THE DISTRIBUTION OF IRON IN *DAPHNIA* IN CONNEXION WITH THE SYNTHESIS AND BREAKDOWN OF HAEMOGLOBIN.

by

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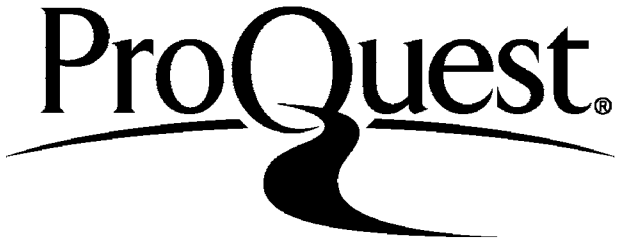
Thesis submitted for the Ph.D. degree in the University of London.



ABSTRACT

THE DISTRIBUTION OF IRON IN *DAPHNIA* IN CONNEXION WITH THE SYNTHESIS AND BREAKDOWN OF HAEMOGLOBIN.

Experimental studies have previously been made on invertebrates with haemoglobin in the body fluids. In spite of this the site of formation and breakdown of the respiratory pigment is, in many cases unknown. The aim of this work has been to show these sites in an arthropod, which possesses haemoglobin in the blood, namely *Daphnia* (Crustacea, Cladocera).

The haemoglobin of *Daphnia* is synthesised in the large fat- and glycogen- containing cells of the fat body. This is an extensive organ which lines the haemocoel and so is everywhere bathed by blood. These fat cells often contain iron and sometimes haemoglobin. A synthesis of haemoglobin may also take place in the ovaries.

A breakdown of haemoglobin occurs in the fat body where iron is found in an unbound form when the blood haemoglobin content falls. The fate of iron released from haemoglobin breakdown, differs in *Daphnia*, from that in mammals. In *Daphnia* iron has been found in the paired excretory organs following a loss of haemoglobin from the blood. Experimental studies and a study of the histological

distribution of iron, have indicated that this iron is excreted.

Mammals retain iron, as stores of ferritin, but ferritin has not been found in Daphnia.

A histochemical test for bile pigments, failed to reveal any such pigments in Daphnia. Green pigments have been found in Daphnia, these are sometimes carotenoid protein complexes, sometimes green derivatives of haemoglobin, which are not bile pigments.

Preliminary results have indicated that a general increase of haem compounds accompanies an increase in the haemoglobin content of the blood.

The work is presented as a histochemical study. The distribution of inorganic iron, organic iron and haem iron, has been studied and it has been found that the distribution of iron is related to the condition of the haemoglobin in the blood.

Some experimental work has been undertaken to confirm histochemical observations.

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SECTION 1.

INTRODUCTION.

1. INTRODUCTION.

Cellular respiration in animals is often aided indirectly by the transport of oxygen by respiratory pigments in the body fluids. These pigments are of four types: chlorocruorins, haemerythrins, haemocyanins and haemoglobins; the two latter pigments are the most widely distributed. Chlorocruorin, haemerythrin and haemocyanin are restricted to animals of certain phyla. Chlorocruorin has been found in polychaetous annelids such as Serpula (Lankester 1867; Fox 1926). Haemerythrin has been found in sipunculids (Lankester 1872), a single annelid Magelona (Benham 1896,) and a brachiopod Lingula (Kawaguti 1941). Haemocyanin is limited in distribution to gastropod and cephalopod Mollusca, decapod Crustacea and arachnids.

Haemoglobin on the other hand has a wider but sporadic distribution and occurs in animals of varying habits and habitats. It is found in almost all vertebrates* and is of widespread occurrence among annelids and entomostracan Crustacea (Lankester 1872; Regnard & Blanchard 1883; Fox 1949). It is less widespread in

* Certain fish have recently been described as having no haemoglobin in the blood (Ruud 1954).

other invertebrates and has only been found in isolated genera such as an ophiuroid Ophiactis (Foetinger 1880) and a gastropod mollusc Planorbis (Lankester 1872). Some larval Diptera, ciliate paramoecia and Nematoda have also been shown to contain haemoglobin. Outside the animal kingdom haemoglobin occurs in the root nodules of the Leguminosae (Kubo 1939).

Haemoglobin may occur in solution in the blood plasma, within blood or other cells, or as myoglobin in muscles. Vertebrates have haemoglobin in red blood cells and in muscles. Invertebrates rarely have haemoglobin in either blood cells or muscles; the pigment is usually in solution in the blood plasma as in the Entomostraca. Some invertebrates have haemoglobin within cells, such as the coelomic corpuscles of Thalassema (Ohuye 1937) and Solen has haemoglobin in blood cells (Lankester 1872).

Vertebrate animals* have haemoglobin throughout life and the concentration in the blood normally shows little variation. In some animals e.g. Daphnia (Cladocera, Crustacea) the amount of haemoglobin in solution in the blood plasma and consequently the colour of the animal varies greatly. This has been shown to be directly influenced by the amount of oxygen dissolved in the

* Except eel larvae.

surrounding water since Daphnia gains haemoglobin in oxygen deficient water, maintains the pigment while the oxygen content of the water remains low, and loses it again in aerated water (Fox 1948; Fox & Phear 1953). The colour of the blood of an individual can change from red to colourless and back to red in ten days.

In mammals, haemoglobin is synthesised in the red bone marrow where the erythrocytes develop. After a life span of approximately 120 days (reviewed by Lemberg & Legge 1949) the erythrocytes are destroyed mainly in the spleen and the contained haemoglobin is broken down. The haem part of the molecule is first split off from the globin. The haem is broken down as follows: iron is removed from the porphyrin and temporarily stored in the form of ferritin (an iron protein complex found in the spleen, bone marrow and liver (Laufberger 1937)); the iron may be used again in further haemoglobin synthesis. The remaining part of the molecule - the porphyrin - is converted to bile pigment in the cells of the reticulo-endothelial system, excreted via the bile duct and so lost from the body.

The site of haemoglobin synthesis and breakdown has been investigated in a very few invertebrates. In Thalassema cells containing haemoglobin circulate in the

coelom. These cells are formed from the peritoneum overlying the gut and can be seen to become pink before being released into the body cavity. It is assumed that haematopoiesis occurs here (Ohuye 1937). The blood capillaries of the young leech Herpobdella are colourless. Later these contain haemoglobin. The botryoidal tissue is pink in newly hatched young and since the botryoidal tissue contains iron, it is assumed that the haemoglobin, which later circulates in the blood vessels, is formed here (van Emden 1929; Lankester 1880).

It has been shown that some invertebrates can break down haemoglobin to green bile pigments. The blood-sucking bug Rhodnius prolixus forms bile pigment from small amounts of ingested haemoglobin circulating in the haemocoel (Wigglesworth 1943). Chironomid larvae have green bile pigment in the fat body. It is thought that these may be derived from haemoglobin in solution in the haemocoel (Possompés 1937).

Thus, among the invertebrates haemoglobin may be synthesised in different organs. It is of interest to discover more of the sites of synthesis and breakdown of haemoglobin in invertebrates. Some arthropods i.e. Cladocera, have the ability both to gain and lose haemoglobin. This gain and loss can be controlled by

altering the dissolved oxygen content of the water. Thus animals may be obtained which are either gaining or losing haemoglobin and so form ideal material for a study of the site of synthesis and breakdown of the blood pigment. For this reason a daphnid has been selected for a histological investigation as to the site of synthesis and breakdown of haemoglobin in an invertebrate.

SECTION 2.

PREVIOUS WORK ON DAPHNIA.

2. PREVIOUS WORK ON DAPHNIA.

The site of synthesis and breakdown of haemoglobin in Daphnia has not yet been investigated, but some previous observations have been made by other workers, on the gain and loss of haemoglobin.

A. Gain of haemoglobin.

It is known that a synthesis of haemoglobin occurs when the surrounding water has a low dissolved oxygen content (Fox 1948; Fox, Hardcastle & Dresel 1949). There is an inverse relationship between the oxygen content of the water and the concentration of haemoglobin in the blood. In oxygen deficient water Daphnia does not however continue to increase the concentration in the blood indefinitely. After a period under these conditions the haemoglobin concentration remains approximately steady while the oxygen content remains unchanged (Fox and Phear 1953).

The haemoglobin concentration is further increased if additional iron is added with an algal diet (Fox & Phear 1953).

Thus, some factors which induce haemoglobin synthesis are known but the site of synthesis is not known.

B. Loss of haemoglobin.

It is known that a loss of haemoglobin occurs when the surrounding water becomes well aerated (Fox 1948). It is not known whether the loss of haemoglobin follows breakdown of the pigment in the body. The loss of haemoglobin could be brought about by excretion of the whole unchanged haemoglobin molecule or, on the other hand, breakdown might occur and be followed by excretion or storage of the breakdown products. By analogy with vertebrate animals these might be haem, iron, bile pigments or porphyrin. Some of these have been looked for by other workers.

The excretory organs are a pair of glands in the maxillary segment commonly called shell glands because they lie within the shell. Haemoglobin is occasionally found accumulated in one (rarely both) of the excretory organs; its tubule is then filled with the red-brown pigment. Fox (1948) points out that this accumulation of pigment could be interpreted as an indication of the normal excretion of haemoglobin in quantities too small to be usually seen; the accumulation being a result of a pathological condition. That this condition is pathological is suggested by the fact that many animals die soon afterwards. Fox however, has been unable to

detect oxyhaemoglobin or haem, in aerated water in which Daphnia is losing haemoglobin. This suggests that the intact haemoglobin molecule or even haem molecule is not excreted as such. Affected animals have less haemoglobin in the blood than normal individuals so that the pigment may have escaped from the blood into the excretory organ. To summarize: haemoglobin in the excretory organ may be a result of (a) normal excretion of the whole haemoglobin molecule or (b) escape of haemoglobin from the blood. There is yet no conclusive evidence to support either hypothesis.

It has been shown (Dresel 1948) that some of the haemoglobin in the blood of Daphnia leaves the body by an entirely different route. Towards the end of each instar haemoglobin passes from the blood into the ovary and leaves the body when the eggs are laid. It is possible that all the haemoglobin leaves the body by this route. Male Daphnia does not have haemoglobin in its testes but I have found that the haemoglobin content of the blood decreases in males if they are placed in well aerated water. This could indicate a loss of haemoglobin other than that lost in eggs but perhaps there is a sexual difference and haemoglobin leaves the body by a different route in males.

A haemochromogen is found in the gut lumen of Daphnia. Fox (1948), who named this compound daphniarubin, suggests that it might be an excretory product of haemoglobin. This might account for the absence of haemoglobin or other haem in water containing Daphnia which is losing haemoglobin. Research on the occurrence of haemochromogens in the gut of invertebrates has been undertaken in this department by Miss E. Phear and is not dealt with here in detail.

Bile pigments have been looked for in Daphnia but these have not yet been found (Fox 1948). Since no bile pigment has yet been found it might be that a haem group after losing its iron appears as a protoporphyrin. Fox (unpublished) has looked for the red fluorescence given by a porphyrin in ultra-violet light but could find none; nor could he extract porphyrin with acetic acid; he therefore concludes that haem must be broken down at once to compounds simpler than bile pigments or porphyrins.

Thus a loss of haemoglobin from the body occurs through the eggs. It is not certain that a breakdown of haemoglobin occurs and no possible breakdown products, other than daphniarubin have yet been found.

SECTION 3.

PRESENT STUDY.

3. PRESENT STUDY.

It is of particular interest to know whether the haemoglobin physiology in Daphnia resembles that of mammals with formation of bile pigments and storage of iron as ferritin. Further it would be interesting to know where synthesis occurs and if breakdown occurs also, then what is the fate of the breakdown products?

With these problems in mind five main studies have been made:

1. A histological study of the distribution of iron (other than haem iron) in animals with varying amounts of blood haemoglobin. This might show the site of intake of iron and perhaps an accumulation at the site of synthesis. If iron is released during breakdown this would be revealed and its subsequent fate indicated. If iron stores are normally present they would be detected.

2. A histological study of the distribution of haem in animals with varying amounts of blood haemoglobin. It seems likely that a positive reaction to haem would be given by the organ responsible for synthesis and breakdown of haemoglobin, as well as by the blood. The strength of the reaction would depend on the rate of transference of haemoglobin from the site of synthesis to the blood. These methods showed other haem pigments in addition to

haemoglobin.

3. Chemical tests for ferritin were made in order to see whether iron stores in Daphnia were of the same nature as those in mammals. If this proved to be true it would be the first reported case of ferritin in an invertebrate.

4. A histological test for bile pigments was made which did not destroy the tissues. This was an improvement on the chemical tests used prior to this investigation, for the identification of bile pigments in Daphnia.

5. In addition to these studies and as a result of them some experimental work has been undertaken to confirm histological observation.

SECTION 4.

MATERIAL AND CULTURE METHODS.

4. MATERIAL AND CULTURE METHODS.

The largest daphnid Daphnia magna Straus, was selected for this investigation and obtained from the gull pond in the London Zoo. Unless otherwise stated all results have been obtained from mature females.

Animals were required in which the haemoglobin concentration was in one of the following four states 1) increasing; 2) decreasing; 3) at a steady level and 4) negligible. These were obtained by controlling the oxygen content of the water. Filtered Regent's Park lake water was used.

Well aerated cultures were obtained in the following way: animals were kept in 500 ml. glass troughs which were covered with glass plates to prevent the growth of a bacterial scum. 200 ml. culture water and 50 animals were placed in each trough. Well aerated water, 89%[x] saturated with air at room temperature, could be maintained by this method. These conditions were required

(a) to maintain animals with a negligible haemoglobin concentration (colourless animals) and

(b) in order that red animals should become colourless (animals losing haemoglobin)

x The oxygen content was kindly determined by Miss A. Taylor and Mr. J. Green using the micro-Winkler method (Fox & Wingfield 1938).

Poorly aerated cultures were obtained in the following way: animals were placed in uncovered 100 ml. or 300 ml. conical flasks. The oxygen content of the water was reduced initially by bubbling nitrogen through it for three minutes. Each flask contained approximately 1 animal to 4 ml. of culture water; 100 ml. flasks contained 22 animals and 90 ml. water; 300 ml. flasks contained 70 animals and 280 ml. water. The oxygen content of the water could be raised if it became too low by lowering the level of water in the conical flask; this increased the surface area. Warning that this was necessary was given when the animals swam very near the surface of the water. The cultures were kept in the dark since green algal food was used; this would raise the oxygen content of the water by photosynthesis in the light. Poorly aerated water, approximately 12% saturated with air at room temperature could be maintained by this method. These conditions were required

(a) in order that animals could be induced to gain haemoglobin (animals gaining haemoglobin) and

(b) in order that animals could maintain a steady high haemoglobin concentration (steady animals).

