m, and -0.50 ($P > 0.1$) for the animals from 70m. The correlation ~~is~~ therefore ~~probably~~ may not be real.

The difference in the haemoglobin concentrations of individuals from the two depths corresponds to a t value of 11.4 ($P < 0.001$).

FIGURE 2 PECTINARIA BELGICA



Family Arenicolidae

Arenicola marina (L)

Animals were dug from mud at low water.

The wide gut lumen contained a clear yellow syrup-like liquid, and a haemochromogen was found in this, and was most concentrated in the mid gut. No haem was found in the hind gut. The oesophageal pouches also contained a haemochromogen (9), and it was noticed that these were most dilated when the haem concentration in the gut was high.

There was much plasma haemoglobin.

A sample of 20 animals was examined immediately after collection (Aug.2). A further sample of 20 animals was transferred to a well aerated tank of sea water. There was a little mud at the bottom of this tank, but the animals were not supplied with other food. They were examined after 6 days (Aug.8). The results of this examination are shown below.

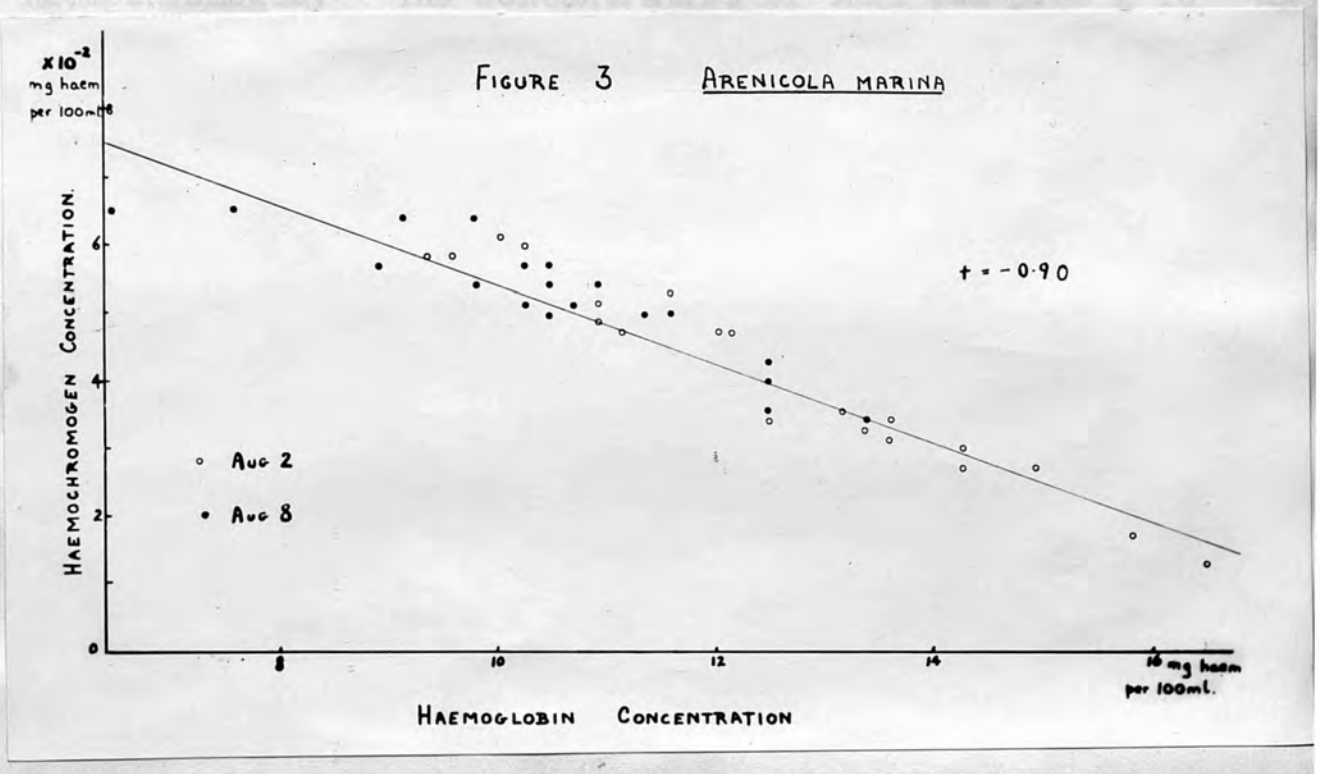
Table VII

Date on which examined	Average length in cms	Average haemochromogen conc. in mg haem / 100ml X 10^{-2}			Average haemoglobin conc. in mg haem /100ml
		Fore gut	Mid gut	Oesophageal pouches	
Aug.2	10.7	4.1	5.2	5.3	12.55
" 8	9.9	5.2	6.6	6.7	10.40

The correlation between the haemoglobin concentration and the haemochromogen concentration in the fore gut of individuals is shown in Figure 3, and since there is no

significant difference between their regressions on the two days, they have been plotted on a single line. The correlation coefficient is -0.90 ($P < 0.001$).

The difference between the individual haemoglobin concentrations on the two days corresponds to a t value of 3.4 ($P < 0.01$), while the difference between the individual haemochromogen concentrations corresponds to a value of 4.6 ($P < 0.001$).



Family Chaetopteridae

Chaetopterus variopedatus (Renier)

5 specimens were dredged from 30m. These specimens varied in length from 8 to 10 cms.

No haem was detected in the gut, and there was no respiratory pigment in the blood.

Spiochaetopterus typicus (Sars)

One specimen of 4 cms was dredged from 70m.

No haem was found in the gut, and no respiratory pigment in the blood.

Family Chlorhaemidae

Stylariodes plumosa (O.F.Müller)

One specimen was dredged from 14m. In this animal of 5.5cms, the gut liquid was a right red colour and contained a haemochromogen. The concentration of this was 5.88 X 10^{-1} mg haem / 100ml. The chlorocruorin concentration of the blood was not measured.

Family Maldanidae

Maldane Sarsi Malmgren

Specimens were dredged from 30 and 70m, and were all 10 cms long.

A haemochromogen was present in the gut, haemoglobin in the plasma, and myoglobin in the muscles of the pharynx, and the body wall. The table below shows the results obtained by examination of these animals.

Table VIII

Animal Number	1	2	3	4	
Depth at which dredged in m.	30	30	30	70	Average
Haemochromogen conc.in mg haem / 100 ml.	2.46	2.52	2.68	3.40	2.76
Haemoglobin conc. in mg haem/ X 10^{-1} 100ml	1.94	1.82	1.94	1.70	1.85

For these two haem compounds the correlation coefficient is -0.54 (P.=0.01).

Family Sabellidaeariidae

Sabellaria spinulosa Leuckart

3 specimens were collected from 14 m. These specimens were of an average length of 2.5cms, and had a haemochromogen in the gut, but no respiratory pigment in the blood.

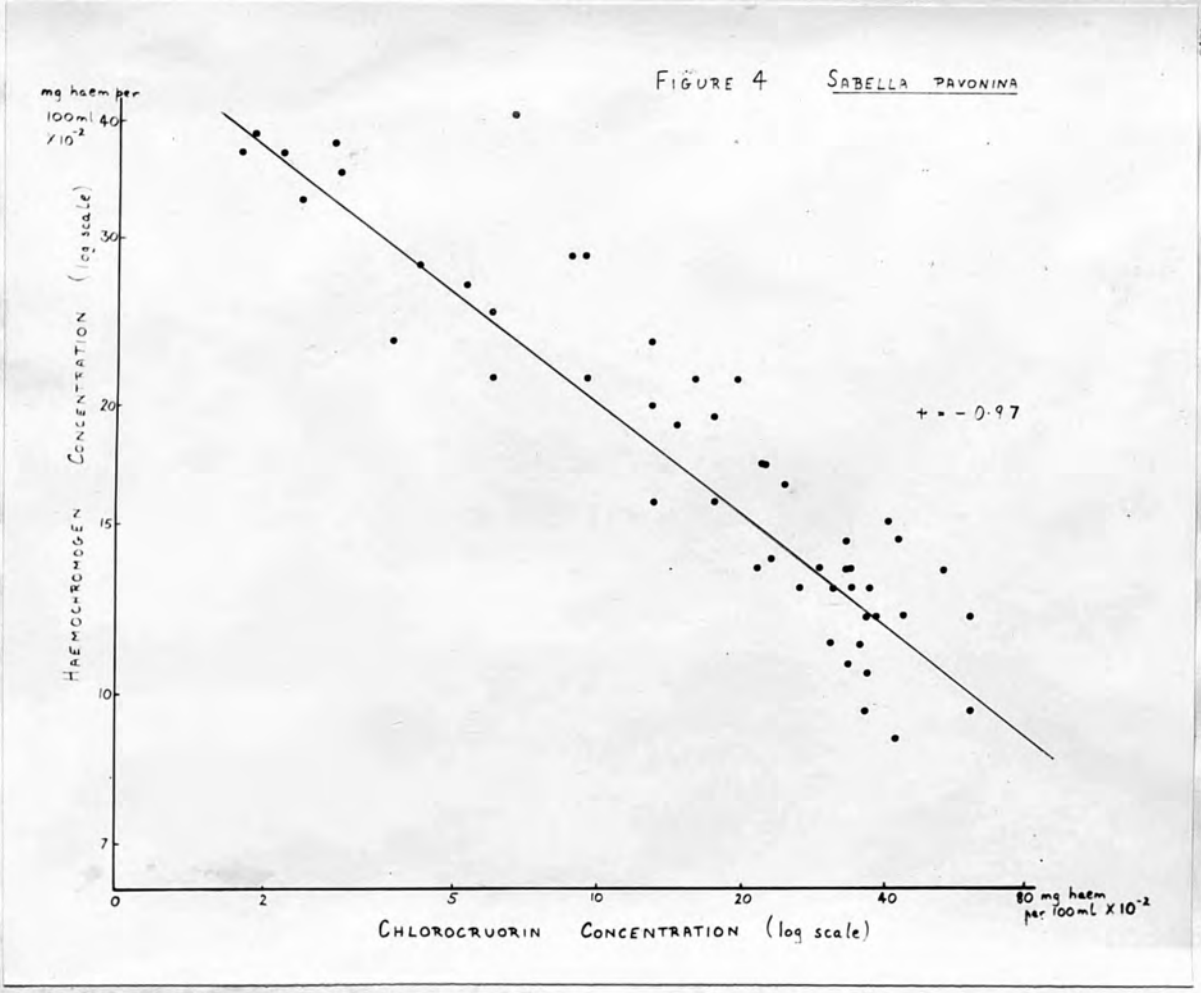
Family Sabellidae

Sabella pavonina Savigny

Animals were dredged from 30m.

The yellowish brown gut liquid contained a haemochromogen, but no other haem. This haem was most concentrated in the mid-gut, and none was detected in the rectum. It was noticed that if the branchial crown had been broken off a few weeks previously, and the animals kept in aerated sea water, the concentration of haemochromogen in the gut was unusually high. However no measurements were made of this phenomenon. The blood contained chlorocruorin in very variable concentration.

The correlation coefficient between the logarithms of the haemochromogen, and chlorocruorin concentrations of 50 individuals was -0.97 ($P < 0.001$), and this correlation is shown in Figure 4. The average length of these animals was 11.1cms.