The experiments of Fox and Phear (1953) have shown that under the conditions in the poorly aerated cultures, a gain in haemoglobin concentration is apparent during the

first 15 days. After this period the haemoglobin concentration remained approximately steady for a further 13 days when their experiment ceased. In the present study animals removed from the poorly aerated cultures after 15 days were assumed to be maintaining a steady haemoglobin concentration.

Two methods of feeding were tried. Bakers' yeast was added to the culture water by means of a damp paintbrush, but proved an unsatisfactory diet since it was impossible to control the oxygen content of the culture water. The unicellular green alga Chlorella vulgaris Beij was then tried. This was grown on agar slopes (medium of Pearsall & Loose 1936). The amount of food given was standardised by adding the alga gradually to the water and measuring the optical density of the suspension with an M.R.C. photometer; 30/40 units of Chlorella was found to be a suitable amount and when the water was changed every five days the food was renewed to this level.

It was desirable that the animals maintained in this artificial way should have adequate food and the means to synthesize haemoglobin. It is known that the number of eggs laid into the brood pouch during any instar is influenced by the quantity of food available in preceeding instars (Ingle, Wood & Banta 1937). Under these experimental

conditions the average egg number was rarely less than fourteen which indicated that adequate food had been given.

While Chlorella seemed an adequate diet as judged by egg number, more haemoglobin was synthesised when extra iron was added (Fox & Phear 1953). It was found that if all cultures were given small amounts of additional iron, the histological distribution of iron could be more easily studied. This iron was given by adding 1.0 ml. of a stable ferrous ammonium sulphate solution (0.14 g. in 200 ml. distilled water) containing 0.1 mg. iron, daily to every 100 ml. culture water. When larger quantities were added, iron was found adsorbed on the exoskeleton, especially on the thoracic limbs and the antennae. Since this might have invalidated the results the added quantity of iron was kept low.

Limitation of the culture method.

The dissolved oxygen content of the culture water was checked once in four days. Fluctuations in oxygen content might have occurred between oxygen determinations owing to the birth of young and temperature changes. Whether these were of sufficient duration or extent to influence the haemoglobin state is not known; this possibility was borne in mind in the interpretation of results.

SECTION 5.

STUDY OF THE HISTOLOGICAL DISTRIBUTION OF IRON (NON-HAEM) IN DAPHNIA.

5. STUDY OF THE HISTOLOGICAL DISTRIBUTION OF IRON (NON-HAEM).

Tissue iron is found in three forms. Firstly as easily detectable iron ("inorganic" or free iron) it may be revealed by chemical tests without previous treatment. Secondly, bound iron ("organic" or masked iron), not so easily detectable, must be treated with acid alcohol before the chemical tests are applied. Thirdly, iron porphyrins in e.g. haematin; haemoglobin; cytochrome, can be revealed by micro-incineration or after treatment with hydrogen peroxide (Brown 1911). Haem iron, though chemically a bound form, is not revealed after acid-alcohol treatment.* The terms used for the three iron forms, in the literature, are all inexact but throughout this work the distinguishing terms inorganic, organic and haem iron will be used.

Animals, in general have more tissue iron in the organic than the inorganic state. Lison (1936) maintains that inorganic iron is rarely found in tissues, although Tompsett (1934) had found that all the iron (presumably non-haem) in the hen's egg was inorganic. Inorganic iron is mostly in the ferric, more rarely the ferrous, state (Bolles-Lee 1950; Pearse 1953).

* Red blood cells failed to give a positive reaction for iron after being subjected to acid-alcohol treatment.

The nature of iron in the tissues of Daphnia is not known, nor is its nature always understood in vertebrates; a large literature exists on this subject. In vertebrates ferric iron forms complexes with non-diffusible phosphorus compounds, e.g. phospoproteins, but not with simple proteins such as albuminin of egg white, gelatin, etc. (Tompsett 1934). In addition iron occurs in tissues as ferric hydroxide in "haemosiderin" and ferritin, as well as iron porphyrin in cytochromes and haemoglobin.

1. METHODS.

The iron distribution in Daphnia has been studied largely by means of simple histochemical tests; a radioactive tracer technique has also been used to confirm some histochemical observations.

The technique used for organic (non-haem) and inorganic iron was that devised by Perls (1867) and used with small modifications by many workers since then as the Prussian and Turnbull's blue technique. Bunting (1949) has recently reviewed the histochemical detection of iron and recommended other modifications which are largely used here.

The principle involved in the detection of iron, using the Prussian blue and Turnbull's blue procedure is as follows:- iron in tissues reacts with acid solutions of potassium ferrocyanide and ferricyanide to form the intense blue ferri-ferrocyanide (Prussian blue) and ferro-ferricyanide (Turnbull's blue) respectively. Ferrous iron can thus be distinguished from ferric histologically so long as fixatives have not altered the state of oxidation of the element.

Other techniques exist for the detection of iron: the ammonium sulphite method (Quinke 1868), the haematoxylin method (Mallory 1938 etc.) and the dinitroresorcinol method (Humphrey 1935). None of these is as sensitive as

the test above (Gomori 1936; Lison 1936; Bunting 1949). The Prussian blue procedure as used in microchemistry is capable of detecting 0.002γ of iron (Lison 1936).

a) Technique for inorganic iron.

Fixation.

Animals were immersed for twenty-four hours in 10% formalin buffered to pH 7.0. (The buffer mixture was added to the fixative in the following proportion: 4g. sodium dihydrogen phosphate and 6.5 g. anhydrous disodium phosphate per litre of fixative).

After fixation the material was washed in 70% alcohol for fifteen minutes (two changes) then quickly hydrated.

Staining.

Animals were left in the staining reagents for thirty minutes (The staining solution was prepared as follows: Equal quantities of a 2% solution of potassium iron* cyanide and 2% hydrochloric acid were mixed immediately before use.) During staining the solution remained a pale yellow colour and was discarded if a green colour developed; (this occurs when iron cyanide radicles in the reagent break down and react with intact radicles (Bunting 1949)).

Dehydration.

The animals were washed in distilled water (two changes)

* For the detection of ferric iron, potassium ferrocyanide was used.
For the detection of ferrous iron, potassium ferricyanide was used.

and then dehydrated as follows: 50% alcohol (five mins.); 70% alcohol (five mins.); two changes 95% alcohol (fifteen mins.); two changes absolute alcohol (fifteen mins. each). The material was then cleared in xylol and mounted in balsam.

Bunting recommends filtering the mixture of acid and potassium iron cyanides but this was found to make no difference to the results and subsequently the mixture was not filtered. He also stained his material for sixty minutes using fresh stain after thirty minutes; this also was unnecessary for Daphnia, since the blue colour reaction became no stronger after thirty minutes. "Clarite" is recommended (Bunting 1949; Lillie 1948) as being a suitable mounting medium after iron staining since balsam preparations fade after a period and "clarite" preparations do not. "Clarite" however, is at present unobtainable in this Country. Gomori (1936) showed that fading of tissues could not be overcome by using neutral balsam; this indicated that fading might be a result of reduction, not of acidity, and could be overcome by using almost dry canada balsam mixed with old resinified oil of turpentine, which contains peroxides. If this was done the colour did not fade over a period of five months. In the present work, fading of Prussian blue slides has not been a serious problem since results have been recorded from freshly mounted material and Gomori's refinement has not been used.

b) Technique for organic (non-haem) iron.

Animals were fixed in formalin as for inorganic iron, after which, animals were left in acid-alcohol (3% concentrated nitric acid in 95% alcohol) for at least twenty four hours, at 35°C. This released the organic iron (unmasked it) so that it could now react with the inorganic iron staining reagent. The unmasking technique was devised by Macallum (1895). Most iron is in the ferric form after acid alcohol treatment. The specimens were then washed in 90% alcohol and in water, and stained in the Prussian blue inorganic iron staining reagent (for five mins. only). Dehydration and mounting were as before.

2. LIMITATIONS OF THE METHODS.

Fixation of iron.

Many fixatives have been used by research workers for iron in tissues and there can be little doubt that some of these have been responsible for artefacts. Certain fixatives although giving good histological definition gave a variable iron distribution. I have found that 10% formalin buffered at neutrality (as recommended by Lillie 1948 and Bunting 1949) gives a reliable consistent distribution, though cytological detail is poor.

Bouin's fluid or Allen's modification of it, was useless if inorganic iron was to be detected in Daphnia, though recommended by Lison (1936) and Gomori (1951) and used successfully with Rhodnius prolixus (Wigglesworth 1943). In Daphnia little inorganic iron remains after Bouin fixation and results are totally inconsistent.

Alcohol, 95% or absolute, gave good cytological fixation but the diffusion of Prussian blue from the animal could be seen both during and after staining. After mounting, the total iron content, of these alcohol-fixed animals was thus less than in formalin fixed specimens. The distribution was similar except for irregular blue patches in alcohol fixed animals at inconsistent sites

which could be seen after mounting. These I interpret as probably caused by diffusing iron which had not left the body.

Formol fixation has one disadvantage: after its use most of the iron is in the ferric state; alcoholic fixation has shown that some of this may have been ferrous iron. (A simple test served to show that ferrous salts are quickly turned to ferric in buffered formol but remained ferrous for more than 24 hours in alcohol.) For this reason the nature of the iron in alcohol fixed specimens was periodically compared with that of formalin fixed specimens and any differences are recorded in the results.

Diffusion of iron.

Though the Prussian blue method is sensitive to 0.002γ of iron, quantitative study is complicated by diffusion of iron from the tissues. Diffusion occurs if acid fixatives are employed and in the lower alcohols during dehydration. These difficulties were overcome by buffering the fixatives, leaving the tissues in the lower alcohols for not more than 5 minutes, and dispensing with 30% alcohol; diffusion of iron was thus largely overcome in the inorganic iron technique. During the unmasking process in acid-alcohol diffusion could not be checked; quantitative studies in the organic iron technique could not therefore

be made. Organic iron shows a constant distribution which can be accurately recorded but quantitative data have not been given.

Adsorption of iron.

Acid fixatives cause certain tissue elements, especially nuclei, to attract iron and give a false localization. This provides a convenient staining method for nuclei (Wigglesworth 1952). Much of the early work on iron in cells has been invalidated because of this adsorption. The neutral fixative used here has overcome this difficulty in the inorganic, but not the organic iron technique. A more difficult problem to overcome is the presence of minute quantities of free iron in reagents. Glass-distilled water, iron-free alcohols and glass instruments have been employed, but hydrochloric acid almost invariably contains minute traces of iron and care has been taken to test the contamination of the acids used. As nuclei have rarely shown blue iron masses in the inorganic iron technique it is assumed that limitation of the method has been overcome.

Bunting (1949) mentioned the phenomenon of positive Prussian blue reactions in the nuclei of cells near the sites of haematin or haemoglobin. This has not been observed in inorganic preparations of Daphnia.

Penetration of reagents.

The constitution of the integument of arthropods varies during each instar and this might affect the penetration of chemical reagents. It is to be expected that the degree of penetration of reagents into animals which were just about to moult, would differ from that in newly-moulted animals. Results showed that animals in either of these stages became so badly distorted when fixed in formalin that they had to be discarded.

It is possible to determine the approximate number of hours which have elapsed since the last moult by examining the stages in the development of embryos in the brood pouch (Fox 1948). Animals of similar "instar age" could thus be selected. When this was done and the iron distribution subsequently recorded, it was seen that no large differences existed between groups of individuals of different instar age. It is thus apparent that penetration of the staining reagent has been accomplished.

3. RESULTS.

Structures in which inorganic and organic (non-haem) iron have been found in Daphnia are indicated in table 1.

Iron has been found in the mid gut walls (including the caeca), the fat body*, the shell glands, the ovaries, the blood plasma, the appendage muscles and the labral glands. Most of these structures may show both inorganic and organic iron but there are just two exceptions; the appendage muscles show inorganic iron alone, the labral glands only organic iron.

No iron has been found in the walls of the fore and hind gut, the blood cells, the heart musculature, the septa limiting the blood channels, the nervous tissue, the eye lenses or the vestigial antennary glands.

The concentration of iron varied greatly between tissues. This was easily judged by eye from the intensity of the Prussian blue reaction. Individual variation occurred, some animals giving no staining reaction and immature animals staining so variably that they were discounted.

* The fat body is the totality of the fat cells (Jäger 1935).

TABLE I.

TISSUES IN WHICH IRON HAS BEEN FOUND IN DAPHNIA.

	INORGANIC	ORGANIC (NON-HAEM)
GUT WALLS (Fore	•	•
GUT WALLS (Mid	+	+
GUT WALLS (Mid gut caeca	+	+
GUT WALLS (Hind	•	•
FAT BODY	+	+
MAXILLARY (shell) GLANDS	+	+
OVARIES	+	+
BLOOD (Plasma	+	+
BLOOD (Cells	•	•
MUSCULATURE (Appendage	+	•
MUSCULATURE (Heart	•	•
LABRAL GLANDS	•	+
SEPTA Limiting blood channels	•	•
NERVOUS TISSUE	•	•
LENS OF EYE	•	•
INTEGUMENT	•	•
Vestigial ANTENNARY GLANDS	•	•

+ indicates iron present.

• indicates no trace.

A. ORGANIC IRON.

The distribution of organic iron in Daphnia with different concentrations of blood haemoglobin, is summarised in table 2. More than 400 animals have shown this constant distribution.

After Prussian blue treatment, organic iron was found in all parts of the mid gut walls, the fat cells, the ovaries and the labral glands of all animals. Thus the distribution in these structures is unrelated to the concentration of haemoglobin in the blood. Iron was found in the shell glands however, only when blood haemoglobin was decreasing and it occurred here at no other time.

The fat cells, in spite of diffusion, gave a brilliant Prussian blue reaction, indicating a large quantity of iron. The Prussian blue reaction was less intense in other structures and the blood plasma sometimes gave only a slight reaction. This may have been because little iron was present at the time of fixation or because diffusion had occurred.