Dasychone bombyx (Dalyell)

2 specimens were dredged from 14m. The results of their examination are shown below:-

Table IX

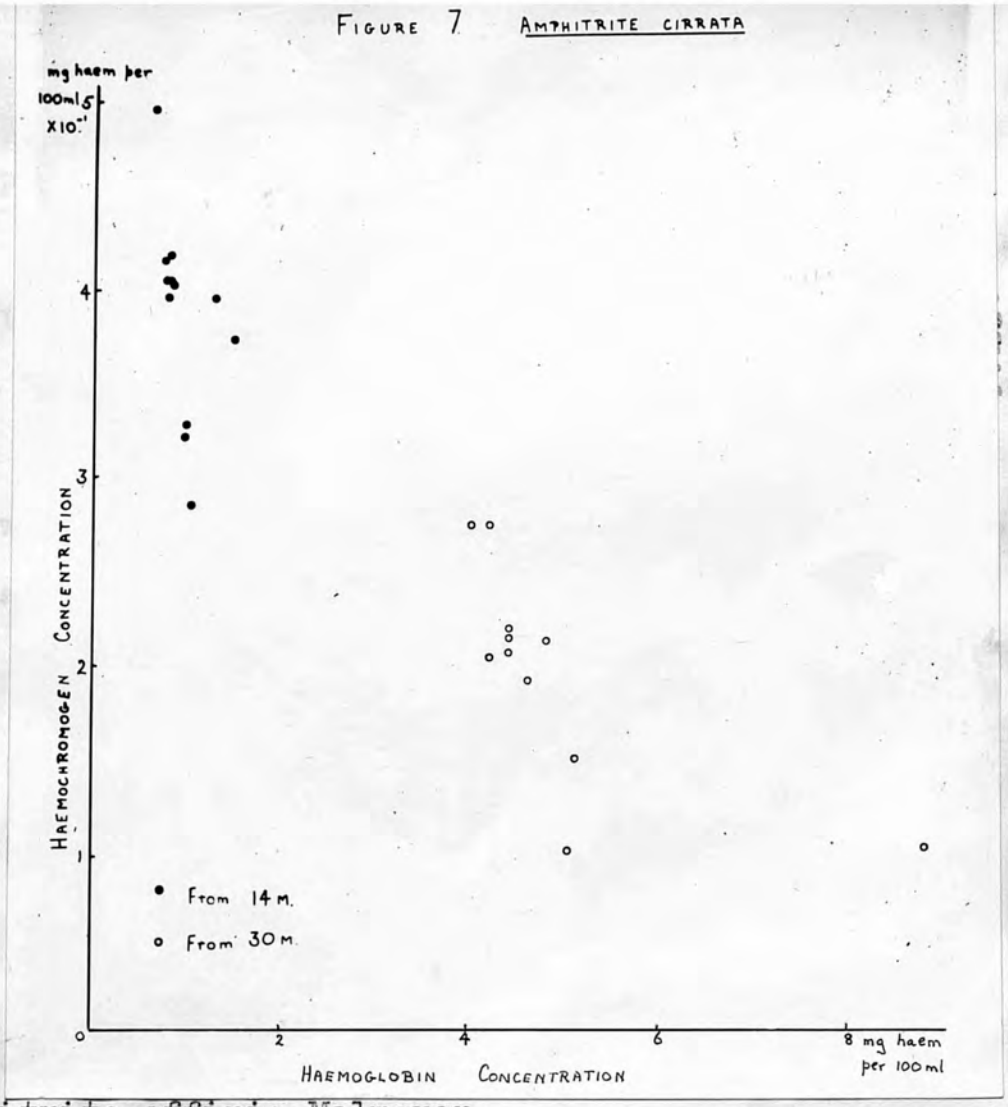
Length in cms	3.0	5.0
Haemochromogen conc. in mg haem / 100ml X 10-1	1.60	3.53
Chlorocruorin conc. in mg haem / 100ml X 10^{-1}	?	11.8

Euchone rubrocincta (Sars)

One specimen was dredged from 14m.

Haemochromogen was present in the gut, and its concentration in the fore gut was estimated as 1.39 X 10^{-1} mg

FIGURE 7 AMPHITRITE CIRRATA



Amphitrite affinis Malmgren

2 specimens were dredged from 80m. The length of these were 8.0 and 6.0cms. The haemochromogen concentration in the fore-gut of these animals was estimated at 8.01 and 7.12×10^{-2} mg haem / 100ml respectively. Their haemoglobin concentrations were not measured.

Pista cristata (O.F.Müller)

Animals were dredged from 30m.

The gut liquid was clear brown and a haemochromogen was found in highest concentration in the fore-gut. The volume

of plasma was small, and contained haemoglobin.

The average haemochromogen concentration in the fore gut of the 24 specimens examined was 2.31 X 10^{-1}mg haem / 100ml, and the average haemoglobin concentration of these animals was 5.22 mg haem / 100ml. The correlation coefficient between their individual concentrations is -0.79 ($P. < 0.001$). This correlation is shown in Figure 8.

FIGURE 8 PISTA CRISTATA

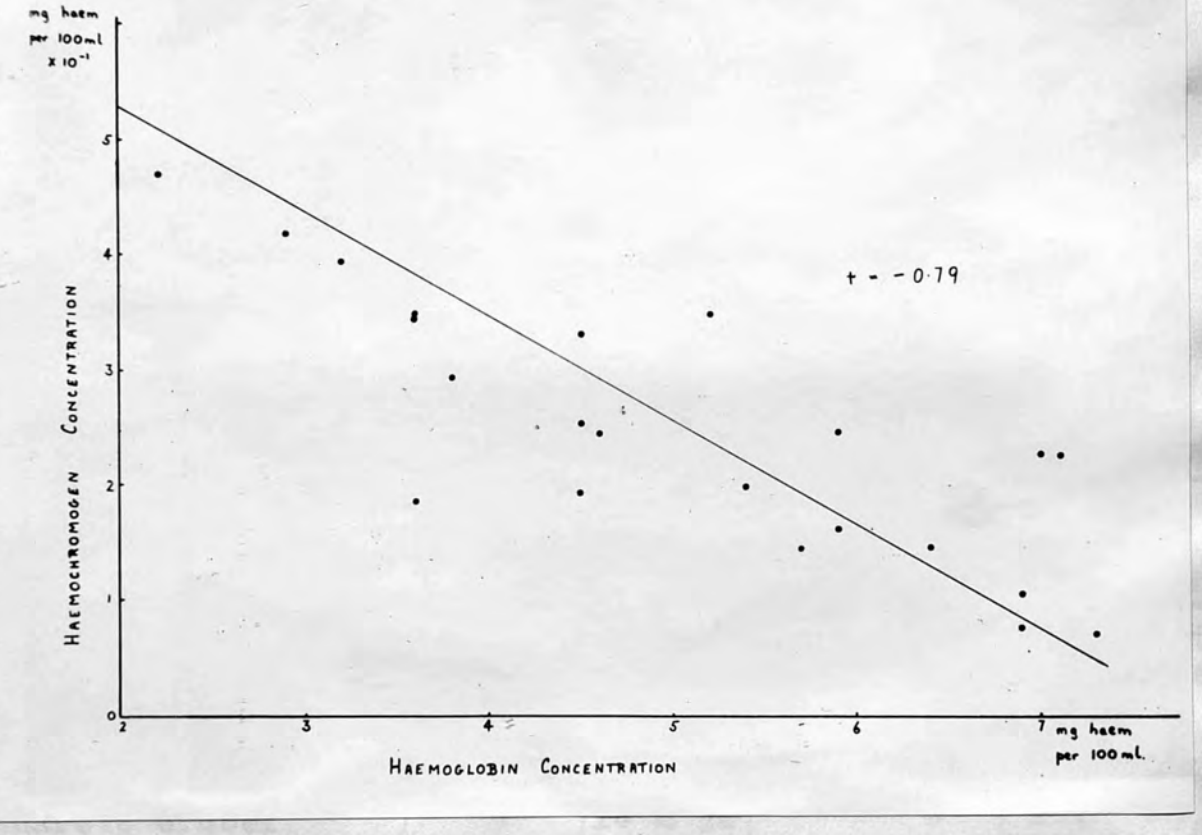


Table XI

Summary of Results

The concentrations of haem compounds are given in mg haem / 100ml X 10^{-2}.

\+ = detected, but concentration not measured.

? = nature of haem compound not investigated.

1. No respiratory pigment in blood, or in corpuscles in coelom

Species	Number examined	Depth from which dredged in metres	Average conc. of haem in gut as haemoch.	haem-atin	meth-aem
Aphrodite aculeata	1	35	-	-	8.0
Hermione hystrix	4	6	5.3	-	-
Harmothoe imbricata	2	45	4.3	-	-
Lepidonotus squamatus	11	6 & 10	-	11.2	-
Panthalis Oerstedi					
a) freshly collected	3	70	-	16.8	-
b) after 2 days in non-aerated aquarium	3	70	-	61.6	-
Phyllodoce maculata	4	6	-	?	?
Phyllodoce lamilosa	9	6	-	35.4	-
Syllis armillaris	1	30	?	?	?
Autolytus prolifer	1	30	?	?	?
Chaetopterus variopedatus	5	30	-	-	-
Spiochaetopterus typicus	1	70	-	-	-
Sabellaria spinulosa	1	14	+	-	-

2. Respiratory pigment in plasma only

A Chlorocruorin

Species	Number examined	Depth from which dredged in metres	Haem-ochr.	Chloro-cruor in	Other haems in gut
Anobothrus gracilis	5	30	-	205	Haematin
Ampharete Grubei	5	14 & 30	-	161	?
Sabellides octocirrata	1	30	+	+	
Amphictenis Gunneri	1	14	2.9	320	
Pomatoceros triqueter	10	6	10.3	92	
Euchone rubrocincta	1	14	13.9	86	
Dasychone bombyx	1	14	35.3	118	
Sabella pavonina	50	30	8.9 to 42.0	62 to 2	
Stylariodes plumosa	1	14	58.8	+	

B. Haemoglobin

Species	Number examined	Depth from which dredged in metres	Haemoch.	Haemo-globin	Haem-atin in the gut
Platynereis Dumerilii	1	80	+	+	?
Nereis diversicolor	1	6	-	+	
Lumbriconereis fragilis	4	70	-	720	55
Nephthys caeca	1	40	-	552	?
	1	14	-	232	?
Nereis pelagica	29	1	-	502	60
Pectinaria belgica	8	70	3.9	576	-
Arenicola marina					
a)freshly collected	20	0 to 2	4.1	1255	
b)after 6 days in aerated aquarium	20		5.2	1040	
Amphitrite affinis	2	80	7.6	+	
Terebellides Stroemi	2	40	7.7	812	
Castalia punctata	2	6	11.6	+	
Melinna cristata	1	30	16.9	62	
Amphitrite cirrata	12	30	21.1	490	
Eumenia crassa	23	40	22.4	548	
Pista cristata	24	30	23.1	522	
Polymnia nebulosa	1	40	23.8	380	
Maldane Sarsi	4	70 and 30	27.6	185	
Pectinaria belgica	7	30	29.6	103	
Amphitrite cirrata	12	14	39.1	103	

The correlation coefficient between these haemoglobin and haemochromogen concentrations is -0.81 ($P<0.001$). This correlation is shown in Figure 9.

3. Haemoglobin in coelomic corpuscles.

Species	Number examined	Depth in metres	Haem in gut	Haemoglobin in coelom.
Glycera convoluta	1	14	No. haemochr.	?
Glycera alba	6	53	Haematin 32.0	49

4. Haemoglobin in cells in coelom, and in plasma.

Species	Number examined	Depth in metres	Haemochr.	Hb. in plasma	Hb in coelom
Terebella lapidaria	3	6	22.3	19.0	7.6

referred to here as such. Unlike Bloch-Raphaël, no haemoglobin was found in the gut caecae of Aphrodite aculeata. Examination of this animal suggested that here the haem was present as methaemoglobin, but this may have been derived from haemoglobin by oxidation. Table XI shows that contrary to the observations of this worker, no haemoglobin was found in the gut of Harmothoe imbricata, or of Hermione hystrix. The other species in which she found haemoglobin were not examined.

The present work is however in agreement with that of Fox (9) except that a protohaemochromogen was found in the gut of Sabella pavonina which he was not able to detect. However Figure 4 shows that its concentration was very variable, and so it may well be that the animals which he examined were for some reason completely devoid of this compound.

No investigation was made of the haem in the gut wall, and so previous work on this subject has not been confirmed.

In the present survey the gut haem has always been found in the form of protohaem, and confirming the work of Fox (9) chlorocruoro-haem has not been found in the gut. The haem was present in various forms, but the reason for this is not known. Possibly the haemochromogen may have been decomposed by digestive enzymes in the gut of some species, giving rise

to haematin. However it seems impossible that methaemoglobin could have been formed from haemochromogen, so that the variations in the form of the gut haem are probably attributable to physiological variations in the animals.

Possibly the absence of gut haem from the Chaetopteridae may have some connection with the symbiotic algae, which are found in the gut wall (3, 12, 20), although of course this is pure speculation.

Distribution of haem pigments in the gut

In the present survey it was found that haem pigments were most concentrated in the mid gut. Fox (9) also had found that in Serpula vermiliaris L, Vermiliopsis infundibulum (Phillipi), Protula intestinum, and Apomatus similis Marion, the gut haemochromogen was most easily detectable in the anterior third of the abdomen, although perhaps because the lumen was wider in this region. In few species during the present work was any haem found in the hind gut. This fact is open to three interpretations, namely:-

1. Antiperistalsis might cause concentration of the haem in the fore-gut, in a similar way to that in which dyes become concentrated in the fore-gut of Daphnia magna, and other Cladocera.

2. The haem might be destroyed in the gut, either by enzymes, bacteria or by autoxidation (1,13). In the latter case the

rate of destruction would be increased by the oxygen entering the gut during the antiperistaltic gut movements, and so would be most rapid in the hind gut.

3. The haem might be reabsorbed in the hind gut. In connection with this possibility it is of interest that Tandan (21) has shown that the fore gut of the earth worm is mainly secretory, while the hind gut has a reabsorbtive function.

(This distribution of haem in the gut seems to be fairly general in the Invertebrata, for it has also been found in the Mollusca and Crustacea, but only further work can elucidate its cause.)

Absorption Spectrum of Gut Haemochromogens

Fox (9) measured the wave length of the α bands of the gut haemochromogens of various Polychaeta. He found that in the Serpulimorpha, and in Polycirrus calendrium these were at 560mμ, while in Arenicola marina the band was at 563mμ, thus suggesting that there were specific differences in these compounds.

Variations in the pH, or the degree of polymerisation might however have accounted for these differences, and it is significant in this connection that the two α bands of helicorubin are separated by 3mμ.

Although during the present work attempts were made to

measure the position of the α bands in the species examined, it was found that the Zeiss spectroscope was not sufficiently accurate for this purpose, and so the records made have not been included here. In any case they probably would not have been of great value unless the compounds had been pure.

Therefore it is uncertain, although it seems likely, that there are specific differences in these compounds.

Connection between the gut haem, and the haemoglobin and chlorocruorin

Table XI shows that all the animals examined, with haemoglobin in the blood plasma or coelom, had either haematin or haemochromogen in the gut. Further the table shows that haematin was found in the 'free living' Polychaeta with haemoglobin in the plasma, while haemochromogen occurred in the 'sedentary Polychaeta' but it is not known why this should have been so.

With the exception of Ampharete Grubei, from which it was not possible to extract the gut liquid, and Anobothrus gracilis which had haematin in the gut, all the animals examined with chlorocruorin in the plasma had a haemochromogen in the gut. These observations again suggest that haemochromogens are found predominantly in sedentary Polychaeta.

It is curious that protohaem rather than chlorocruorohaem should have been found in animals possessing chlorocruorin in the plasma, for as Figure 4 and 6 suggest there is a relation between the concentration of these two compounds.

gut increased, and that in many animals a correlation was found between the concentration of haem in the gut and that of the respiratory pigment in the blood.

This work also suggests that the gut haem is either formed from tissue protohaem, or that it is formed by an alternate synthetic path to that of the respiratory pigments of the blood. Evidence for this is firstly that the correlation between the concentration of gut haem and that of the respiratory pigment was inverse rather than direct, and secondly that, although there was an inverse correlation between the concentrations of haemochromogen and chlorocruorin in Sabella and Pomatoceros the haemochromogen was proto-, not chlorocruorohaem, and chlorocruorohaem was never found in the gut.

Summary

1. A survey has been made of the distribution of haem pigments in the gut lumens of the Polychaeta.
2. Protohaem has been found in all the families examined with the exception of the Chaetopteridae. Chlorocruorohaem was not found in the gut.
3. This haem was found to be present either in the form of prothaemochromogen, haematin, or methaemoglobin.
4. The haem was most concentrated in the anterior part of the gut.
5. Among those species examined with a respiratory pigment in the plasma, haematin was found predominantly in free living, while haemochromogen only in tubicolous animals.
6. A significant inverse correlation was found between the concentration of haematin in the gut, and the concentration of haemoglobin in the plasma of *Nereis pelagica*.
7. A significant inverse relation was found between the concentration of haemochromogen in the gut and the concentration of -
   - a. chlorocruorin in the plasma of *Sabella pavonina* and *Pomatoceros triqueter*.
   - b. haemoglobin in the plasma of *Arenicola marina*, *Eumenia crassa* and *Pista cristata*
8. A significant inverse correlation was found between the average concentrations of haemoglobin in the plasma, and of haemochromogen in the gut of all the species examined.

9. A combination of high oxygen, and partial starvation in the laboratory has been found to decrease the haemoglobin concentration, and to increase the haemochromogen concentration of Arenicola marina.

10. Chlorocruorin has been found for the first time in the blood of three species of the Ampharetidae: Ampharete Grubei, Anobothrus gracilis and Amphicteis gunneri + Sabellides octocirrata.

11. The significance of the results has been discussed.

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Results of Survey

Species	Habitat	Respir. Pig.	No. Examined	Haemochr. Conc.
BRANCHIOPODA				
Artemia salina (L)	M	H	4	3
Daphnia hyalina Leydig.	F	H	5	1-3
D. longispina O.F.Muller	F	H	5	1-2
D. magna Straus	F	H	Many	1-3
D. obtusa Kurz	F	H	5	3
D. pulex (De Geer)	F	H	5	3
Moina rectirostris (Leydig)	F	H	5	3
Triops cancriformis (Bosc)	F	H	3 (2 immature)	1
Scapholebris aurita Fischer	F	H	1	3
Simocephalus vetulus (O.F.Muller)	F	H	5	3
Evadne nordmanni Lov.	M		Many	1
Podon leuckarti Sars.	M		Many	1
COPEPODA				
Calanoids spp.	M		Many	1
Cyclops vicinus Kiefer	F		Many	1
Ascidicola rosea Thorell	M		2	1
Caligus curtus O.F.Müller	M		1	1
Clavella deliciosa Leigh-Sharpe	M		5	1
Modiolicola insignis Aurivillius	M		Many	1
Notodelphys rufescens Thorell	M		6	1
CIRRIPEDIA				
Balanus hammeri (Asc) Brown	M		3	3
B. balanus (L)	M		3	1 (Methb)
Peltogaster paguri Rathke	M	H	2	1
Sacculina carcini Thompson	M	H	1	1
OSTRACODA				
Heterocypris incongruens (Ramdohr)	F	H	10	2 - 3
LEPTOSTRACA				
Nebalia bipes (Fabricius)	M		35	1 - 2
ISOPODA				
Idotea granulosa Rathke	M		5	1
Jaera albifrons Leach	M		11	1
Ligia oceanica (L)	M		5	1
MYSIDACEA				
Gastrosaccus spinifera Goes	M		1	1
Praunus inermis Rathke	M		2	1
CUMACEA				
Leucon nasicoides Lilljeborg	M		15	1
AMPHIPODA				
Acidostoma obesum (Bate)	M		1	1
Amphithoe rubricata (Montagu)	M		4	1
Corophium bonelli G.O.Sars	M		Many	1
Gammarus locusta locusta (L)	M		5	1
G. locusta zaddachi Sexton	M		5	1
G. pulex (L)	F		10	1
Haploops tubicola Lindström	M		10	1
Leucothoe spinicarpa (Abildgaerd)	M		1	1
Maerella tenuimana (Spence Bate)	M		10	1
Parajassa pelagica (Leach)	M		1	1
Pontoporeia affinis Lindström	M		1	1
Socarnes erythrophthalmus Robertson	M		1	1
Sympleustes glaber (Boeck)	M		10	1
DECAPODA				
Astacus pallipes Ler.	F	C	2	2
Cancer pagurus L	M	C	2	1
Carcinus maenas (L)	M	C	12	1
Eupagurus bernhardus (L)	M	C	4	1
E. cuanensis (Thompson)	M	C	2	1
E. pubescens (Krøyer)	M	C	1	1
Hyas araneus (L)	M	C	1	1
H. coarctatus Leach	M	C	1	1
Inachus dorhynchus Leach	M	C	4	1
Leander adspersus (Rathke)	M	C	Many	1
Paelemonetes varians (Leach)	B	C	1	1
Portunus arcuatus Leach	M	C	1	1
P. pusillus Leach	M	C	4	1

Discussion

The results of this survey are in agreement with those of previous workers. During the work, animals from ten of the twelve orders of the Crustacea have been examined. Gut haemochromogens have only been detected with certainty in members of five of these orders, namely the Branchiopoda, Cirripedia, Ostracoda, Leptostraca, and the Decapoda, and in these orders have been found not to be of universal occurrence. Thus gut haemochromogens are not widely distributed in the Crustacea.

Although animals of widely different modus vivendi have been examined, no obvious correlation has been found between the occurrence of gut haemochromogens and any other factor.

The concentration of gut haemochromogen in different species of the Anomopoda seems to vary in parallel with that of the haemoglobin in the blood (cf. 10). This is not however true of the Branchiopoda in general, since Triops has a high concentration of haemoglobin in the blood, but apparently no haemochromogen in the gut. Fox (unpublished) has recently found much haemochromogen in the oviduct of this species, so it may be that haemochromogen accumulates here in this animal, while in most other Branchiopoda it passes into the gut. However, since no haemochromogen was found in immature animals before the ~~glands~~ gonads had developed, if

A. A series of ponds (I to VIII) in the Bedford College Botany Garden, Regent's Park, London.

B. The pond in the Eastern Aviary, Regent's Park Zoo, London.

C. A tank containing washings from the secondary filters at Hampton Water Works, London.

Letters A, B, and C will be given to indicate the source of the material in the experiments described below.

METHODS

1. Culture of Daphnia in the laboratory.

Daphnia were cultured at high and low oxygen concentrations by the standard methods used in this laboratory (13, 14, 15), the oxygen content of the water being determined by the Micro-Winkler Method of Fox and Wingfield (12, 14).

Cultures were either fed with Chlorella vulgaris Beij (14), or with bakers' yeast, and the feeding level measured with an M.R.C. Grey-wedge Photometer (19), using the red, number 1 filter. When Chlorella was used as food, its concentration was daily brought to an optical density of 0.25 by the addition of fresh algae. When the animals were fed on yeast, they were transferred daily to fresh suspensions of optical density 0.15, a feeding level which gave similar egg numbers to those of the Chlorella cultures. It was necessary to renew the water daily, for the rapid growth of bacteria in the yeast cultures made it otherwise impossible to maintain a steady oxygen concentration. In a few experiments described in Part I, animals were fed on Banta's medium,

(3) prepared in the normal way ~~from~~ with soil and camel's dung.

The volume of water allowed per animal varied in individual experiments, and so has been indicated in the 'Culture Condition' section, of each experiment. A volume of more than 5ml per animal was considered an uncrowded, and less than this as a crowded culture.