The intensity of the Prussian blue reaction in the tissues varied directly with the quantity of iron available to the animal (and with the amount of diffusion). This was shown by modifying the standard culture conditions

TABLE 2.

DISTRIBUTION OF ORGANIC IRON IN DAPHNIA.

	COLOURLESS ANIMALS	ANIMALS GAINING HAEMOGLOBIN	STEADY ANIMALS	ANIMALS LOSING HAEMOGLOBIN
MID GUT WALLS (Ant.	+	+	+	+
MID GUT WALLS (Mid.	+	+	+	+
MID GUT WALLS (Post.	+	+	+	+
CAECA	+	+	+	+
FAT CELLS	+	+	+	+
SHELL GLANDS	.	.	.	+
OVARIES	+	+	+	+
BLOOD PLASMA	V	V	V	V
LABRAL GLANDS	+	+	+	+

+ = present

. = not present

V = variable

in the following way. Colourless animals were deprived of algal food and iron and left in well aerated water for one week. These animals contained little to no iron in the tissues after that period. When iron was present in the animals it was located in the ovaries. Colourless animals were then placed in water containing Chlorella but no iron, for seven days. These animals showed more iron in the tissues, than starved animals after that period. When animals were placed in water containing Chlorella and additional iron (as in culture methods) a more intense Prussian blue reaction was noted in the tissues after a further seven days. The organic iron content of colourless Daphnia is therefore related to the quantity of available iron.

Thus Daphnia absorbs iron when haemoglobin synthesis is not taking place, if the tissues contain little iron. When large iron stores were initially present it was impossible to detect, with this technique, whether the addition of iron to the environment increased the iron in the body; since though the reaction given at the end of feeding was intense in such animals, a similar intense reaction was given before the addition of iron. It is thus impossible to say that the addition of iron in all cases increases iron stores. This could be tested quantitatively with an ashing technique.

The fact that iron is absorbed when synthesis of haemoglobin is not occurring is interesting since this differs from the condition in mammals where uptake of iron from the food is slight and regulated. Granick (1946) suggests that ferritin in the gut wall acts as a regulator. He further suggests that when iron is required by the body it is released from ferritin in the gut wall. Apoferritin, i.e. ferritin minus iron, is left in the gut wall. Apoferritin is quickly turned back to ferritin by absorption of iron from the food. As much iron as is required to saturate the apoferritin can be absorbed and no more.

The chief call for iron in mammals is for haemoglobin synthesis. Iron is normally provided from stores in the spleen, the bone marrow and the liver. A small daily uptake occurs to meet a similar small daily loss of iron; the uptake may be increased in certain pathological anaemias. Uptake of iron in Daphnia is unrelated to the amount of haemoglobin in the blood since iron is absorbed when there is no call for haemoglobin synthesis.

Colourless Daphnia has apparently no means of regulating the uptake of iron in the concentrations used here. (0.1 mg. added daily to every 100 ml. of culture water.) Regulation of iron intake may perhaps occur at higher iron

concentrations. The iron content of pond waters has been found to range between 2 mg. per litre and no detectable iron. (One isolated value of 50 mg. iron per litre has been recorded (Chandler unpublished)). The standard cultures used had therefore less iron than is sometimes available to animals in ponds.

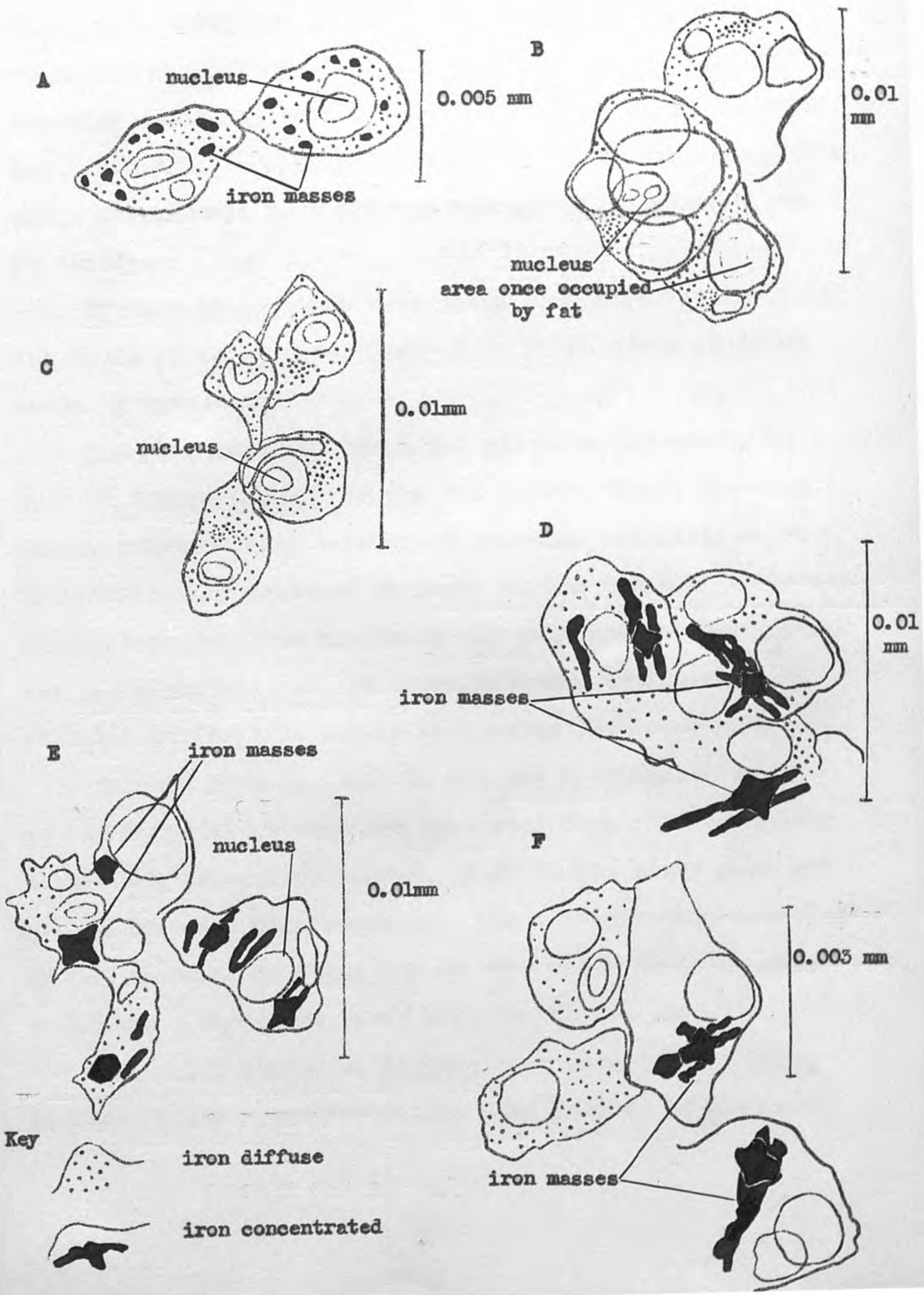
The distribution of organic iron in Daphnia, with the exception of the shell glands, is thus independent of the concentration of haemoglobin in the blood.

The histological form of the iron.

The histological form of organic iron in the fat body is related to the concentration of haemoglobin in the blood. The histology of the fat body was studied by Jäger (1935) who described the large nuclei of the fat cells. He also describes a light "space around the nucleus" which has now been seen in Prussian blue preparations. The cytoplasm of the fat cells reacts variably with the Prussian blue reagents:- it may stain uniformly or show granular blue deposits. Sometimes the cytoplasm remains unstained but in it lie deep blue, single or multiple strands overlying another blue crystalline structure. Colourless animals and those in which the haemoglobin concentration is increasing have fat cells in which the cytoplasm stains diffusely (Fig. 1. B,C). Granular iron is present in the fat cells, (Fig. 1.A)

Figure 1.

Appearance of organic iron in fat cells of Daphnia losing haemoglobin.



of animals which are losing haemoglobin and those in which the haemoglobin concentration is steady; similarly the blue irregular strands of iron (Fig. 1. D, E, F) have been seen only in many animals which are losing haemoglobin and a few animals in which the haemoglobin concentration is steady.

Strands or crystals have never been observed in living fat cells of Daphnia, so that those shown after staining could be artefacts.

Numerous small or large fat globules can easily be seen in living Daphnia in the fat cells. These globules may be coloured with carotenoid pigments naturally or can be artificially coloured by means of the Sudan IV technique. During Prussian blue treatment all colour disappears from the fat globules. The position occupied by the fat globules during life can be seen after the treatment.

Organic iron is found in all the cytoplasmic parts of the ovary after Prussian blue staining. The cytoplasm stains a uniform blue colour. Yolk in the ovary does not give a Prussian blue reaction. The position once occupied by fat globules in life, can be seen after Prussian blue treatment. These are fewer than in the fat body.

The shell glands of Daphnia were described by Claus (1875). These excretory organs each consist of a closed

internal saccule, a long winding labyrinth and a shorter vesicle leading to the orifice in the region of the vestigeal maxilla. D. magna shows no histological distinction between the labyrinth and the vesicle. Organic iron is found in the shell gland when the haemoglobin content of the blood is decreasing. Granular iron is located in the walls of the labyrinth. The granules are irregular, more rarely spherical. The walls of the labyrinth have not been observed to show the strand formation of iron or a uniform blue colour. A uniform blue colour has been observed in the lumen of both the labyrinth and the vesicle. The saccule has not been observed to contain iron.

The shell glands often contain accumulations in the walls and the lumen during life. These are sometimes brown or they may be white by reflected light and black by transmitted light.

B. INORGANIC IRON.

The structures in which inorganic iron has been observed have already been indicated (table 1). They are: the mid gut walls, the fat cells, the shell glands, the ovaries, the blood plasma and the muscles of the appendages.

The observed distribution of inorganic iron in relation to the concentration of blood haemoglobin is shown in table 3. Unlike the distribution of organic iron (table 2), that of inorganic iron is related to the concentration of blood haemoglobin.

This histochemical technique does not allow accurate quantitative data to be given. Differences in the intensity of the Prussian blue reaction have therefore been recorded in the following way:- A strong blue reaction was recorded as +++ in the table 3. A blue reaction, obvious, but not intense was recorded as ++ in table 3. Some tissues gave a slight pale blue-green reaction. This was recorded as + in table 3. This last colour reaction might indicate traces of iron normally present or might represent iron which has diffused from another site. A negative reaction was recorded as . in table 3.

During the inorganic iron technique, no blue material was observed leaving the animals, either during staining

or subsequent dehydration. Diffusion of iron had evidently been partially overcome.

The results obtained from the inorganic iron technique are presented in the following way:-

The distribution of iron and the intensity of the iron reaction are first recorded for each tissue during each of the four states of blood haemoglobin.

This distribution is illustrated in Figures 2, 3, 4 & 6 and summarized in table 3 at the end of this section. (page 60).

The figure pages (2, 3, 4, & 6) are arranged as follows: each contains a simple anatomical sketch of Daphnia. Two outline drawings below indicate the distribution of ferric and ferrous inorganic iron. Traces of iron are omitted from the figures and dotted structures are added for clarity.

a. INORGANIC IRON IN COLOURLESS ANIMALS (Fig. 2. Table 3 column 1).

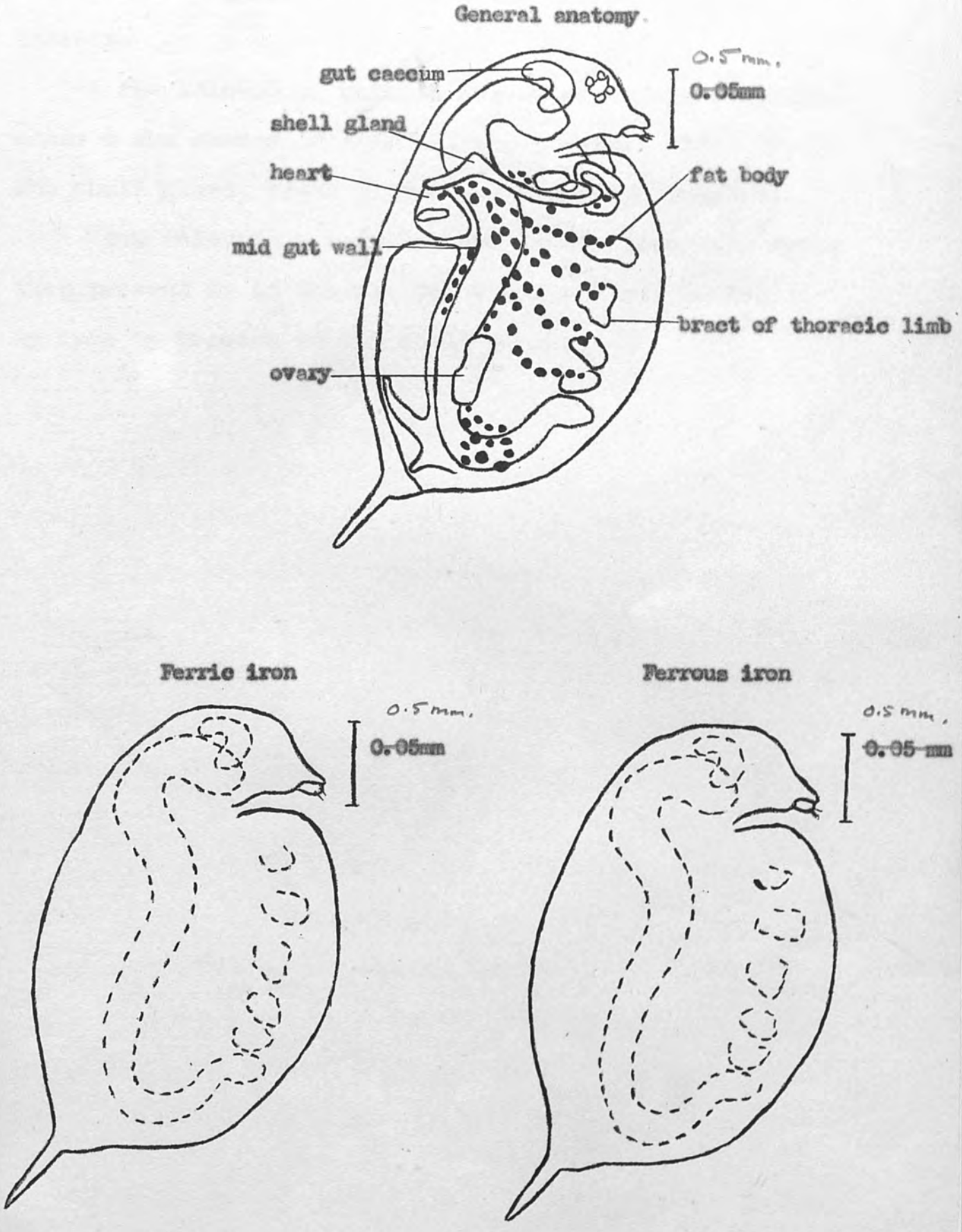
The majority of colourless animals gave slight Prussian and Turnbull's blue reactions. About 200 animals have been tested. Parts of the mid-gut walls and the fat cells gave a pale blue reaction. No reaction was observed in the mid gut caeca, the shell gland, the ovaries, the blood plasma or the appendage muscles.