Animals were either cultured in pond water, or in water from a tap which had been allowed to run for some time, and the cultures were kept at room temperature (15 to 24° C) either in a dark cupboard, or under a mercury stip light. Well aerated cultures were kept in wide glass troughs, while cultures at low oxygen were maintained in conical flasks.

Iron was added to some cultures by the method described previously (15).

Variations from these standard procedures will be indicated in the text, in connection with the individual experiments.

2. Measurement of the haemoglobin concentration.

The haemoglobin concentration in the blood was measured by the index method (10, 13, 14, 15) and at the same time the length of the animal from the crown to the base of the tail spine was measured. It is obvious that a greater depth of blood is examined when measuring the concentration of haemoglobin ~~in~~ of a large animal, than when measuring that of a small one, and that the estimate is therefore proportionately

higher. Mr. J. Green of this laboratory (unpublished) has found that the length of the body is directly proportional to its width at the position where the heamoglobin concentration is measured. The haemoglobin concentrations of individual animals were therefore corrected for size. The standard size chosen was arbitarily fixed at 2.37mm body length, this being equivalent to 10 microscope eye-piece scale divisions under the conditions used during the measurement. All indices were based on samples of 10 animals.

3. Measurement of the concentration of dyes in the gut.

When it was necessary to measure the activity of the gut, animals were introduced into solutions of dye of known optical density (measured with the M.R.C. Grey-wedge Photometer) and when the ~~pigments~~ dyes had accumulated in the gut (10, 11) indices were obtained by measuring their concentration in the fore gut. These measurements were made by a method identical with that used for haemoglobin determinations. A solution of the dye was introduced into the wedge-trough, and moved on a 160-divisions scale in front of the microscope, until its strength matched that of the dye in the gut. It was seldom possible to obtain a standard of the same colour, for the dyes changed colour in the acid gut, and the gut wall itself was usually coloured, and acted as a light filter. However, it was possible to match the intensity rather than the colour of the dyes, and as repeatable results

were obtained, the method must have been satisfactory.

Nigrosin was usually used for the work, since it was found to be absorbed only slowly from the gut. Differences in its concentration were, however, only measurable after about 24 hours, so for rapid determinations of the activity of the gut the dye methylene blue was used. This was found to be absorbed more rapidly from the gut (cf.21) but when it was used, the experiments were of short duration, and it was assumed that during the experimental period, negligible amounts of dye were absorbed, and that its concentration in the gut did give a true index of the activity of the gut. The concentrations of dyes normally used for these experiments are shown below, and departures from the normal procedure are indicated in the text.

Table A.

Dye	Optical Density of solution into which animals introduced.	Optical Density of Standard used for measuring index.
Nigrosin	0.35	0.25
Methylene Blue	0.15	0.15

4. Measurement of the concentration of daphniarubin.

The absolute concentration of daphniarubin could not be measured, but a comparative value for this was obtained by the method of Elliott and Keilin (9). This method is

identical to Method 2, Section II, and to that described in Section III. and readers are referred to the latter section. (p 80 et seq.)

The figures for daphniarubin concentrations given below are in terms of the side arm scale (100 divisions), and unless otherwise stated, have been corrected for the size of the animal. As with the haemoglobin concentrations the standard size was chosen as 2.37mm.

5. Estimation of the total haem in eggs, embryos, and mature animals.

Known dilutions of a standard solution of globin haemochromogen were prepared by a similar method to that used in the preparation of the standard for the comparator method above. These were introduced into a Graham type compressorium (16) and the screw-head of this instrument racked down to 1.5 turns from its lower limit. The intensity of the α band of this solution was matched with that of the normal haemochromogen standard (0.3% blood in 60% N sodium hydroxide) in the wedge-trough on the calibrated side stage of the microscope. A calibration graph was then plotted of the concentrations of globin haemochromogen in the compressorium (in mg haem) against the corresponding scale reading, Since the extinction coefficient of pyridine haemochromogen is the same as that of globin haemochromogen (6, 20) it was possible to estimate the strength of solutions of this from the graph.

The total haem in eggs (E) was determined after dissect-

ing them from the brood pouch, and drying them lightly with filter-paper, and homogenising them with 0.1ml of pyridine, and a few crystals of sodium hydrosulphite. This homogenate was introduced into the compressorium, and the strength of it α band estimated at 1.5 turns of the head screw from its lower limit. The total haem per egg (e) was then calculated by dividing E (in mg haem per 100ml) by the total number of eggs (N) used for the estimation.

$$\text{Thus} \quad e = \frac{E}{N}$$

The total haem per animal was not measured in absolute terms, but a comparative estimate of its concentration (H) was made. This was done after drying 5 or 10 animals, depending on their size, with filter-paper, counting their total number of eggs and embryos (n) and measuring their individual lengths (L). This done, they were homogenised with 0.1ml of pyridine and a little hydrosulphite, and the haem concentration of the homogenate (h) estimated as above. Then H was calculated from the following equation:

$$H = \frac{h - ne}{\sum L^3}$$

This value was considered a satisfactory estimate of their total haem concentration because, as the length is proportional to the width and breadth (Green, unpublished) it must also be proportional to the volume of D. magna, and because the haem concentration of embryos was found not to

3. Some substance(s) is present in pond water, but not tap water, which is necessary for daphniarubin production, and this is partially removed, or no longer formed in filtered pond water.

Various experiments were undertaken to investigate these three possibilities.

Investigation of the rate of loss of dyes by the gut -

a. - in pond and tap water.

The rate of accumulation and loss of nigrosin at high oxygen by male and female Daphnia was measured by the standard methods described previously. No difference was found between the rates of these two processes in pond and tap water.

b - in crowded and uncrowded conditions.

Methylene blue, rather than nigrosin was used for this work, since it was necessary to measure the rate of loss of dye before excretory products had accumulated in the water. The details and results of the investigation are shown below.

Table 2.

Source. A Pond V.

Culture conditions.

Culture No.	Water	Crowding (Volume / animal in ml)
1	Pond water, previously containing 1 animal/15ml for 2 days.	7.5
2	Pond water, previously containing 1 animal/2ml for 2 days	7.5
3	Fresh Pond water	2.5
4	Fresh Pond water	7.5

Animals were kept in a solution of methylene blue for 1 hour and then introduced into these cultures.

Results:

Culture No.	Methylene Blue index at intervals after transfer to cultures.				
	0 hrs.	2 hrs.	Difference	4 hrs.	Difference
1	28	18	10	13	5
2		24	4	17	7
3		23	5	15	8
4		5	23	0	5

Thus crowding Daphnia, or introducing them into water which has previously contained animals, decreases the rate at which dyes are lost from the gut. This effect is probably partially due to the accummulation of some excretory product in the water, since the effect persisted in the water in cultures 1 and 2.

Thus the effect of crowding on the rate of loss of materials from the gut, cannot explain the effect of crowding on the daphniarubin index. That is, it disproves possibility 1, page 147.

Next was investigated the possibility that some factor present in normal pond water was necessary for daphniarubin production.

Salt content of Pond and Tap water.

It was thought that the decrease in the daphniarubin index of animals cultured in tap water might be caused by a difference in the salt content of this water from that in the pond. The Water Pollution Board at Watford kindly made an analysis of the salts in the laboratory tap water, and in pond water from two sources. The results are shown below.

Table 3.

Figures in parts / mill. of each kat&nion.

Water	Ca	Mg	Na	K	Cu	Co
Source A (Pond V)	75	5	15	4.8	< 0.05	< 0. 05
Source B	65	4	16	4.4	"	"
Tap Water	92	5	15	4.3	"	"

Thus there is no great difference in the kat-anion content of the pond and tap water.

The pH of these waters was tested, and found to be essentially similar (7.5 - 8.0) Thus the difference in daphniarubin index in the two waters seemed to be due to the presence of some organic substance(s) in the pond water.

Further investigation of this substance was facilitated by the discovery that light affected the daphniarubin index of animals cultured in pond water.

Effect of Light on the Daphniarubin Index in pond water.

When cultured in the dark, in frequently renewed pond water, the daphniarubin index can be maintained at, but not increased much above its initial value (cf. however Part II). Animals in the field may often have a very high index. The only difference between the two conditions seemed to be that in the field Daphnia are exposed to light. The effect of light on the daphniarubin index was therefore investigated, and since preliminary experiments showed that it did indeed have an effect, the following experiment was made.

the iron having an effect on the haem of yeast, but not on that of the Chlorella.

It is not known why the daphniarubin index fell in the Chlorella cultures. Perhaps this fall was in some way connected with the increase in the egg numbers of the animals during the experiment.

This experiment shows that light increases the daphniarubin index. Its effect may either be a direct one, on the Daphnia themselves, or an indirect one, due to changes in the composition of the medium.

Effect of 'Old' Chlorella on the Daphniarubin Index

Many experiments showed that the daphniarubin index of animals cultured in tap water in the light is low, when they are fed on fresh Chlorella. It was found, however, that if the water was not renewed, the daphniarubin index began to increase after a few weeks. This effect on the index, which did not occur in cultures kept in the dark, is shown in the table below.

Table 5

Source C

Culture Conditions Light. Chlorella. High oxygen 1 animal /10ml Tap water, not renewed. No fresh Chlorella added.

Initial and Final Haemoglobin indices 10

Days from beginning of experiment	0	7	11	12	14
Daphniarubin Index	10	14	15	17	23

When the tap water, but not the Chlorella was renewed every second day, a similar effect on the daphniarubin index was observed. This is shown below.

Table 6

Source, Culture Conditions and Haemoglobin indices as above, but Chlorella filtered off every two days, and reintroduced into fresh tap water.

Days from beginning of experiment	0	7	14	22	26
Daphniarubin Index	10	12	13	16	19

Table 6 proves that the low daphniarubin index of animals fed on fresh Chlorella cannot be due to the possible failure of tap water to counteract factors produced by Daphnia, inhibiting the production of daphniarubin. That is it disproves possibility 2, page 147 .

The results shown in Tables 5 and 6 suggested that bacteria might have grown in the Chlorella when this was not renewed, and that these might have caused the increase in the daphniarubin index. It was therefore thought, that the presence of bacteria, might be necessary for a high daphniarubin index, and that this could explain the higher indices in pond than in tap water cultures.

Banta medium contains more bacteria than pond water, and so the effect of this medium on the daphniarubin index of animals cultured in light and dark, was investigated.

Investigation of the effect of Light on the Daphniarubin Index in Banta Medium

Banta medium was prepared, and was 'grown' for 4 days in two portions, one in the dark, the other in a position exposed to the sun. Then 25ml of these two media, and of bacterially free filtrates from them, were introduced into a series of cultures in the light and the dark. The water, and media in these cultures was renewed daily. The daphniarubin indices after 5 days together with other details of these cultures, are shown below.

Table 7

Source C

Culture Conditions. Tap water. High oxygen. Yeast. 1 animal / 10ml.
Initially Haemoglobin index 53. Daphniarubin index 16.
Egg number 2.

L= light. D= dark. A= medium kept in light. B= medium kept in dark. Af and Bf = bacterially free filtrates from these media.

Added medium	-				A		Af		B		Bf	
Added iron	-		+		-		-		-		-	
Cultured in	L	D	L	D	L	D	L	D	L	D	L	D
Dr Index	10	10	12	12	26	20	20	20	19	13	13	13
Hb Index	21	23	32	37	31	23	37	35	34	32	22	36
Egg Number	15	14	14	13	13	17	10	14	15	12	11	13
O_2 in ml / L	5.5	5.5	5.9	5.8	5.4	5.5	6.0	6.0	5.9	5.9	5.9	6.1
pH	7.8				7.6				7.7			

This experiment shows that Banta medium produces a similar, but not a greater effect than pond water, on the daphniarubin index, and that its effect is not entirely due to the presence of iron. Since examination showed that this medium was much richer in bacteria than pond water, the experiment suggests that a high daphniarubin index is not entirely attributable to the presence of many bacteria in the medium.

decrease the rate of this process, evidence suggesting that this latter effect is probably due to the accumulation of some excretory products in the water. The above results cannot, therefore, be explained in terms of the rate at which daphniarubin is lost from the gut under the various conditions.