The organic iron study indicated that iron is absorbed by colourless animals. The inorganic iron results do not substantiate this though they do not contradict it. Perhaps inorganic iron, absorbed through the gut walls, rapidly becomes bound in the body. Unmasking with acid alcohol is then required to reveal it.

The gut lumen contains Chlorella and iron, and frequently gives an intense colour reaction for inorganic iron. The gut contents normally occupy the middle and posterior third of the mid gut. The anterior gut lumen is normally free of solid contents and does not give an inorganic iron reaction. The iron of the gut contents is usually ferric iron in colourless animals but some colourless animals have ferrous iron throughout. There is no indication of an oxidation of iron near the hind gut where anal swallowing has been in progress. This is interesting since it has recently been suggested (Fox 1952) that anal

Figure 2.

The distribution of inorganic iron in
COLOURLESS DAPHNIA



swallowing is not a mechanism to aid respiration in Crustacea though it has this function in some aquatic insects.

A few colourless animals showed no colour reaction, while a few showed iron in all the possible sites except the shell gland, blood plasma and appendage muscles.

Thus, colourless animals have little inorganic iron; that present is in the fat cells and mid gut walls. No iron is present in the shell glands.

b. INORGANIC IRON IN ANIMALS WHICH ARE SYNTHESISING HAEMOGLOBIN. (Fig. 3. Table 3, Column 2).

During haemoglobin synthesis inorganic iron is present in most organs. More than 200 animals have been tested. In the majority a positive reaction for ferric iron was given but the mid gut caeca and a less marked ferric reaction by the fat cells. A positive reaction for ferrous iron was given by the remaining mid-gut walls, the fat cells and the ovaries. Sometimes ferrous iron was observed in the blood plasma and in the especially prominent sarcoplasm (Binder 1932) of the antennal and mandibular muscles.

Animals fixed in buffered formalin gave a more intense reaction for ferric iron, than those fixed in alcohol. Animals fixed in alcohol showed ferric iron in the gut caeca but mostly ferrous iron elsewhere. This indicates that there is ferric iron in the gut caeca, but probably largely ferrous iron elsewhere. The Turnbull's blue reaction of specimens fixed in alcohol often acquired a purple-blue hue.

No inorganic iron was observed in the shell glands.

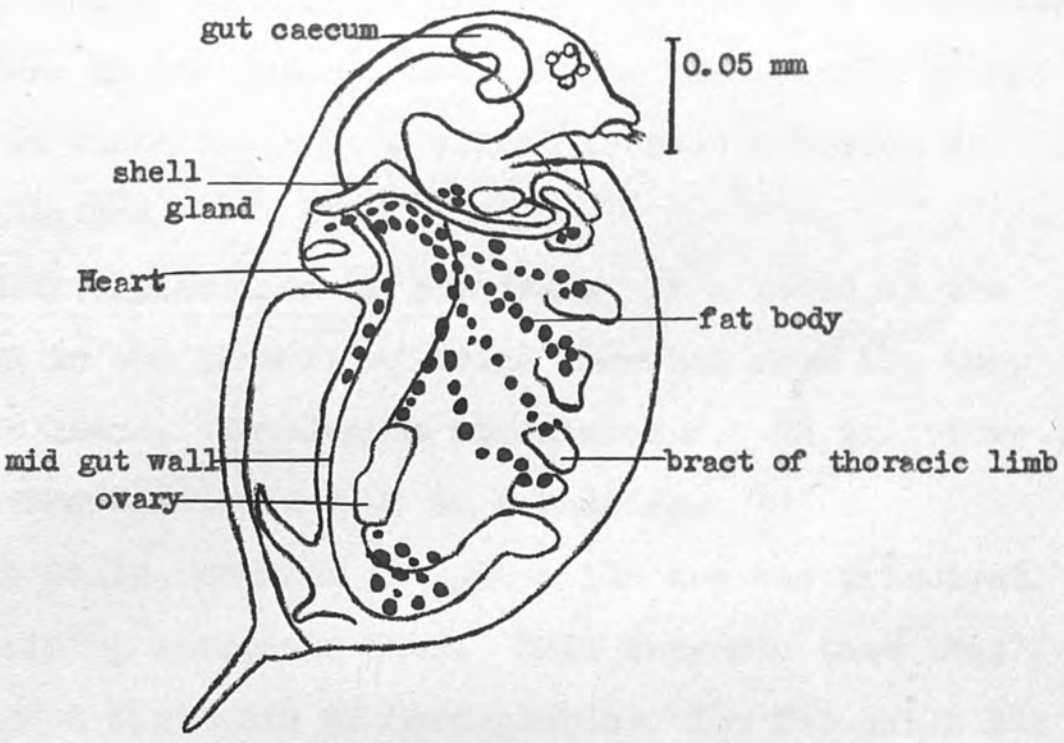
It thus appears that during synthesis of haemoglobin iron may be absorbed through the gut wall where it appears in the ferrous form except in the anterior caeca. The different states of oxidation of iron in the caeca and the remaining gut walls is striking and at present unexplained.

Figure 3.

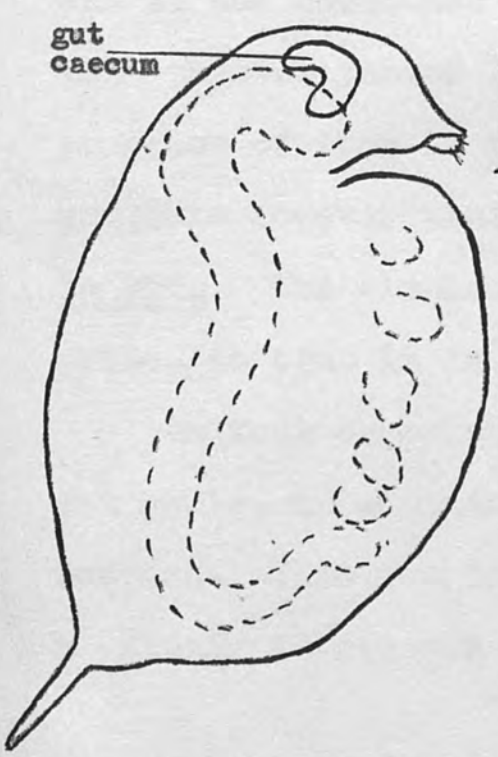
The distribution of iron in

ANIMALS GAINING HAEMOGLOBIN.

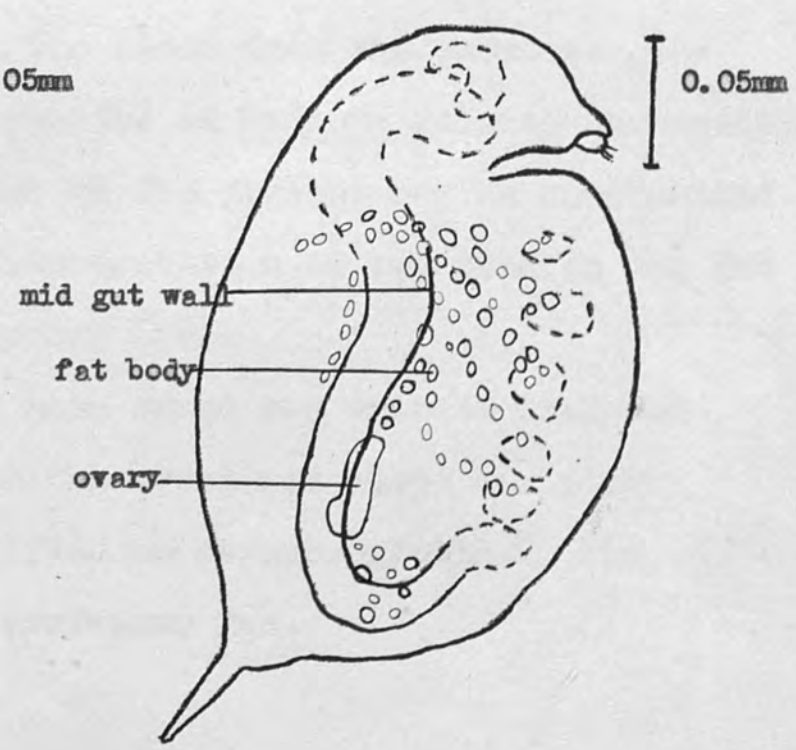
General anatomy



Ferric iron



Ferrous iron



In man it has been suggested (Lancet 1951) that iron is absorbed by the intestinal mucosa in the ferrous form. In Daphnia, if ferrous iron is absorbed and rapidly converted to ferric form in the caeca, then ferrous iron should always be present in these organs; a slight ferrous reaction is sometimes obtained.

A further explanation is possible. Iron found in the caeca may be in the process of being excreted from the body into the gut lumen. Homologous structures act as excretory organs in other Crustacea e.g. in barnacles.

The fat cells, ovaries and gut walls are the principal organs containing inorganic iron. This suggests that they may be sites of synthesis of haemoglobin. The fat cells lie in the haemocoel and are constantly bathed by the blood. The ovaries as well as the blood normally contain haemoglobin and it was concluded (Fox, Hardcastle & Dresel, 1949) that this pigment passed from the blood into the oocytes. The presence of iron in the ovaries of Daphnia gaining haemoglobin suggests however that some of the pigment may be synthesized in situ. The simplest interpretation of the iron in the gut walls, is that it is absorbed here.

On four occasions I have found red animals with red fat cells, in addition to the usual red blood and pink ovaries. This was identified as oxyhaemoglobin.* The

* Kindly identified by Professor Fox.

animals came from two different sources and showed that haemoglobin may be present in these cells and may also be synthesised here.

Iron is found in the non-yolky regions of the ovary i.e. in the oocytes arranged in groups of four cells.

A few of the animals tested gave a negative reaction, while a few showed no reaction in the anterior gut diverticula. This was unrelated to the stage of the instar.

Thus, animals which are gaining haemoglobin have inorganic iron chiefly in the gut walls, fat cells and ovaries. Most of the iron is ferrous iron but that in the mid-gut caeca is in the ferric form. Other structures sometimes have inorganic ferrous iron i.e. blood plasma and appendage muscles.

c. INORGANIC IRON IN ANIMALS WHICH ARE LOSING HAEMOGLOBIN.
(Fig. 4, 5, Table 3, column 3.)

Animals which are losing haemoglobin show a strikingly different iron distribution. More than 400 have been tested. Parts of the shell gland now give an intense and granular ferric iron reaction in the majority of animals. The gut caeca frequently give an intense but non-granular blue reaction. The fat cells similarly show an intense blue reaction for ferric iron.

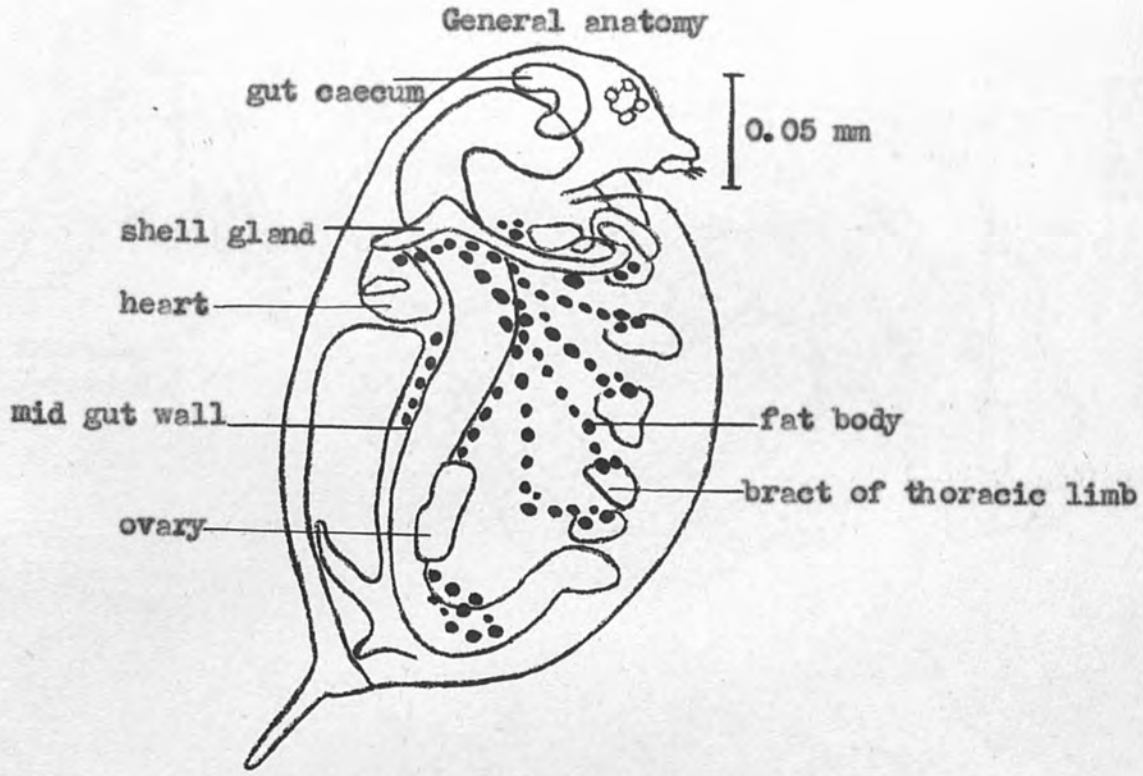
Iron is not found throughout the shell glands (Fig. 5a); it has never been observed in the closed internal saccule, but the walls of the labyrinth show large and small irregularly shaped blue masses. Its lumen shows non-granular blue contents, which can be moved along the lumen by pressing on the animal gently, with the flat edge of a Borradaile needle. The vesicle leading towards the orifice has non-granular blue contents and these are disposed in patches along its length.

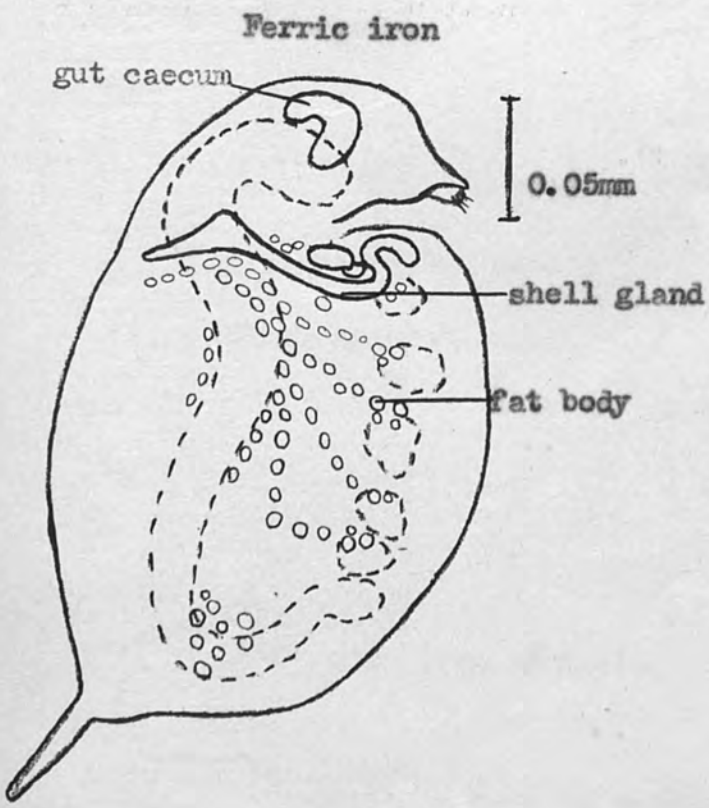
The distribution suggest that iron is being excreted into the lumen of the labyrinth and then leaving the body through the vesicle. A second interpretation is that iron in the wall of the labyrinth is being reabsorbed back into the body. This latter assumes that an iron-containing compound has first of all been passed into the lumen. This

Figure 4

The distribution of iron in

ANIMALS LOSING HAEMOGLOBIN.





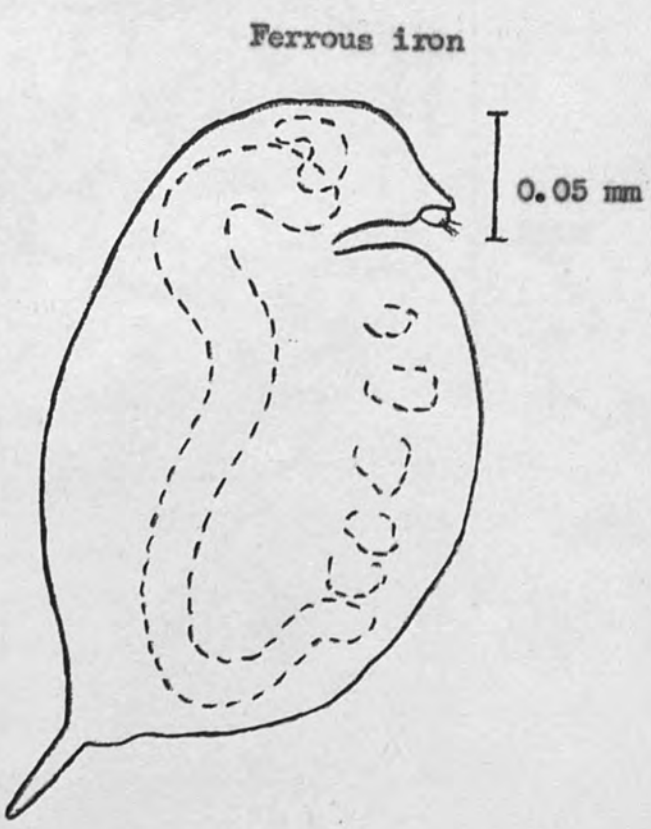
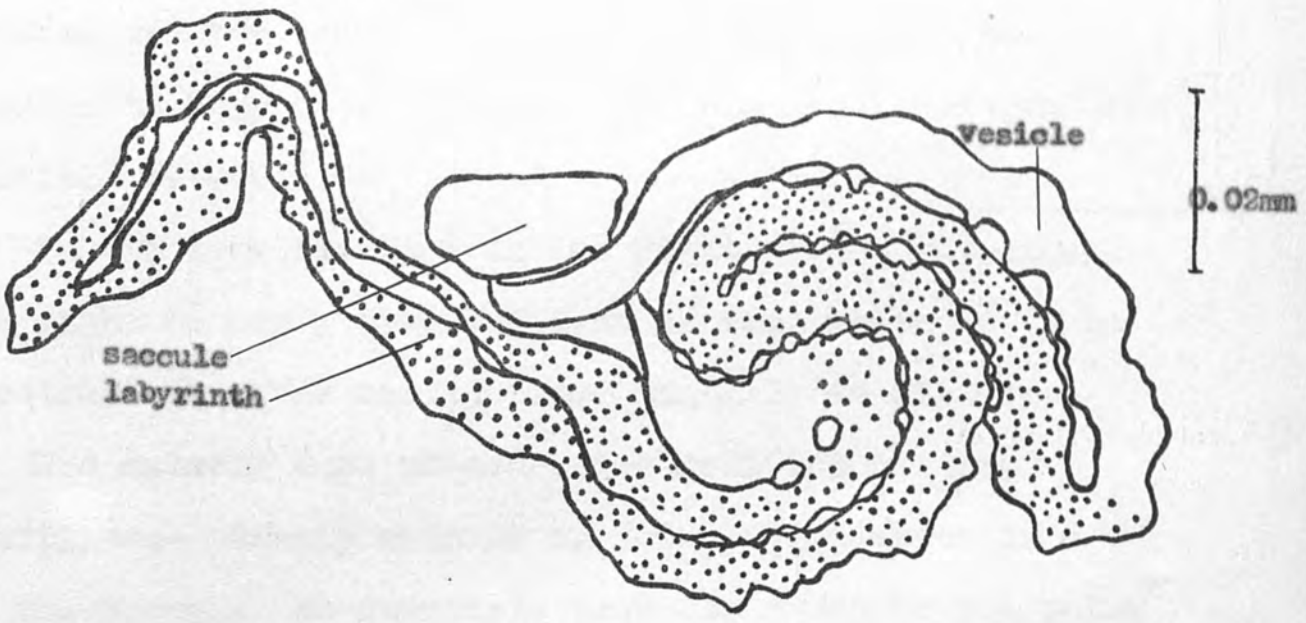


Figure 5.

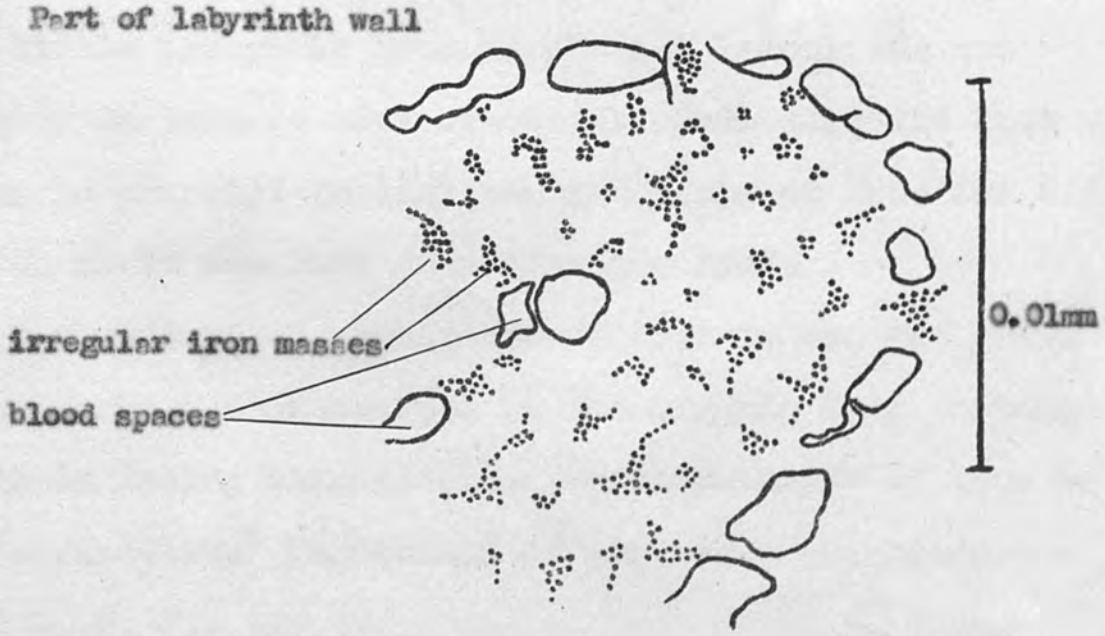
The distribution of iron in the shell glands of

DAPHNIA LOSING HAEMOGLOBIN.

A One shell gland

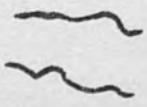


B Part of labyrinth wall



Key

with iron in walls



without iron in walls

is discussed more fully later. If iron is leaving the body, the concentration of iron in the surrounding water should increase and the amount in the body should decrease. Experiments have been made to test this hypothesis and are described later.

Ferric iron is found in the walls of the gut caeca. This might be newly absorbed iron or iron which is to be excreted. This was tested experimentally as follows.

Red animals were placed under modified culture conditions:- twenty animals in 100 ml. tap water in a 250 ml. trough. No inorganic iron was added to the water but a little Chlorella, which contains no detectable inorganic iron, was added as food. After five days these were fixed and stained in the usual way. The animals showed a very little inorganic iron in the gut lumen, the gut caeca gave an intense blue reaction. This suggests that iron may be excreted through the gut caeca at this time since this iron could not have come from the food.

The histological appearance of iron in the fat cells was similar to that described in the organic iron technique for animals losing haemoglobin. Small granules of iron or large "strand-like" formations of iron have been observed (Fig. 1 D, E, F).

The fat body is an organ of definite form (Jäger, 1935).

It consists of the thoracic mass with extensions into the haemocoel. Processes of the organ extend into the five pairs of thoracic limbs; others pass posteriorly on both dorsal and ventral sides of the mid and hind gut. Another extends into the carapace blood sinus and comes into intimate contact with the loops of the shell gland. Limb extensions of the fat body sometimes show no iron in this state of blood haemoglobin while the remainder of the fat body does so. The fat body varies in size and is said to be constantly broken down and replaced (Jäger 1935). Though the organ varies in size according to its fat content, no cytolysis of cells has been observed during this study.

A weak Turnbull's blue reaction is given by the middle and posterior mid-gut walls and sometimes by the caeca. There is no inorganic ferrous iron in the ovaries, fat cells, shell glands, blood or appendage muscles. Animals fixed with alcohol show a similar distribution of ferric and ferrous iron.

Thus, most iron is present in the mid-gut caeca, the fat cells and the shell glands. This iron is ferric iron; iron in the ferrous form is present in small quantities in the gut walls.

d. INORGANIC IRON DISTRIBUTION IN ANIMALS WITH A STEADY HAEMOGLOBIN LEVEL. (Fig. 6. Table 3, column 4.)

These animals showed a distribution of iron similar to that in animals gaining haemoglobin. About 200 animals have been tested. The fat cells again appear to contain both ferric and ferrous iron with more ferric iron than occurs in animals gaining haemoglobin, but no intense blue reactions were obtained.

In the majority of animals the gut caeca gave a pale blue reaction for ferric and ferrous iron. The rest of the gut and the ovaries showed ferrous iron alone. It was interesting to observe that the shell glands contained no iron. Iron only appears in that organ when the haemoglobin content of the blood is decreasing.

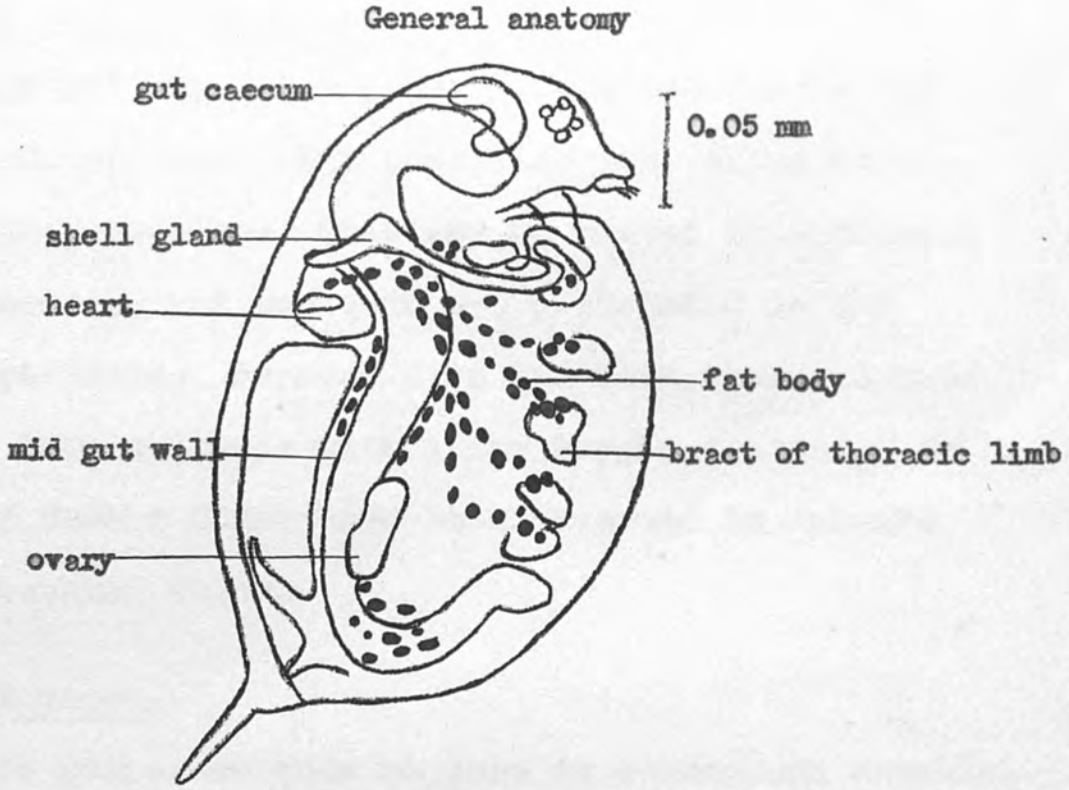
A few specimens have been observed to contain inorganic ferrous iron in the blood and in the sarcoplasm of the antennal and mandibular muscles.