The effect of light on the daphniarubin index has been shown to be an indirect one, caused by the formation of some substance(s) in the medium, which is necessary for daphniarubin production.

The production of this substance(s) has been studied. It has been found that it does not become available to Daphnia during lysis of bacteria, or of algae. Probably the effect of light is produced by the acceleration of some photochemical reaction in either algae or bacteria. During this reaction a substance (or substances) is produced, which is, at least partially liberated into the water, and which is necessary for daphniarubin production. It has been found that at least one of these substances is thermolabile.

PART II.

Relationship between the Haemochromogen Concentration in the gut, and that of the body Haems.

In Part I, it was shown that the maintenance of a high daphniarubin index in laboratory cultures seemed in many cases to be dependent on the presence of some external factor(s). In this part, it will be shown that the daphniarubin index is also governed, to a large extent, by that of the body haems, and that the presence of an external factor is not always necessary for daphniarubin production. Evidence for these two facts is given below.

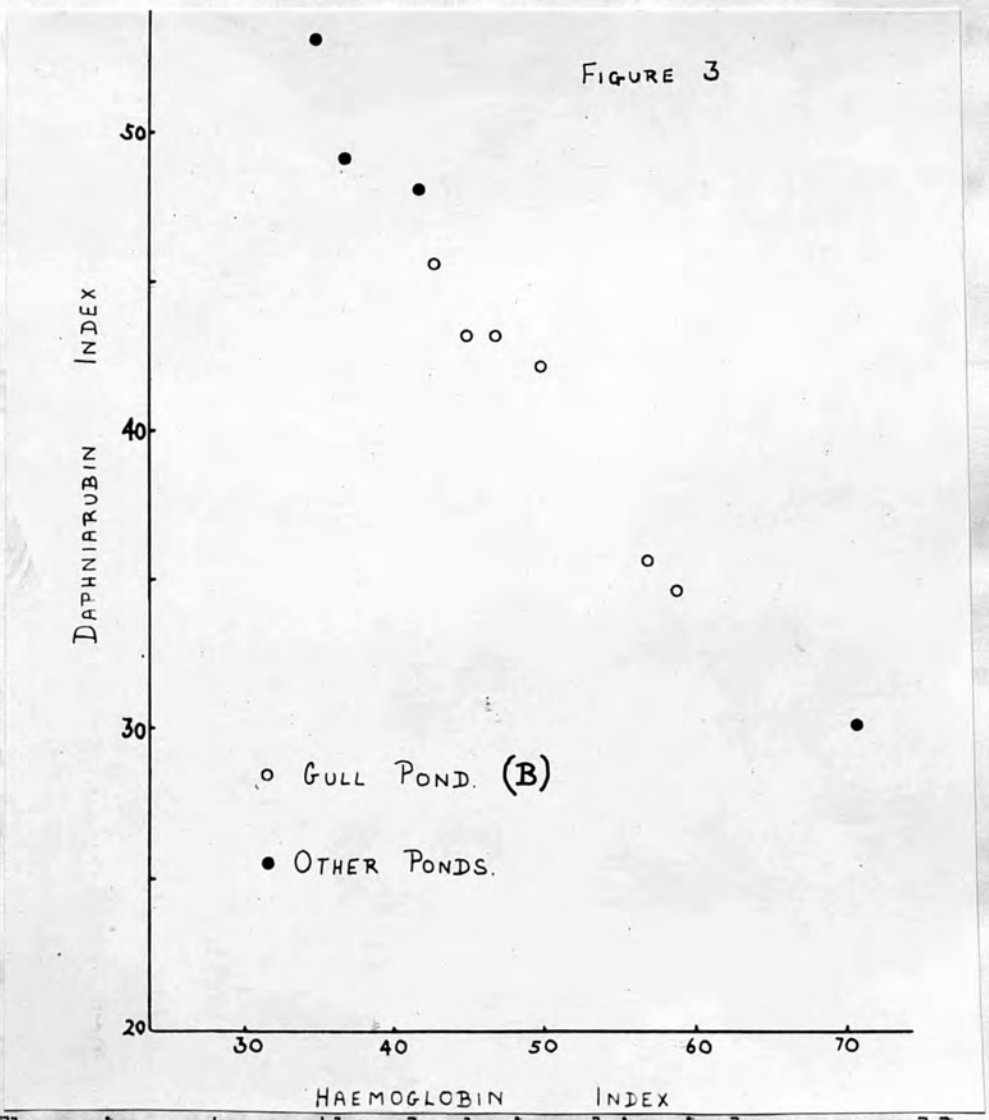
1. Relationship between the daphniarubin and haemoglobin indices of natural populations.

Measurements were made of the daphniarubin and haemoglobin indices of natural populations, feeding on a variety of different predominant foods. These indices based on samples of 20 animals, were measured immediately after the animals had been brought into the laboratory, since, as shown in Part I, the daphniarubin index may decrease rapidly. The results are shown below.

Table II.

Source	(Date 1952)	Size in mm.	Egg no.	Indices of Haemoglobin	Indices of Daphniarubin
A, Pond V	17. 3	3.25	21	35	53
A, Pond V	25. 2	3.56	14	37	49
Home Pond, Whipsnade	25. 2	3.02	0	42	48
B	22. 2	3.25	16	43	46
B	13. 3	3.46	27	47	43
B	11. 3	3.45	28	45	43
B	3. 3	3.49	17	50	42
B	29. 2	3.26	16	57	36
B	26. 2	3.46	17	59	34
Holly Frindle III, Whipsnade	25. 2	3.99	30	71	30

The coefficient of correlation between the logarithms of the haemoglobin and daphniarubin indices of these populations is $-0.98 (P < 0.001)$, and this is greater than that between the actual indices. The correlation between the actual indices is shown below.



Thus in nature the daphniarubin index normally varies inversely with the haemoglobin index.

It is of interest in connection with the results given in Part I, that after tap water had been run through Source B for one day, it was found that the daphniarubin index was 42

haemochromogen concentration is correlated, not only with the haemoglobin concentration at Stage 5 of development, but also with the initial haem concentration.

c. Effect of low oxygen tension on the haemochromogen concentration of late embryos.

Since the oxygen content of the water effects the haemoglobin concentration of mature Daphnia (10,13), its effect on the appearance of haemochromogen in late embryos was investigated.

Source B.

Details of Experiment. Mature animals (from the same source) cultured at high and low oxygen concentration for 3 days, and the haemoglobin and haemochromogen indices estimated on 10 embryos, immediately after liberation of these animals from the brood pouch, on the 2nd and 3rd days. (These embryos would have had the same initial haemoglobin, and haem contents). The results are shown below. The temperature was 23° C.

Table 13

Hb = haemoglobin index, measured with haemoglobin standard } By Comparator
Hr = haemochromogen index, measured with haemochromogen standard } Method.

Average O_2 of cultures in ml O_2/L	6.5		3.6	
Day of experiment	Hb	Dr	Hb	Dr
2	8	26	16	8
3	6	26	15	8

This experiment shows that less haemoglobin is lost, and less haemochromogen formed at low oxygen tensions.

d. Effect of light, dark, pond and tap water on the haemochromogen concentration in late embryos

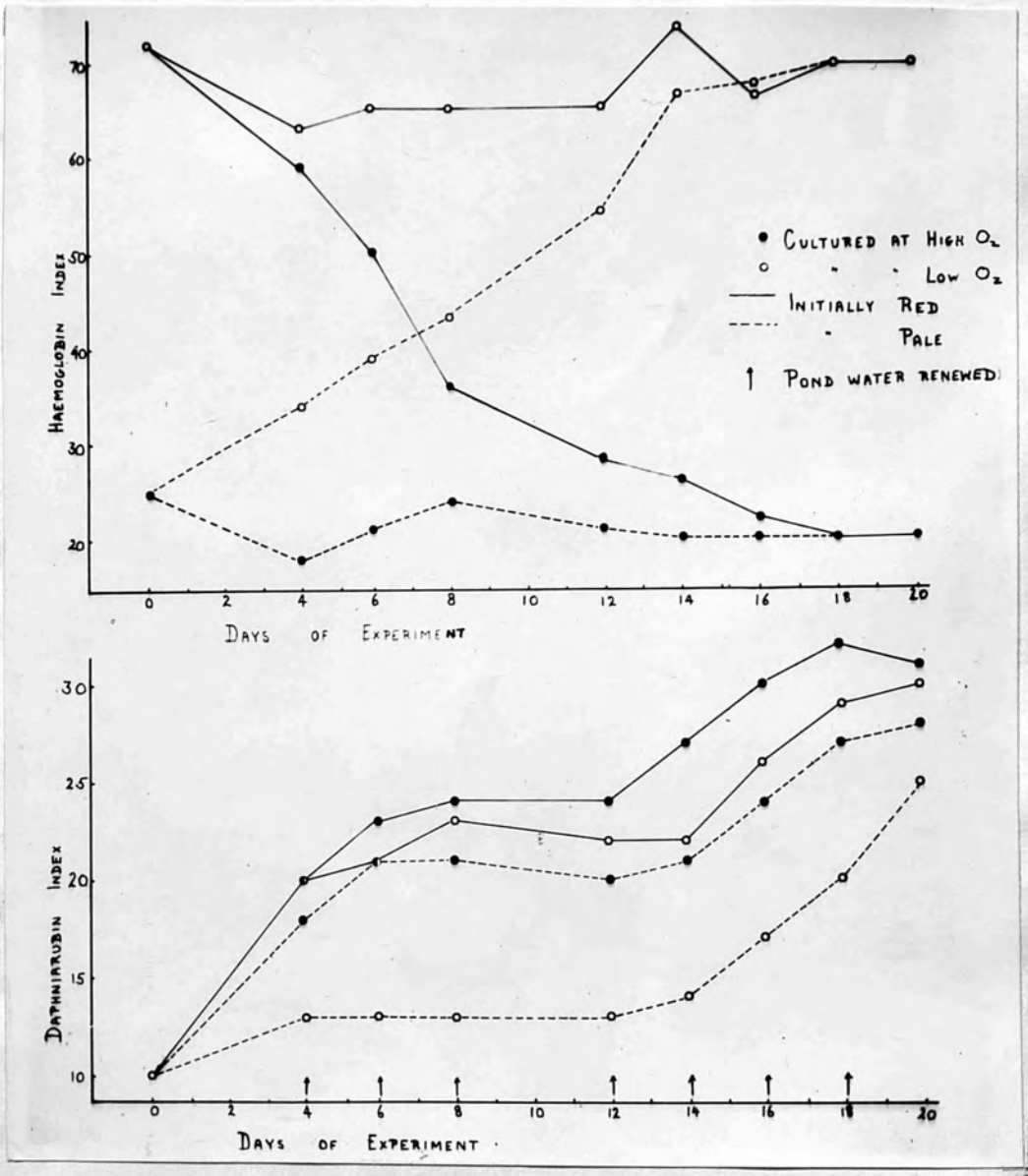
Experiments with dyes showed that the haemochromogen appears in the gut of embryos after their mouths have opened. The experiments described in Part 1 showed that light, dark, pond and tap water all effect the haemochromogen concentration of mature animals, and that their effect is probably due to the variations in the amount of some external factor required for the synthesis of the haemochromogen. It was thought that as the embryos were feeding, at the time when the haemochromogen appeared in the gut, an external factor might be necessary for its production. Accordingly the effects of light, dark, pond and tap water on the haemochromogen production of late embryos was studied. All these were found to be without effect. The addition of Chlorella and yeast also was found to have no effect on its production.