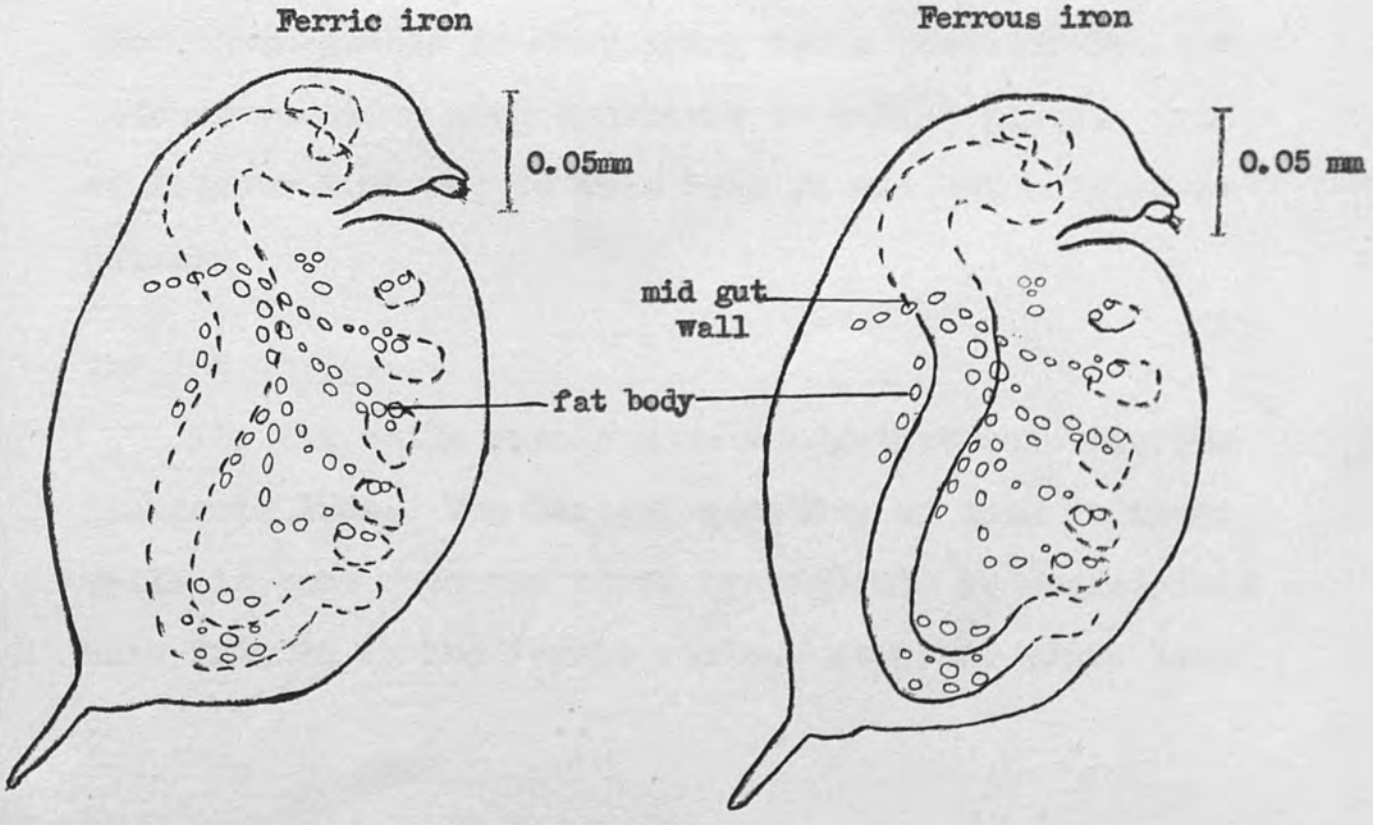
Thus the fat cells have more iron, the gut caeca and ovaries less iron than appear in these organs in animals which are gaining haemoglobin. The iron distribution in animals with a steady haemoglobin concentration therefore resembles that of animals gaining haemoglobin, rather than that of animals which are losing the blood pigment.

Figure 6.

The distribution of iron in

ANIMALS WITH "STEADY" HAEMOGLOBIN.





A SUMMARY OF THE MAIN RESULTS OF THE INORGANIC IRON STUDY.

The mid gut walls (except the caeca).

The mid gut walls may contain both the ferric and ferrous forms of iron; the posterior part shows more iron than the anterior; this may be caused by diffusion of iron since the gut contents are restricted to the posterior position. Ferrous iron has been observed here in animals from cultures with a low oxygen content. Both ferrous and ferric forms have been observed in animals from well aerated water.

The mid gut caeca.

The mid gut caeca show no iron in colourless animals, and none in a small proportion of other animals. An intense reaction for ferric iron is observed here when blood haemoglobin is decreasing and a positive but not intense reaction when synthesis is taking place. Traces of ferrous iron may be seen here in all but colourless animals.

The fat cells.

The fat cells rarely give a negative reaction for inorganic iron. The largest quantity of iron in these cells is seen when the blood haemoglobin is decreasing: this iron is in the ferric state. At other times less

intense reactions are obtained. The fat cells show a positive reaction for ferrous iron when haemoglobin synthesis is in progress and for ferrous and ferric iron when a steady level has been attained.

The shell glands.

The shell glands give a negative reaction for ferrous iron. Iron in the ferric form is found in some parts of the organs when blood haemoglobin is decreasing.

The ovaries.

The ovaries have not been observed to give intense reactions for inorganic iron. Ferrous iron occurs during synthesis of haemoglobin and when the steady level has been attained.

The blood and the mandibular and antennal muscles.

These do not give intense reactions for inorganic iron. Ferrous iron has been seen in animals from cultures of low oxygen content. But specimens may all show a positive reaction and others from the same flask, on the next day fail to show this - results are quite inconsistent.

TABLE 3.

DISTRIBUTION OF INORGANIC IRON IN DAPHNIA.

	COLOURLESS ANIMALS		ANIMALS GAINING Hb.		ANIMALS LOSING Hb.		ANIMALS WITH STEADY Hb.	
	Ferric	Ferrous	Ferric	Ferrous	Ferric	Ferrous	Ferric	Ferrous
MID GUT WALLS (Ant. 1/3)	.	.	.	+	.	.	.	+
MID GUT WALLS (Mid. 1/3)	+	+	.	++	+	+	.	++
MID GUT WALLS (Post. 1/3)	+	+	.	++	+	+	.	++
MID GUT CAECA	.	.	++	+	+++	+	+	+
FAT CELLS	+	+	+	++	+++	.	++	++
SHELL GLANDS	.	.	.	.	+++	.	.	.
OVARIES	.	.	.	++	.	.	.	+
BLOOD	.	.	.	+	.	.	.	+
APPENDAGE MUSCLES	.	.	.	+	.	.	.	+

Reaction with inorganic reagents

+++ intense blue

++ blue but not intense

+ pale blue

. negative

SECTION 6.

STUDY OF THE HISTOLOGICAL DISTRIBUTION OF HAEM IRON IN DAPHNIA.

6. STUDY OF THE HISTOLOGICAL DISTRIBUTION OF HAEM IRON IN DAPHNIA.

Haem compounds are known to occur in Daphnia; haemoglobin is found in solution in the blood plasma (Lankester 1872) and is present in the eggs (Tessier 1932). Haemoglobin has also been identified in the fat cells (see page 49) and a haemochromogen in the mid gut lumen (Fox 1948).

During haemoglobin synthesis a positive haemoglobin reaction might be detectable at the site of synthesis. The distribution of haemoglobin has therefore been studied histologically in animals in which the concentration of blood pigment is 1) increasing, 2) decreasing, 3) at a steady level and 4) negligible.

There is no specific stain for haemoglobin although eosin, safranin and orange G, show an affinity for the pigment. Acid fuchsin has recently been claimed as a specific stain for haemoglobin (Hirschler 1949), good results having been obtained with vertebrate (amphibian) and invertebrate (tubificid and chironomid) haemoglobin. The principle of the process is not explained chemically and in the original paper other structures which are not known to contain haemoglobin also stain, for example, yolk of oocytes.*

* Acid fuchsin stains all tissues of Daphnia.

Haemoglobin can, however be identified histologically; firstly by means of the peroxidase reaction which is given by haem compounds and secondly, spectroscopically. The distribution of haem compounds in Daphnia has been studied by a peroxidase technique.

1. METHODS.

The two methods most widely used by previous workers for the identification of peroxidases are, the benzidine technique of Adler and Adler (1906) and the cyanol or zinc-leuco technique of Lison (1936; 1938). The principle of the benzidine technique is as follows: under slightly acid conditions and in the presence of a peroxidase, benzidine is oxidised by hydrogen peroxide to an unstable blue and finally a brown compound. The benzidine technique has been widely used but recently the cyanol technique has been used because it has some histological advantages. The principle of the cyanol technique is as follows: reduced, colourless cyanol is converted into a stable blue compound, by means of the breakdown of peroxide by peroxidase. The advantages of the cyanol method are that, a) the colour reaction given is more intense and stable than that of the benzidine method, b) animals can be dehydrated in the usual way and mounted in balsam without loss of colour and c) cyanol is said (Lison 1936) not to give a reaction with some thermostable tissue peroxidases which thus makes it a more specific test for haemoglobin than benzidine. Gomori (1951), reviewing the histochemical staining methods for peroxidases says "Lison's zinc-leuco technique is so vastly superior to all other methods that

it should be used exclusively. It is highly selective for haemoglobin."

Lison's method has been modified by other workers in an effort to make it a specific test for haemoglobin. Fautrey & Lambert (1937) used a technique employing cyanol which they maintained would selectively stain vertebrate haemoglobins and not other tissue haems. This method relies on the fact that the isoelectric point of vertebrate haemoglobin is more alkaline than the majority of tissue proteins (Fautrey & Lambert 1937). Unfortunately the isoelectric point of invertebrate haemoglobins is more acid and the method would not selectively stain these. Dunn (1946) simplified the technique of Fautrey & Lambert, and his modification was used in this study. The details are:

Fixation. Daphnia was fixed for 24-28 hours in 4% formaldehyde buffered at neutrality. (The buffer mixture is given on page 25).

Staining. After hydration, animals were stained in a cyanol "working" solution (see below) for not more than five minutes and washed quickly in distilled water.

Dehydration. Animals were dehydrated in a series of alcohols, cleared in xylol and mounted in canada balsam.

The cyanol "working" solution was made up from the

stock solution as follows:

Stock solution. A mixture of 10 g. zinc dust and 2.0 ml. glacial acetic acid was added to a solution of 1.0 g. cyanol in 100 ml. distilled water. This mixture was boiled so that the cyanol became reduced, and the mixture remained colourless for a few months.

"Working" solution. Just before use, 10 ml. stock mixture were filtered. To the filtrate 2 ml. glacial acetic acid and 1 ml. 3% hydrogen peroxide were added. The working solution was then ready for use.

Tests to determine whether the Dunn method for haemoglobin is specific.

Dunn (1946) states that his cyanol method stains haemoglobin; he makes no mention of other haem compounds which may also react with cyanol. Since it was important to know if other compounds did this, a selection of materials was subjected to his test for haemoglobin.

1. Chemical compounds. Human haemoglobin in solution and substances derived from it were tested. These were: pyridine haemochromogen, haematin, haemin. Pure non-human crystalline biliverdin and bilirubin were subjected to the treatment in addition to glycine, ferric chloride and ferrous lactate.

2. Whole animals. The animals selected were of two categories a) those known to have haem compounds in the blood, i.e. Chironomus plumosus larvae; Tubifex tubifex and red Daphnia magna,

b) those without haem compounds in the blood, i.e. Nebalia bipes; Gammarus sp. and various Copepoda.

3. Tissues or organs. The tissues and organs selected were of two categories a) those known to have haem compounds, i.e. human red cells; buccal mass of Helix pomatia; heart muscle of Carcinus maenas and flight muscles of a locust,

b) those in which haem compounds have not been identified, i.e. haemocyanin in blood of Helix pomatia; claw muscle of Carcinus maenas and eye of Palaeomonetes varians.

Results. Table 4 lists the reaction of all materials tested to cyanol. Substances, and animals known to contain haem compounds gave positive reactions. Haemoglobin and its haem derivatives in solution gave peroxidase reactions but haemocyanin, as expected, did not do this. Glycine, iron salts, bile pigments and tissues not known to contain haem compounds, all gave negative reactions. From these tests it may be inferred that all haem compounds will react with reduced cyanol and peroxide to give a blue derivative. This is not therefore a specific stain for haemoglobin.

TABLE 4.

REACTION OF VARIOUS MATERIALS TO CYANOL.

MATERIAL TESTED	HAEM COMPOUND	REACTION TO CYANOL
Haemoglobin	Haemoglobin	+
Pyridine haemochromogen	Haemochromogen	+
Haematin	Haematin	+
Haemin	Haemin	+
Biliverdin	•	•
Bilirubin	•	•
Glycine	•	•
Ferric chloride	•	•
Ferrous lactate	•	•
Chironomus	Haemoglobin	+
Tubifex	Haemoglobin	+
Daphnia (red)	Haemoglobin	+
Nebalia	•	•
Gammarus	•	•
Copepoda	•	•
Human red cells	Haemoglobin	+
Buccal mass-Helix	Cytochromes *	+
Heart muscle-Carcinus	Cytochromes	+
Flight muscle-locust	Cytochromes	+
Blood - Helix	•	•
Claw muscle-Carcinus	•	•
Eye - Palaeomonetes	•	•

• indicates no haem present and negative reaction to cyanol
+ indicates a positive reaction to cyanol
* bands at 605 ; 566 and 550 were identified by Miss E. Phear.

2. LIMITATIONS OF THE METHODS.

The Dunn method for the identification of haem compounds was applied to Daphnia. It was seen that some factors limited the success of the technique. When whole animals were placed in the reagents after staining, a little blue material was sometimes seen leaving the animals, some diffusion evidently takes place. In other animals it was clear that the cyanol reagents were not penetrating the animals; this was indicated by the following observation: after fixation in 4% formaldehyde, red Daphnia became red-brown in colour. This pigment sometimes accumulates in the carapace blood sinus, from where in a living animal, blood is returned to the heart (Hérouard 1905). The red-brown haemoglobin derivative sometimes failed to give a cyanol positive reaction. This was certainly owing to lack of penetration of the reagents since after piercing the blood space with a sharp needle an immediate blue reaction could be obtained. Fortunately the number of animals showing this was low and such animals were discarded. There was one further limitation. The blood in the haemocoel gave a positive reaction to cyanol when haemoglobin was present. It was therefore often necessary to view internal structures through a blood layer which was cyanol positive. Organs with

quantities of haem would be seen through this but others with less haem would be more difficult to see. This limitation was overcome in part, by sectioning the material, so that internal structures could be examined.

3. RESULTS.

Haemoglobin in Daphnia has previously been identified spectroscopically in the blood, the ovaries and the fat cells. The expected positive reaction to cyanol was given by these structures. Individuals with red blood, pink ovaries or red fat cells produced a strong blue colour reaction after fixation and subsequent cyanol treatment. A haemochromogen has been found in the intestinal lumen of Daphnia. This would be expected to react to cyanol like other haem compounds, but it is not certain that this was so, since the overlying gut wall gave a blue reaction and this could mask a reaction in the lumen. Though the gut wall did not invariably contain a peroxidase (see page 73) the gut lumen was never observed to react when a negative reaction was given by the gut walls.

When selected animals with different haemoglobin concentrations in the blood were tested with cyanol, two observations were made. Most tissues and organs have shown a positive peroxidase reaction; this is more intense and widespread when the haemoglobin content of the blood is high but less so when the haemoglobin content is low. There seems to be some correlation between the presence of haemoglobin in the blood and haem pigments in other tissues. It follows that colourless animals showed a

negative reaction with cyanol. This could indicate that a synthesis of haemoglobin is accompanied by a general synthesis of haem.

The distribution of haem, shown by the cyanol technique is summarized in table 5. In order to simplify this, columns 2, 3 and 4 show the distribution of red animals, for each of the three haemoglobin phases.

a. Colourless animals (Table 5, column 1).

The majority of colourless animals gave negative reactions to cyanol in all organs, but in some, the heart and appendage muscles, and anterior mid-gut walls contained small quantities of haem.

b. Animals gaining haemoglobin (Table 5, column 2).

Animals living in water containing little dissolved oxygen increase the haemoglobin content of the blood, and hence their capacity to react with cyanol. At this time strong peroxidase reactions were given by the heart and appendage muscles, the anterior parts of the mid-gut, including the caeca, and the lenses of the compound eye. Less intense reactions were given by the fat cells, the labral glands, the nervous tissue and the posterior mid-gut walls. An occasional weak reaction was seen in the shell glands. The fore and hind-gut walls did not give a peroxidase reaction. The blood cells,

ovaries and integument* reacted inconsistently. Since this occurred in all but colourless animals the inconsistent reactions are described more fully at the end of this section (page 76).

Pink animals appear to contain less haem than red animals, which may be approaching the steady, high, haemoglobin concentration. This was suggested by the observation that pink individuals gave less intense reactions to cyanol in most organs, than did red individuals.

The haemoglobin in red fat cells gave an intense reaction to cyanol; this is a rare occurrence, however, and red fat cells have not been observed in animals from the standard laboratory cultures (page 15), but only in specimens collected recently from ponds.

The reaction in the heart and appendage muscles was especially interesting, since muscle haemoglobin and cytochrome have not yet been reported in Daphnia, although they have been looked for (Fox 1948). The peroxidase reaction is not given by the sarcoplasm but only by the myofibrils.

Since many tissues contain haem, the number of possible sites of synthesis of haemoglobin is large. The anterior

* The cuticle itself gives a negative reaction, stellate cells underneath the cuticle react inconsistently.

gut caeca, fat cells and ovaries are the only organs which consistently have haem, and inorganic non-haem, iron, during haemoglobin synthesis. This suggests that synthesis might occur in some or all of these organs. The fact that the gut caeca contain more haem than the other structures does not necessarily imply that synthesis only occurs here. The strength of a cyanol reaction at a site of synthesis, would be determined by the rate of transference of pigment from the site. If the rate of transference were high then a weak reaction could be given by the organ of synthesis. All these structures could therefore be organs of synthesis.

c. Animals losing haemoglobin. (Table 5, column 3).

Animals losing haemoglobin gave varied reactions, and these appeared to be related to the haemoglobin content of the blood. After a few days in aerated water the distribution of haem resembled that in red animals. After two weeks when the blood haemoglobin had disappeared the distribution was that of colourless animals. The shell glands of many animals now gave a positive reaction to cyanol; in others, a positive reaction in the carapace blood space, made it impossible to be sure that the excretory organ also contained haem. When the positive reaction in the shell

gland was clearly seen, the haem appeared to be in the walls of the labyrinth. The fat cells gave a more intense reaction to cyanol, when haemoglobin was being lost than at other times.

d. Animals with a steady high haemoglobin concentration (Table 5, column 4).

Red animals reacted in the same way as those which were gaining haemoglobin.

It was reported above (page 74) that the blood cells, the ovaries and the integument gave inconsistent reactions. Sometimes, intense blue reactions, sometimes negative reactions were given. It was expected that the ovaries would show variable amounts of haem, since the contained haemoglobin varies in quantity (Dresel 1948). The blood cells and integument have not been seen to contain inorganic or organic (non-haem) iron and so are unlikely to be sites of synthesis of the blood pigment. Nevertheless their variable reactions were interesting and are at present unexplained.

Thus, haem compounds appear to be more widespread in Daphnia than was once thought. The greatest quantities of haem have been found in the blood, the heart and appendage musculature, the anterior mid gut walls and the

eye lenses. The haem in the blood is haemoglobin; that in the gut walls could be daphniarubin but the nature of the haem compound in the muscles and eye lenses is not known.

The cyanol technique indicates that a) colourless animals have little haem, b) a synthesis of haem may accompany the synthesis of blood haemoglobin, c) the shell glands have a haem compound in all but colourless animals and d) the fat cells contain less haem than the blood or muscles but have the largest quantities when haemoglobin is being lost.

TABLE 5.

THE DISTRIBUTION OF HAEM COMPOUNDS IN DAPHNIA

ANIMALS	COLOURLESS	RED, GAINING HAEMOGLOBIN	RED, LOSING HAEMOGLOBIN	RED, WITH STEADY HAEMOGLOBIN
BLOOD PLASMA	•	+++	+++	+++
MUSCLES HEART	•	+++	+++	+++
APPENDAGE	•	+++	+++	+++
MID GUT WALLS Ant.	•	+++	+++	+++
Mid.	•	+++	+++	+++
Post.	•	+	+	+
MID GUT CAECA	•	+++	+++	+++
EYE LENSES	•	+++	+++	+++
FAT CELLS	•	+	++	+
LABRAL GLANDS	•	+	+	+
NERVOUS TISSUE	•	+	+	+
FORE GUT WALLS	•	•	•	•
HIND GUT WALLS	•	•	•	•
SHELL GLANDS	•	V	V	V
BLOOD CELLS	•	V	V	V
OVARIES	•	V	V	V
INTEGUMENT	•	V	V	V

Reaction with cyanol.

+++ intense blue
++ blue
+ pale blue
• negative
V inconsistent.

SECTION 7.

TESTS FOR FERRITIN AND BILE PIGMENTS.

7. TESTS FOR FERRITIN AND BILE PIGMENTS.

A. TESTS FOR FERRITIN.

Stored iron in mammals is largely in the form of ferritin, a crystallisable compound containing 20% - 24% ferric iron linked to a protein - apoferritin. Ferritin is not found in all tissues, but is concentrated in the liver, spleen and red bone marrow, the sites of breakdown of red blood cells (Granick 1943). Ferritin cannot be crystallised from all mammals (Michaelis 1944). Until recently it had not been found outside the class Mammalia, but Tecce (1952) found ferritin and crystallised it from the liver of an elasmobranch, Scyllium canicula. He could not find it in the spleen of this dogfish or from the liver of teleostean fishes. It has not yet been found in any invertebrate.

The study of the distribution of iron in Daphnia has not indicated that the metabolism of haemoglobin in Daphnia was similar to that in mammals. Nevertheless it was of interest to know whether iron stores, in the form of ferritin were at any time present. The Prussian blue technique has shown that ferric non-haem iron is sometimes present. Ferritin was therefore looked for in Daphnia after preliminary tests on vertebrate tissues.

The method of identification of ferritin.

Laufberger (1937) first crystallised ferritin but most of the subsequent work on the role of ferritin has been done by Granick and his school. The ferritin of horse spleen is crystallised by teasing a small portion on a slide, with a 10% solution of cadmium sulphate. Crystals of ferritin form under the coverslip after three minutes. These are of two types: small, yellow octahedra and large orange-brown twinned octahedra.

Results.

Crystals of ferritin were obtained from horse and human spleen. The test was then applied to a large sample (500 animals) of teased pink Daphnia, from which the surrounding water had been removed; no crystals formed under the coverslip. Colourless and red animals were then used but no ferritin could be identified. Other concentrations of cadmium sulphate, namely 3%: 5%: 8%, also failed to crystallise any ferritin. The teased samples of Daphnia contained a high proportion of exo-skeleton which reduced the quantity of material in which ferritin was likely to be found. A method of extraction and isolation of ferritin from horse spleen is known (Granick 1942). The extraction and subsequent isolation of ferritin was therefore attempted using a large sample

of Daphnia from which the surrounding water had been removed.

The method of extraction and isolation of ferritin.

With horse spleen the method is as follows: a portion of horse spleen is thoroughly teased with distilled water. The brown aqueous extract so formed is then filtered. The filtrate is made up to 50 ml. with distilled water and heated to 80°C. Most proteins are coagulated by heating to this temperature but ferritin is not. Most other proteins are now coagulated and thus ferritin is separable from them by filtering the heated filtrate; the precipitate is thus removed. The filtrate so formed is measured and the remaining proteins are salted out with ammonium sulphate (30 g. ammonium sulphate added to every 100 ml. of filtrate). Salting out takes place in a refrigerator at 0°C. overnight. The suspension so formed is then centrifuged down and the supernatant fluid removed. The coagulum is redissolved in water and the volume measured. Cadmium sulphate is added to crystallise the ferritin (5 g. cadmium sulphate to every 100 ml. solution). The isolation is completed by centrifuging down lightly.

Results.

The isolation of ferritin was successfully attempted using horse spleen and was then tried on, red, pink and

colourless Daphnia. No crystals of ferritin were obtained.

Thus the non-haem iron of Daphnia is apparently not in the form of ferritin. This is as in some vertebrates. Non-haem iron which is ferric and bound to a protein is found in the liver and spleen of ox, sheep, deer and chicken; ferritin cannot however be crystallised from these animals with cadmium sulphate (Michaelis 1944). It seems therefore that the storage iron in the liver and spleen of vertebrates, need not always be in the form of ferritin and it is therefore not surprising to find that it cannot be crystallised from Daphnia.

B. TESTS FOR BILE PIGMENTS.

Vertebrates continuously break down the haem of blood haemoglobin to bile pigments and iron. The formation of bile pigments has been less studied in other animals, but they seem to be of widespread occurrence. It has been shown that some invertebrates e.g. blood sucking bugs (Wigglesworth 1943), form bile pigments from haemoglobin; others have haemoglobin but no bile pigments.

It has not yet been shown that a breakdown of haemoglobin occurs in Daphnia, and no bile pigments have been found (Fox 1948). A study of the iron distribution of Daphnia, has however indicated that breakdown does occur, since a release of unbound iron is apparent in the body, during haemoglobin loss. For this reason bile pigments have again been looked for in Daphnia.

The method of identification of bile pigments.

The method used was a histological adaptation of the iodine reaction used to detect the presence of bile pigments in urine. These pigments become green in colour when tincture of iodine is added. Stein (1935) used the same reagent in a histological technique, which was followed in this study. The chemical basis of the reaction is not understood but it is said to be sensitive and tests have shown it to be specific for bile pigments (Stein 1935).

The tests for bile pigments were made in the following way: Daphnia was fixed in 10% formalin, embedded in paraffin wax and sectioned at 8μ. After being deparaffined and hydrated, sections were left in the iodine reagent (3 parts Lugol's solution: 1 part tincture of iodine) for 10 hours, then washed in distilled water and decolorized in a solution of sodium hyposulphite (30 secs.). After washing, sections were counterstained in carmalum for three hours, washed again in distilled water, then dehydrated with acetone*, cleared in xylol and mounted in canada balsam. Emerald green granules indicate the presence of bile pigments.

Results.

The method was successfully attempted on sectioned human kidney which had contained bile pigment, but dull, not emerald green granules were seen. Pearse (1953), similarly has not been able to obtain emerald green, but only dull green granules, with this method. A control without bile pigment showed no such granules after staining.

No green granules were seen in sectioned Daphnia which had been losing haemoglobin, or in red, or colourless animals.

* Alcoholic dehydration is impossible since alcohol dissolves the green colour produced if bile pigments are present.

Thus, there seem to be no bile pigments detectable, by this method in Daphnia but green pigments have occasionally been seen in the blood and shell glands of live animals. On one occasion, three animals with "green blood" were heated to above 70°C and they became bright orange in colour, this green pigment was not therefore a bile pigment, but a carotenoid protein complex. Another live animal, with green blood was re-examined after 15 minutes and the pigment was no longer in the blood but in the shell glands. This also became bright orange in colour when heated to above 70°C. The sudden appearance of the carotenoid complex in the shell gland was interesting and has only once been observed during this study.

All green pigments did not become orange on heating, however. On almost all occasions when a green pigment has been seen in the shell glands, it has been associated here with concentrated haemoglobin. After the animals had been subjected to the histological test for bile pigments (Stein 1935), the green colour disappeared and the contents of the shell gland i.e. haemoglobin and the green pigment, became brown. This green substance was not therefore a bile pigment.