Thus firstly, there is no evidence for the necessity of the presence of an external factor for the appearance of haemochromogen in the gut, and secondly, the haemochromogen must be derived entirely from the body of late embryos.

e. The changes in the haemoglobin and daphniarubin indices of maturing animals.

The daphniarubin concentration of mature animals

FIGURE 5


HAEMOGLOBIN INDEX
70
60
50
40
30
20
0
2
4
6
8
10
12
14
16
18
20
DAYS OF EXPERIMENT
● CULTURED AT HIGH O₂
○ " " LOW O₂
INITIALLY RED
" PALE
↑ POND WATER RENEWED
DAPHNIARUBIN INDEX
30
25
20
15
10
0
2
4
6
8
10
12
14
16
18
20
DAYS OF EXPERIMENT

This experiment shows that the daphniarubin concentration of red animals cultured at high and low oxygen concentrations, and of pale animals cultured at high oxygen concentrations, begins to increase soon after the animals are introduced into pond water, but that that of pale animals at low oxygen concentration does not begin to increase until haemoglobin synthesis is complete.

The experiment therefore suggests that daphniarubin may be synthesised as an alternative to haemoglobin, and that possibly these compounds may therefore have a common precursor, or a constituent which is only available in limited quantities for their formation.

5. <u>The relationship between the haemoglobin and daphniarubin concentrations of animals in crowded conditions</u>.

Male animals were chosen for this experiment, so that the haem content of the body would not be effected by differential egg production under crowded and non-crowded conditions. The details, and results of the experiment are shown below.

<u>Table 16</u>

<u>Sources</u> A Pond VII & C.

<u>Culture Conditions</u> Pond water, renewed every 2nd day. High oxygen. Light. Crowding - (a) 1 animal / 2ml.
(b) 1 animal /15 ml.

<u>Initially</u>

Source	A. Pond VII	C.
Haemoglobin index	52	95
Length in mm.	1.88	1.91

Equal numbers of animals from each source were introduced

into each culture, so that a wider range of final haemoglobin concentrations would be obtained. The experiment lasted 14 days.

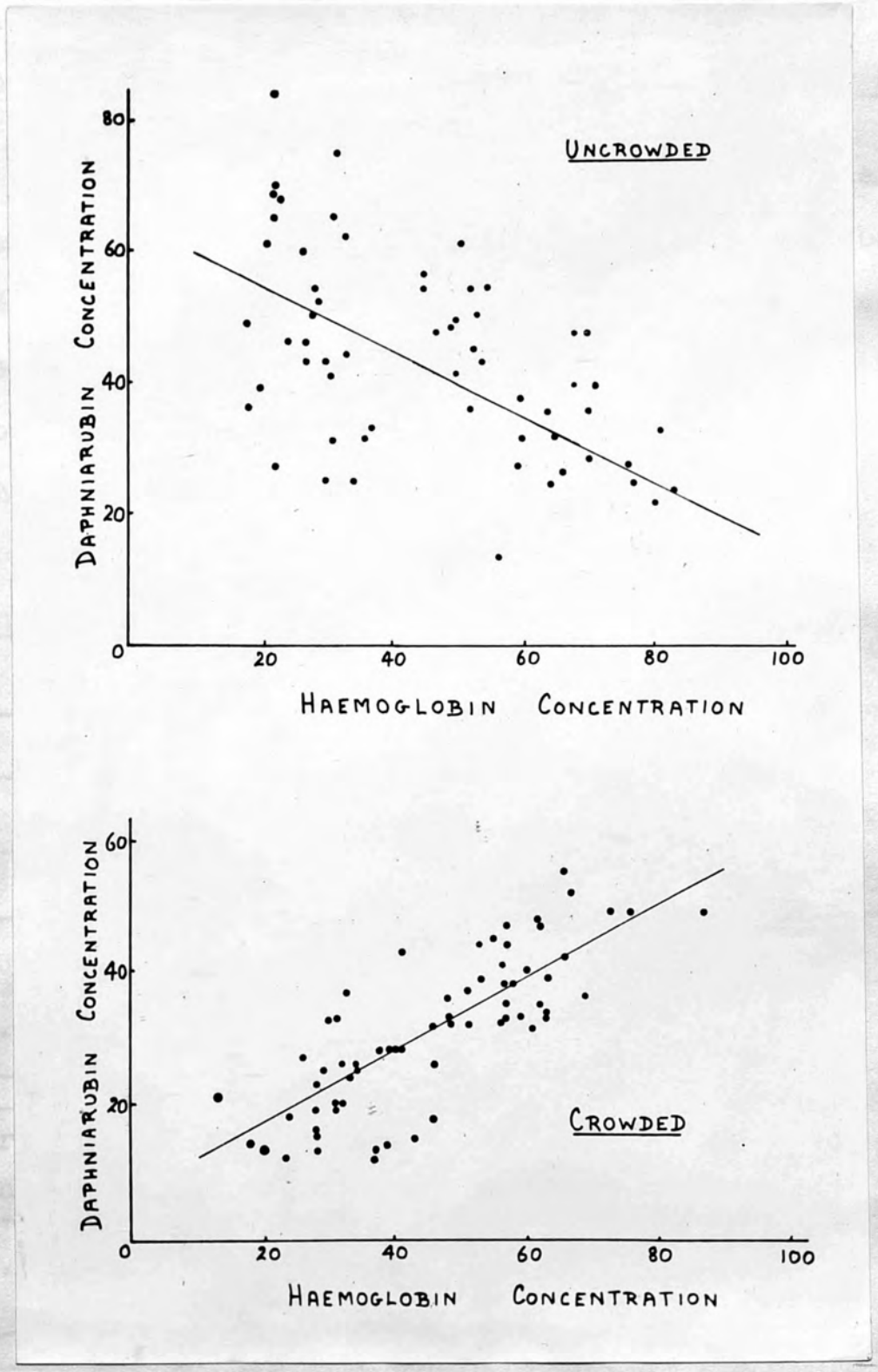
Indices on 14th day.

Culture conditions	Haemoglobin index	Daphniarubin index	Correlation coefficient	Probability level (P)
Crowded - (a)	46	31	+0.76	< 0.001
Uncrowded - (b)	45	44	-0.58	< 0.001

The correlations between the haemoglobin and daphniarubin indices of individuals from the two crowding conditions is shown below.

Figure 6.

FIGURE 6


UNCROWDED
DAPHNIARUBIN CONCENTRATION
0
20
40
60
80
100
HAEMOGLOBIN CONCENTRATION
CROWDED
DAPHNIARUBIN CONCENTRATION
0
20
40
60
80
100
HAEMOGLOBIN CONCENTRATION

Thus in crowded cultures the daphniarubin concentration varies directly, and in uncrowded culture inversely, with the haemoglobin concentration.

Investigation of the total haem of animals from crowded and uncrowded cultures.

The above experiment suggested that the haem of daphniarubin might be derived from the general body haem in animals in crowded conditions, since its concentration varied directly with that of the haemoglobin. The total body haem under the two culture conditions was therefore investigated. The results are shown below.

Table 17

Initially Each figure is the average of three determinations on 10 animals.

Source	H	Hb Index	Dr Index	Av. Length in mm.
B	1.4	83	31	1.95
C	0.9	40	26	1.82

Culture conditions as in Table 16.
After 6 days.
A. Animals from Source B.

Culture	H	Hb Index	Dr Index	Length in mm.
Crowded (a)	1.0	65	32	1.95
Uncrowded (b)	1.4	76	50	2.02

B. Animals from Source C.

Culture	H	Hb Index	Dr Index	Lenght in mm.
Crowded (a)	0.9	33	21	1.88
Uncrowded (b)	1.1	37	27	1.89

Thus the haem content of Daphnia is lower in crowded, than in uncrowded conditions. The results support the suggestion that the haem. of daphniarubin is derived from the body in crowded conditions.

6. The relation between the concentration of haemoglobin and daphniarubin in starved cultures.

The egg number of animals in crowded conditions is lowered (23) and since this is a fair index of the level of nutrition of Daphnia (18, 24) it seems probable that, even when supplied with excess food, animals in crowded conditions feed less. The effect of starvation on the relationship between the concentrations and haemoglobin was therefore investigated, again using male Daphnia.

Table 18.

Source. C.
Initially Length 1.78mm. Haemoglobin index 33.
Daphniarubin index 24.
Culture Conditions. Pond water, renewed every 2nd day.
1 animal / 12 ml.

Feeding level (Figure 7)	1	2	3	4
Optical density of added yeast	0.14	0.09	0.05	0.0
After 6 days				
Average O_2. % at 18° C	88	86	85	89
Haemoglobin index	30	30	30	20
Daphniarubin index	20	20	15	20
Correlation coefficient between Hb and Dr concs	-0.90	-0.88	-0.09	+0.76
P	<0.001	<0.001	>0.1	<0.001

Figure 8

This experiment shows that added calcium both decreases the rate, and the amount of haemoglobin synthesised at a steady, low oxygen concentration.

Since the daphniarubin concentration is abnormally high in the presence of calcium, this experiment suggests that normally daphniarubin passes back into the animal, and that it is used to synthesise haemoglobin. However, as will be shown in Part III the high daphniarubin concentration in the gut in the presence of calcium can be explained at least partially by the action of the latter on the gut musculation. The low haemoglobin synthesis in the presence of calcium in this experiment is probably due to impairment of normal feeding and absorption of food, and hence to partial starvation of the animals, caused by

a decreased permeability of the gut. This explanation is supported in the above experiment by the fact that the animals in the cultures containing calcium had lower final egg numbers.

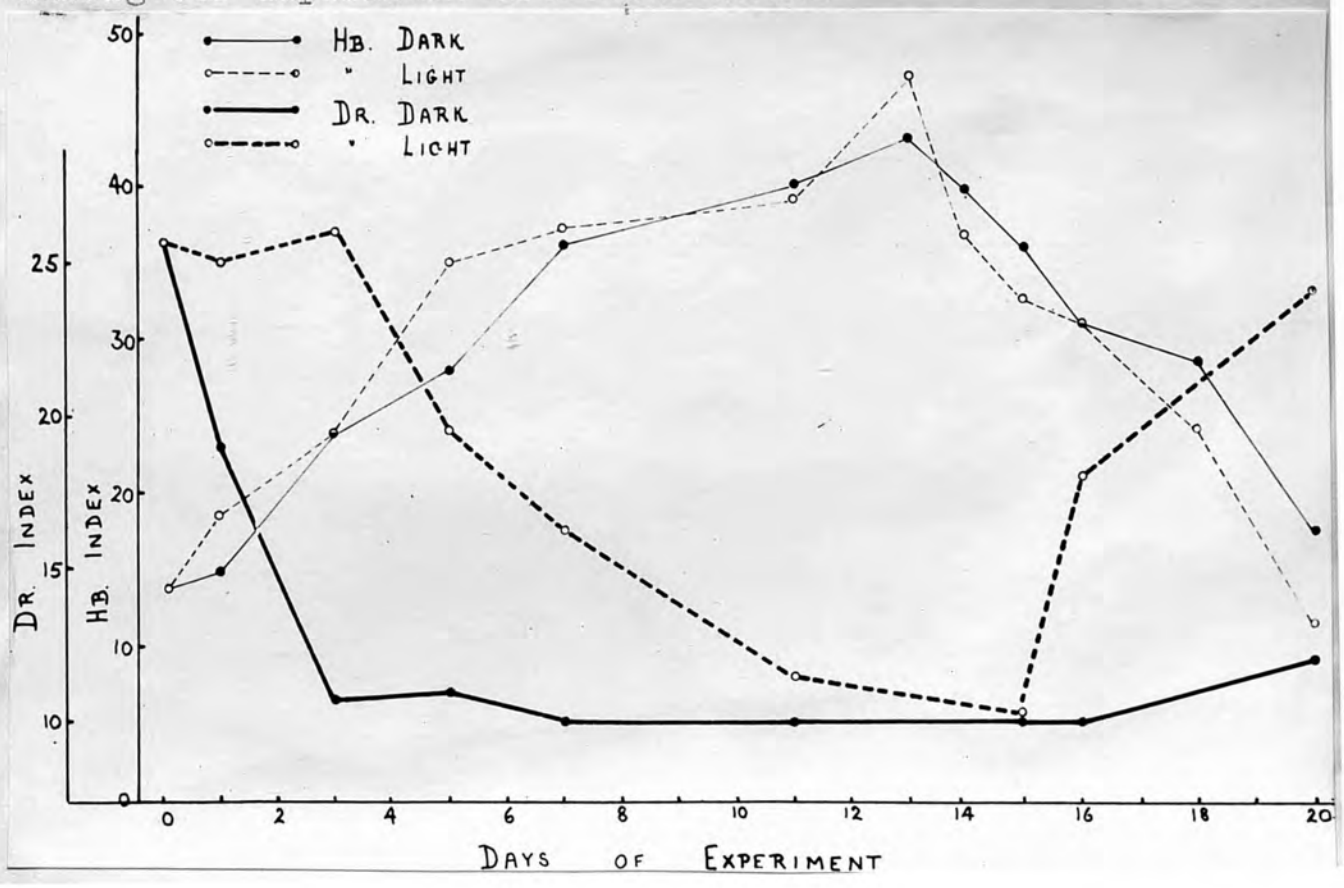
This experiment therefore does not prove that diaphnarubin is reabsorbed from the gut.

b. Effect of light on haemoglobin synthesis and loss

It has been shown in Part I, that in pond water cultures, light increases the daphniarubin index. The effect of light on haemoglobin synthesis was therefore investigated.

Source B.
Culture Conditions Pond water, renewed every 2nd day. Yeast. Iron. *
Initially Haemoglobin index 15. Daphniarubin index 35. Egg number 7. * On Day 13 cultures transfered from low to high Oxygen

The changes in the daphniarubin and haemoglobin indices during the experiment are shown below.



Thus light has no effect on the haemoglobin synthesis of animals in pond water and this suggests either that daphniarubin is not reabsorbed, or if reabsorbed is not utilised in haemoglobin synthesis.

Summary

The results given in this part, show that the haemochromogen concentration in the gut is related to the haem concentration of the body.

A haemochromogen has been detected in the wall of the fore-gut, when the concentration of daphniarubin in the gut is high.

An inverse relation has been established between the concentration of haemoglobin, and that of the daphniarubin in natural populations, and in favourable culture conditions in the laboratory. A direct relation has been found between the concentrations of these compounds in crowded, and starved cultures in the laboratory, when an external factor necessary for ~~its~~ daphniarubin production must have been present in negligible concentration, and in these latter conditions, it has been shown that the total body haem decreases.

It has been shown that a haemochromogen appears in the guts of embryos at the end of the brooding period, and that its concentration increases at the exact time when the haemoglobin concentration of these embryos is decreasing. The final concentration of this haemochromogen

is not effected by light, dark, pond and tap water, but it has been found that at low oxygen tensions, less haemoglobin is lost, and less haemochromogen formed by the embryos, than at high oxygen tensions. It has also been found that their final haemochromogen concentration is directly proportional to the haemoglobin concentration of the embryos at Stage 5.

It has been found that the increase in the daphniarubin concentration of initially pale animals with a low daphniarubin index is delayed when they are cultured at low oxygen in frequently renewed pond water cultures in the dark, until haemoglobin synthesis is completed, suggesting that in these last conditions haemoglobin is synthesised in preference to daphniarubin.

The possibility that daphniarubin is reabsorbed from the gut has been investigated, on the supposition that its reabsorption would effect the rate of haemoglobin synthesis. Light, which increases the daphniarubin concentration of animals in pond water, was found to be without effect in these conditions on haemoglobin synthesis. Added calcium, which also increases the daphniarubin concentration, was found to decrease haemoglobin synthesis but at the same time to lower the ~~rate and amount of~~ egg numbers. From these experiments no ~~satisfactory~~ conclusive evidence has been found to indicate that the haemochromogen is absorbed from the gut.

PART III

Variation in the concentration of Daphniarubin with the Physiological Activity of the gut.

Two observations suggest that the concentration of daphniarubin varies with the physiological activity of the gut. These are, firstly, the observed variation in the daphniarubin concentration during an instar, and secondly the effect of the addition of calcium on the daphniarubin index. These variations and the reasons why they are believed to be association with variations in the activity of the gut, are shown below.

1. Variation in the concentration of daphniarubin during an instar.

The haemoglobin concentration of Daphnia is known to vary considerably during an instar (7). Since the results of Part II suggest that the daphniarubin concentration might sometimes be related to that of the haemoglobin, its concentration was also studied at stages during an instar, to see if parallel variations with that of the haemoglobin could be detected.

Source A Pond VII

Av. 2.9mm, Egg No. 14.

Figure 10 shows the variations in the concentrations of daphniarubin and haemoglobin at stages during the instar,

and suggests that these variations are not related.

Variation in the total body haem during an instar

The experiments described in Part II suggested a possible relation between the daphniarubin concentration and the total body haem. The variation in the latter during an instar was therefore next investigated, to see if this could be related to the variations in the daphniarubin index.

Source 1) A. Pond III.
Haemoglobin index 56, Daphniarubin index 54,
Egg No. 23, Av. 2.6mm.

2) A. Pond VII.
Haemoglobin index 48, Daphniarubin index 62,
Egg No. 14, Av. 2.5mm.

The total body haem was estimated on samples of 10 animals, and the values of H. shown in Figure 10 are the average of 4 estimates from each source. This Figure shows that the total body haem increases more or less steadily during an instar, and suggests that its variation in concentration during this period bears no relation to the variation in the concentration of the daphniarubin.

attributable to the presence of the calcium ion.

The variations in this basal level of the daphniarubin with various concentrations of added calcium (as $CaCl_2$) after 7 days, are shown below.

Source. Gull Pond. Regent's Park Zoo. (B)

Culture Conditions. Distilled water. Yeast. High oxygen Dark. 1 animal / 10ml.

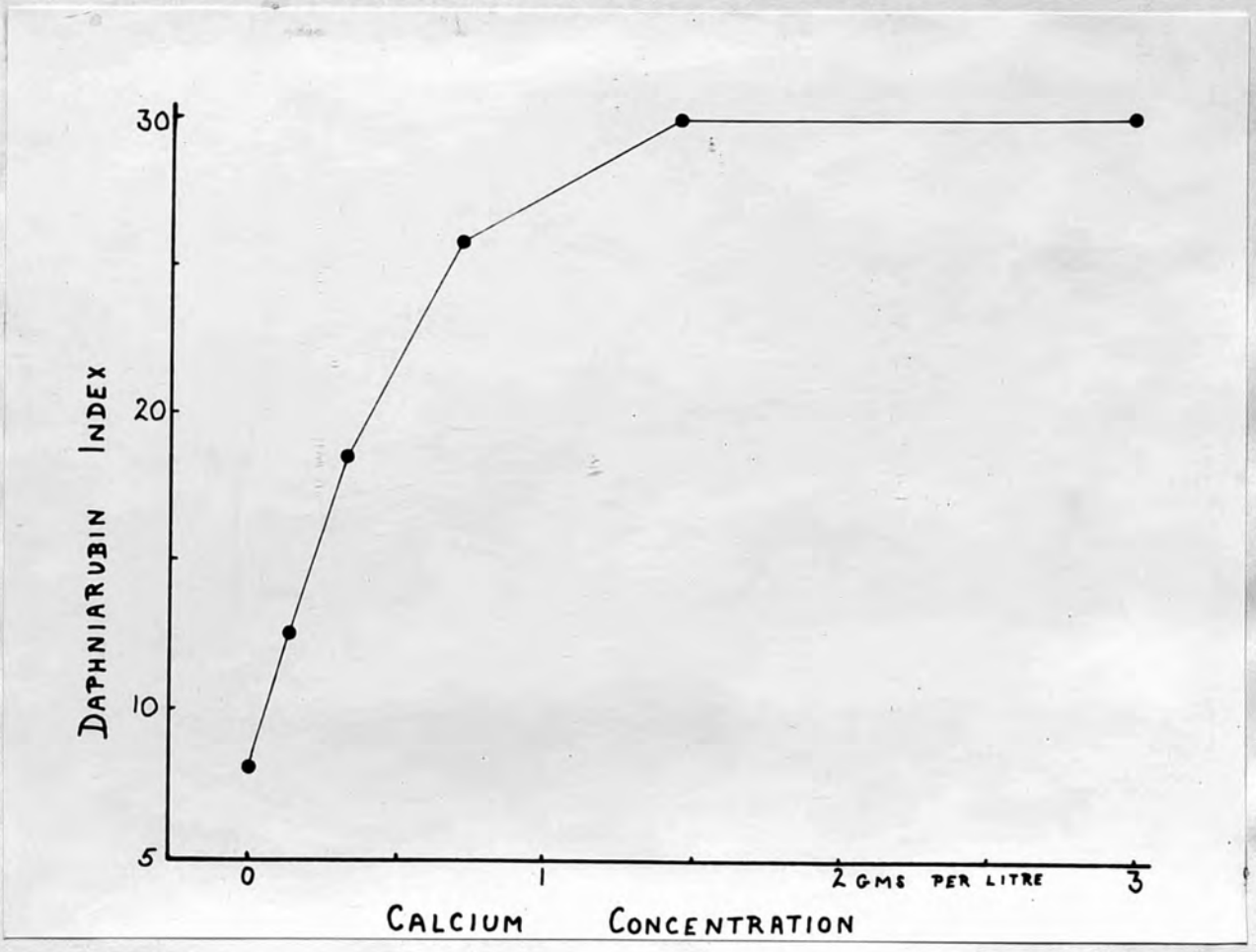
Initially Haemoglobin index 27, Daphniarubin index 44. Egg No. 33.

On 7th day Haemoglobin index 10.

Final egg no.	13	14	15	15	12	8
Gms Ca /L	0	0·15	0·36	0·72	1·45	2·90

The daphniarubin indices of the cultures are shown in Figure 12

FIGURE 12.



Thus the daphniarubin index increases with increasing amount of added calcium. The relationship is asymtotic, and the maximum effect is produced by I.5g or more Ca/L.

The effect of calcium on the rate of accumulation of dyes by the gut.

Since calcium is known to effect permeability, and muscular activity (17) its effect on the rate at which dyes are concentrated by the gut was studied. The results are shown below.

Table 21

Source. Gull Pond. Regent's ParkZoo.(B)
Conditions of Experiment. Tap water (=0.9g Ca / L) ± 2g Ca (as $CaCl_2$) High oxygen. Light. Methylene blue. (O.D.=0.06) Indices measured with standard of O.D.= 0.35.

Time after introduction into dye in hrs.	2	4
Nigrosin index. No calcium added	23	71
Calcium added	6	14

Thus the presence of calcium decreases the rate at which dyes accumulate in the gut. Since the concentration of the dye in the gut is probably due to swallowing followed by absorption of water, this effect of calcium could either be due to a decrease in the rate at which the solution is swallowed, or to a decrease in the rate at which the water is absorbed. That is, its effect could either be on the gut musculature, or on the gut permeability.

The effect of calcium on the rate of loss of nigrosin from the gut.

Table 22

Source Pond V. Bedford College Botany Garden. (A)
Conditions of experiment. Tap water. Nigrosin. (O.D.?) High oxygen Light. Nigrosin index estimated with a standard of O.D.=0.35.

After 24 hrs. animals transferred to pond water (≡ 0.75cg Ca/L) ± 2g/L Ca (as $CaCl_2$)

Time after transfer to pond water in hrs.	0	24	48
Nigrosin Index No added Calcium	69	6	0
Calcium added		61	56

Since nigrosin is not absorbed from the gut, the results of this experiment indicate that the action of calcium is, in part at least on the gut musculature.

Confirming this, it was found that added calcium decreased the rate of swallowing of animals in pond water.

Summary

The results given in this part show that the daphniarubin concentration is effected by the physiological activity of the gut.

Under natural conditions, it has been found that the daphniarubin concentration varies during an instar. It is high just before and after the moult, and minimal at stages 4 and 5. This variation does not seem to be connected with that of the haemoglobin, or total haem of the body. It has

been found however that parallel variations occur in the rate at which dye solutions become concentrated during the instar. This suggests that the variation in the index during an instar is connected with the activity of the gut.

It has been found possible to increase the daphniarubin index under experimental conditions by the addition of sea water, and of Mammalian Ringer. The effect of the latter has been found to be caused by the presence in the solution of the calcium ion. The maximum effect of calcium has been found to be produced by 1.5g or more / L of this ion (added as $CaCl_2$). It has been shown that calcium decreases the rate at which dye solutions become concentrated by, and are lost from, the gut, and suggested that its action is in part at least on the gut musculature.

Discussion

Fox (10) suggested that the haemochromogen which he found in the gut of *Daphnia magna* might be derived from the haemoglobin of the blood, since he thought that its concentration was proportional to that of the haemoglobin, and since he found that a haemochromogen was present in lower concentration in the gut of *Chirocephalus diaphanus* where the blood contained less haemoglobin, than that of *Daphnia magna*. He also detected similar compounds in *D. hyalina*, and *Astacus pallipes*, and suggested that in these animals the haemochromogen might be derived from other tissue haems, since little or no haemoglobin was present in the blood. Apart from this suggestive evidence, previous to this work nothing has been published on the origin, or on the variations in the concentration of gut haemochromogen in the gut of *Daphnia* (daphnia rubin).

Although in Part I, it was shown that under favourable conditions, some external factor(s) was necessary for the maintenance of a high daphniarubin index, this does not always seem to be necessary, for much haemochromogen was present in the guts of starved animals and embryos, under conditions when such a factor could not have been present.

Although under certain conditions this external factor(s) may be necessary during its formation, the results of this Part show that the gut haemochromogen is formed by the animal.

Much evidence supports this: no similar haemochromogen was detectable in the medium, haemochromogen appeared in the guts of starved animals, and many observations and experiments show that the concentration of haemochromogen in the gut is related to the haem concentration of the body.

In developing embryos, and in starved animals, the evidence obtained from this work suggests, not only that the haemochromogen is formed by the animal, but also that it is actually derived from the haem in the body. However, at the present time, it is impossible to know from which of the body's haems it is formed in these conditions, since Miss W. Hosking, of this laboratory (unpublished) has found that the concentration of tissue haem varies directly with the haemoglobin concentration of the blood in D. magna. Therefore during the present work, when a correlation was found between the haemochromogen concentration in the gut, and that of the haemoglobin, this may have been only an apparent correlation, the true relation being with the concentration of tissue haem. Even the decrease in the concentration of haemoglobin in Stage 8 embryos, as the haemochromogen concentration in their guts increased, although suggestive, does not prove that the haemochromogen was derived from haemoglobin, for it may have been formed from cell haems, while the haemoglobin was being lost by another route, and this suggestion is supported by the fact that no correlation was found between its concentration, and the loss

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SECTION V

DISCUSSION OF THE ORIGIN, FUNCTION & FATE OF THE HAEMOCHROMOGENS OF INVERTEBRATES

DISCUSSION OF THE ORIGIN, FUNCTION, AND FATE OF GUT HAEMOCHROMOGENS IN THE INVERTEBRATA

My work has contributed little to our knowledge of the origin, function and fate of gut haemochromogens in the Invertebrata. As the work proceeded, it became apparent that quantitative investigations, and experiments with tracer elements, were necessary to solve these problems, and such experiments were not possible in the time available for this work. Moreover, it now seems unlikely that any conclusions will be reached on these matters, until much more work has been done in the general haem metabolism in Invertebrates. By way of conclusion to this thesis therefore, a brief review will be given of previous opinions on the origin, function and fate of gut haemochromogens, and these opinions will be discussed in the light of the results of the present work.

1. Origin of gut haemochromogens

Previous literature on these compounds is unanimous in attributing their formation to the animals in which they are found, although little evidence has been advanced in support of this suggestion, and their exact site of origin is not known. In the Mollusca, it has been generally considered that these compounds are formed in the liver, and MacMunn (20) was tempted to associate the ferment cells of the liver of *Helix*

with the formation of helicorubin, although he was unsuccessful in proving this.

Many facts arising out of my work support the suggestion that gut haemochromogens are formed by the animals in which they are found. For instance, gut haemochromogens were found in a variety of animals with different foods and habitats, while similar haemochromogens were not found in the environment or food of these animals. In Daphnia magna it was found that a haemochromogen appeared in the gut of embryos deprived of food and that its concentration increased exactly at the time when their haemoglobin concentration was decreasing. In this species too, an inverse correlation in favourable conditions, and a direct correlation in unfavourable conditions, was found between the haemochromogen concentration in the gut, and the haemoglobin concentration in the blood of mature animal. These findings, together with the inverse relation found between the concentrations of gut haemochromogens and of haemoglobin or chlorocruorin in the blood of various Polychaeta, would be difficult to explain on the theory that gut haemochromogens are formed outside the animal.

The present work has contributed nothing to the knowledge of the exact site of formation of gut haemochromogens in the animal. Histological work, and studies with tracer elements are needed to solve this problem.

adequate buffering capacity, without the necessity for the addition of further buffers. Thus it is unlikely that this is the primary function of helicorubin, although it is just possible that it is a function of some other gut haemochromogen.

Influenced by the work of Darwin, Sorby (25) suggested that evolution could be traced in present day animals, not only in their morphological structures, but also in their biochemical compounds. He thought that the most stable form of haematin appeared first in the form of helicorubin, and then, by progressive evolutionary changes, animals became capable of modifying this haem so that it would combine reversible with oxygen. This stage of evolution of the haem he thought was represented today by myoglobin and by the plasma haemoglobin of Planorbis. (Later according to his theory, the haemoglobin became enclosed in 'discs'). It is unnecessary to discuss this theory fully here, since it is based on the fact that, at the time, no gut haemochromogen had been found in any animal with plasma haemoglobin. Subsequent work has shown that gut haemochromogens are often found in animals possessing myoglobin or plasma haemoglobin. Perhaps therefore, their formation is more comparable with the formation in man of Type 1 proto-porphyrin concurrently with the Type 111 proto-porphyrin utilised in haemoglobin synthesis (6). However, both the Type 1 porphyrin, and the gut haemochromogens may have an as yet undiscovered function.

Semper (quoted by MacMunn (18)), Anson and Mirsky (1) and MacMunn (18) were among those who suggested that gut haemochromogens were true respiratory pigments, although MacMunn later changed his mind on this matter. This suggestion was based on the fact that no haemochromogen was found in the gut of Planorbis, where these authors considered that the abundant haemoglobin in the blood was adequate for the animal's respiratory requirements, and also on the beliefs of the authors that the occurrence of gut haemochromogens was 'dependent on aerial respiration' (18) and that these compounds could combine reversibly with oxygen. The work of Morena (21), and that described in Section I, has shown that helicorubin does not combine with oxygen, and that it cannot therefore be a true respiratory pigment. It seems likely that if it is in any way connected with respiration, it is more comparable with the cytochromes, and that it therefore ~~acts~~ functions in the body tissues not in the gut (8).

Finally, it has been suggested that gut haemochromogens are excretory products. Krukenberg (16) suggested that they were derived from haemocyanin, but this obviously cannot be correct, for they are found in animals not possessing haemocyanin. Again Verne (27) thought that they were derived from chlorophyll, but his arguments in proof of this are not convincing. Moreover, during the present work, these compounds have been found in animals not feeding on

chlorophyll-containing foods, and green foods were not found to increase the concentration of haemochromogen in the gut of Helix or Daphnia relative to that of non-chlorophyll-containing foods.

MacMunn (19) wrote of helicorubin '...it is probably the mother substance of those histohaematins which are found in animals in whose liver it is built up. Another view might be held, namely that it is an excretion and represents the form in which the above colouring matters are got rid of, but its very instability is against this view'. Opposing MacMunn's first suggestion, it seems unlikely that Helix would pass a precursor of cytochromes into the gut, many Crustacea have been found which possess cytochromes, but no gut haemochromogen, and the work described in Section IV suggests that the gut haemochromogen is lost from the body of Daphnia.

MacMunn's second suggestion, namely that gut haemochromogens might be the break down product of tissue haems was postulated again later by Fox (11,12). Frenzel's work (quoted by MacMunn) (19) does not support this idea however, for he found that Helix voided colourless faeces when fed on filter paper. The work described in Section 11 confirms this observation of Frenzel, and suggests that helicorubin is not lost from the animal. Moreover, it seems curious that some Invertebrates should excrete haem in such a specialised form, and probably in combination with a protein, while in

Vertebrates haemoglobin is known to be broken down *in vivo* (17), and catalase, peroxidase and myoglobin *in vitro* (14,15) into the simpler form of bile pigments. The necessary enzymes for this latter conversion cannot be absent from all Invertebrates (or even all plants) since bile pigments are known to be formed from haemoglobin in *Rhodnius* and the louse (29), in the Rhizocephala (3,22), and in certain chironomids (5), possibly in *Aphrodite* (2), and in root-nodules of leguminous plants (28), and they have been found, although in the absence of haemoglobin, in many other invertebrates (4,10,13,24, etc.) However, with the exception of integumentary pigments, bile pigments have not yet been found in any animal possessing a gut haemochromogen. Moreover, the work described in Section IV suggests that in *Daphnia magna*, gut haemochromogen is formed from, or as an alternative to body haem, and further that it is normally lost from the gut. Finally in support of MacMunn's suggestion, it has been shown in Section I, that helicorubin is not unstable as MacMunn had thought.

From the survey of the distribution of these compounds in the Mollusca, Polychaeta, and Crustacea, it seems probably that, if they are excretory products from body haem, they must, at least in part be derived from tissue haems as MacMunn had suggested, and that they are not always, if ever, formed from haemoglobin, for they have been found in animals devoid of haemoglobin (cf.11).

If they are excretory products, then they probably have no function in the gut, and this could therefore explain why individual, apparently healthy Helix, Polychaeta, and Daphnia have been found with abnormally little, or no, detectable gut haemochromogen. If it is assumed that the haemochromogen nature of these compounds is not essential to their function as excretory products, then it could also explain why haematin or methaemoglobin, rather than haemochromogen was found in the gut of some Polychaeta, and Crustacea. Finally, if it is assumed that there are other excretory paths for body haem, it could also explain why many species of invertebrates seem to be quite devoid of gut haemochromogens.

Thus the significance of gut haemochromogens is still unknown. They may represent an evolutionary 'cul-de-sac', or be formed by an alternative synthetic path to tissue haems. It seems probably, however, that they are derived, at least in part, from haem in the animal, and that they are true excretory products, having no function in the gut.

3. Fate of gut haemochromogens.

The fate of gut haemochromogens in the Mollusca is not known. The work described in Section II suggests that in Helix helicorubin is either broken down by bacteria in the gut, or passes into the distal diverticulae of the digestive gland where it is possibly reabsorbed, but that it