On four separate occasions, haemoglobin concentrated in the lumen of the shell glands has become green/brown in

colour overnight. This suggests that the green pigment might have been a haemoglobin derivative. Since bacteria are almost always present in the shell glands together with accumulated haemoglobin, which has become green/brown in colour, one is reminded of the "viridans effect" first observed by Schottmüller (1903). This is shown when some micro-organisms grown on blood agar plates cause a green discoloration around the colonies. Similarly oxyhaemoglobin solutions can become greenin colour by bacterial action e.g. by Staphylococcus aureus (Lemberg & Legge 1949). Perhaps bacteria which have often been seen in the shell glands, are acting on the contained haemoglobin in a similar way. Holden (1947), on the other hand, has discovered that such simple chemical substances as ammonia, urea, pyridine, phenols, could promote the formation of green pigments such as pseudohaemoglobin and cruoralbin from haemoglobin without bacterial intervention.

When more specimens are available for examination it may be possible to determine the nature of the green pigment, spectroscopically. Such specimens are rarely found however, "haemoglobinuria" occurs in about 1% of a population, and of this 1%, only an occasional specimen shows the green pigment in the shell glands.

Thus, bile pigments have not been found in Daphnia. Green pigments in the blood are sometimes carotenoid complexes; those in the shell glands are sometimes carotenoid complexes, sometimes apparently green haemoglobin derivatives.

SECTION 8.

THE SIGNIFICANCE OF THE PRESENCE OF IRON IN THE SHELL GLANDS.

8. THE SIGNIFICANCE OF THE PRESENCE OF IRON IN THE SHELL GLANDS.

Experimental work on the loss of haemoglobin in Daphnia.

Histochemical tests have indicated the iron distribution in Daphnia. Ferric iron has been identified in the shell glands, (among other organs) of animals which are losing blood haemoglobin. This iron is present here at no other time. It was of interest to know the significance of this iron; is it lost from the body through the excretory organ, or does reabsorption of the iron occur, so that no iron leaves the body? When does iron first appear in the shell gland and when does it cease to be found there? Some experiments have been made to determine the significance of this iron in the shell glands.

The time of the first appearance of iron in the shell gland was determined, after red animals had been left in aerated water in order to lose haemoglobin, and had been fixed and stained for iron at intervals. Further the time of disappearance of the iron from the shell glands was ascertained. These experiments are described in section 1. Other experiments to test the hypothesis that iron is excreted are described in section 2.

SECTION 1. APPEARANCE OF IRON IN THE SHELL GLANDS.

Three experiments were made in order to discover when iron was present in the shell glands during the loss of haemoglobin from the blood.

Experiment 1. Red animals were collected from the gull pond at the London Zoo; 50 animals were placed in troughs of well aerated water, a little Chlorella being added as food. Fixations and tests for iron were then made at convenient intervals up to 50 hours after transference to the experimental conditions.

The results are summarized in table 6. Out of a total of 44 animals, 38 showed iron in the shell glands; iron was already in the shell gland after 1 hour. In this experiment the state of haemoglobin, in the animals at the beginning of the experiment was not known; they could have been losing haemoglobin, so that the presence of iron in the shell gland within an hour of transference to aerated water might be a result of conditions before the experiment began.

TABLE 6.

The first appearance of iron in the shell glands.

Time, in hours spent in open troughs.	Number fixed.	Number with iron in the shell glands.
1	2	2
4	2	2
6	2	2
8	4	4
10	4	4
23	4	4
24	8	4
50	18	16

A second experiment was made in which it was known that the animals were not losing haemoglobin before the experiment began.

Experiment 2. Twenty-five colourless animals were placed in each of six experimental conical flasks, containing 90 ml filtered lake water and with Chlorella as food. The oxygen content of the water was reduced by bubbling nitrogen through it before the experiment began. The animals became pink and then red in colour. When the animals were red in colour they were transferred to troughs of well aerated water. Each of these troughs contained 25 animals in 100 ml.

filtered lake water with Chlorella as food. The percentage air saturation of these cultures was high, initially 89%. During the following six days the red animals became pink in colour. Fixations and tests for iron were made after the animals had been in the troughs for 1, 2, 4 and 6 days. The number of animals with iron in the shell glands was recorded.

The results of one such experiment are given in table 7. No iron was observed in the shell glands during the first two days in aerated water but by the fourth day nearly all animals showed iron here. Therefore 3-4 days after red animals have been transferred to aerated water, iron appears in the shell glands. It is thus clear that in experiment 1. the appearance of iron in the shell gland, before 3-4 days, was a result of the conditions before the experiment began, i.e. the animals could have been losing haemoglobin.

TABLE 7.

The first appearance of iron in the shell glands.

Time, in days spent in open troughs.	Number fixed.	Number with iron in the shell glands.
1	10	0
2	30	0
4	15	13
6	30	17

By the sixth day about half of the animals tested showed no iron in the shell glands, a possible explanation of this is suggested below. In a laboratory population of Daphnia, living in constant culture conditions, a range of values for the haemoglobin content of the blood can be obtained. Genotypic variation may, in part, account for this. Another possibility is that the laboratory population contains animals of different ages, some having been born since the beginning of the experiment. These animals having had less time in the culture conditions show corresponding differences in haemoglobin content. Thus a "red" population contains some "pink" animals and these pink individuals will lose their blood pigment before the red. Therefore a random selection of animals from a red population, which is losing haemoglobin in well aerated water, will soon include individuals which have lost all their haemoglobin. This hypothesis is supported by the following observation. When the reddest animals were selected for the Prussian blue test during haemoglobin loss, more animals showed iron in the shell glands than showed it when a random selection was made.

Thus, iron appeared in the shell glands 3-4 days after haemoglobin loss had begun*. A longer experiment was now

* Fox and Phear (1953) p.188 show that adecrease in the haemoglobin content of the blood occurs, during the first day in well aerated water.

made, in order to see when iron ceased to occur in the shell glands during haemoglobin loss.

Experiment 3. This experiment was set up as in experiment 2 but the red animals remained in open troughs until they were colourless. The water in the troughs was changed after every six days and Chlorella was added.

The results are summarised in table 8. Most animals showed iron in the shell glands during the first 3-10 days after transference to the open troughs. When the blood had become colourless, no iron was observed in the shell glands.

TABLE 8.

The disappearance of iron from the shell glands.

Colour of the blood	Time, in days spent in open troughs	Number fixed	Number with iron in the shell glands.
Red	3	10	7
Red	4	20	20
Pink	10	10	8
Pale pink	15	10	6
Very pale pink	16	10	3
Colourless	18	13	0
Colourless	19	5	0

It is thus clear that iron appears in the shell glands when animals are losing haemoglobin, 3 - 4 days after the initiation of haemoglobin loss. Iron disappears from this organ w hen the blood haemoglobin has been lost.

It may therefore be that the iron in this organ is that released from haemoglobin breakdown. An apparent decrease in haem compounds occurs in other tissues and accompanies the loss of haemoglobin from the blood so that the iron in the shell gland could have originated from these other haem compounds or from blood haemoglobin.

THE SIGNIFICANCE OF THE PRESENCE OF IRON IN THE SHELL GLANDS.

SECTION 2. EXPERIMENTAL WORK TO TEST THE HYPOTHESIS OF EXCRETION OF IRON, DURING HAEMOGLOBIN LOSS.

Iron is present in the lumen of the shell glands of Daphnia, during haemoglobin loss, and may be excreted from the body into the surrounding water. If iron is excreted, it may be possible to detect it in the water and to show that the iron content of the water increases, as haemoglobin is lost from the blood. The iron content of water containing Daphnia which is losing haemoglobin, has therefore been measured.

The thiocyanate method of estimation of iron in natural waters.

The iron content of the water was estimated by means of the thiocyanate method, proposed by Ossian (1837). The method has recently been critically reviewed by Woods and Mellon (1941). Potassium thiocyanate in solution gives an immediate colour reaction with ferric iron in acid conditions. The intensity of the colour reaction is an indication of the quantity of iron present. When this colour is compared with that of iron standard solutions, similarly treated with thiocyanate, the quantity of iron in the unknown solution can be estimated.

The estimation proceeds in two stages. The iron standards, and then the solutions of unknown iron content are prepared for colorimetric comparison. After preparation

the thiocyanate reagent is added to each solution and colorimetric comparison follows immediately.

The preparation of iron standards.

The standards of known iron content were prepared as follows: 0.722 g. ferrous ammonium sulphate, $FeSO_4(NH_4)_2SO_4.6H_2O$, were dissolved in 50 ml. glass distilled water and 20 ml. concentrated sulphuric acid. The solution was warmed, and potassium permanganate solution (0.2 N. = 6.3 g. in 1 litre glass distilled water) added, until a faint pink colour persisted. This solution was then diluted to 1 litre; 1 ml. now contained 0.1 mg. of iron. This constituted the stock ferric iron solution, and standards for use were prepared from it in the following way: 0.5 ml. and 1.0 ml. stock iron solution were each diluted to 40 ml. with distilled water. Three drops of 0.2 N. potassium permanganate solution and 0.5 ml. of 6N. nitric acid were added. Each solution was then diluted to 50 ml. with distilled water; the iron concentrations of the standards thus prepared were 1.0 mg. iron per litre and 2.0 mg. iron per litre.

The preparation of the solutions to be tested.

The water of unknown iron content was first boiled with nitric acid, (50 ml. water were boiled with 5 ml. of 6N. nitric acid for 5 mins.); this released iron from

organic combination in algal cells etc. To this solution 3 drops of 0.2 N. potassium permanganate solution were added. The sample was then left to cool.

Estimation of iron content.

Potassium thiocyanate solution, (5 ml. of a solution containing 2 g. potassium thiocyanate in 100 ml. glass distilled water) was added to the cooled sample of unknown iron content and to each standard iron solution. The colour reaction was compared immediately by means of a Dubosq colorimeter. The colour fades quickly on standing, is deepened by an excess of thiocyanate and lessened by an excess of acid (Woods and Mellon 1941).

Experiments.

Red animals were obtained and left to lose haemoglobin, in covered troughs of well aerated tap water. Colourless animals were similarly placed in well aerated water and left for the same period. The colourless animals with very little blood haemoglobin acted as controls. The iron content of the water was estimated, both at the beginning and end of the experiment. Tap water, instead of filtered lake water, was used, in order that the bacterial content of the water should be low. A high bacterial content could have altered the iron and oxygen concentrations.

It was desirable that the faeces of the control and experimental animals should be similar, since these would be added to the water during the experiment and so could change the iron content of the water. Before the experiment began therefore, animals were left for 4 days, in separate troughs of water, with Chlorella as food (50 units, as measured by the M.R.C. colorimeter). It was assumed that after this period great differences in the gut contents and faeces of control and experimental animals, would have been overcome. During the experiments dead animals were removed morning and evening; this was necessary since after their decay iron would have been added to the culture water.

The first experiment was made using 50 animals in 200 ml. of water, which was kept artificially aerated. Chlorella (30 units) was added as food and the experiment lasted for 7 days. The results are summarized in table 9. The initial iron content of the water could not be measured. No matching of colour was possible, even when the lowest iron standard was used. After 7 days the iron content of the water, in which Daphnia was losing haemoglobin, exceeded that of one control but not that of the other. The range of readings between the two control troughs, was greater than the difference between the experimental and control troughs.

TABLE 9.

The iron content of water containing Daphnia after 7 days of haemoglobin loss.

Animals	Colourless		Losing haemoglobin.	
Trough	1	2	1	2
Vol. of water, ml.	200	200	200	200
No. of animals				
beginning	50	50	50	50
end	39	25	22	32
deaths	11	25	28	18
Iron content at beginning, mg./litre	NOT DETECTABLE		NOT DETECTABLE.	
Iron content at end, mg./litre	0.64	0.43	0.61	0.67

The experiment was twice repeated, but the number of animals used was doubled, 200 ml. of water now contained 100 animals. The artificial aeration of the water was dispensed with since the red animals became pale without this. At the beginning of the experiments the iron content of the water and Chlorella was estimated, since there was no detectable iron in the water alone. The quantity of Chlorella added to the water was reduced to 20 units in these two experiments; this reduced the excess food, on which iron could be adsorbed, to a minimum. The water and contents were stirred vigorously, before iron estimations were made since the iron could have settled on the bottom of the troughs. The second experiment lasted for 5 days, the third, for 6 days.

The results of experiments 2 and 3 are summarized in tables 10 and 11, respectively. In both experiments the initial iron content of water and Chlorella was 0.53 mg. iron per litre. At the end of experiment 2, the iron content of the water in the experimental troughs exceeded that in the controls. At the end of experiment 3, this was not so however, and the iron content of the experimental troughs exceeded that of one control but not the other.

TABLE 10.

The iron content of water containing Daphnia after 5 days of haemoglobin loss.

Animals	Colourless		Losing haemoglobin.	
Trough	1	2	1	2
Vol. of water, ml.	200	200	200	200
No. of animals				
beginning	100	100	100	100
end	74	73	77	81
deaths	26	27	23	19
Iron content at beginning, mg./litre	0.53	0.53	0.53	0.53
Iron content at end, mg./litre	0.04	0.42	0.48	0.49

TABLE 11.

The iron content of water containing Daphnia after 6 days of haemoglobin loss.

Animals	Colourless		Losing haemoglobin	
Trough	1	2	1	2
Vol. of water, ml.	200	200	200	200
No. of animals				
beginning	100	100	100	100
end	58	47	69	59
deaths	42	53	31	41
Iron content at beginning, mg./litre	0.53	0.53	0.53	0.53
Iron content at end, mg./litre	0.24	0.19	0.24	0.21

It is thus clear that an excretion of iron by Daphnia which is losing haemoglobin, has not been revealed by this technique. It may well be that an excretion of iron does occur, but that it has been beyond the limits of the method to reveal it.

The accuracy of the method was restricted by two factors. Firstly, a high death rate, which was possibly a result of damage sustained during the setting up of the experiment and secondly by the low iron concentration of the water, which was never greater than that of the lowest standard (containing 1.0 mg. iron per litre).

The range of readings between the control troughs was greater than the difference between these and the experimental troughs. It is thus likely that the values obtained at the end of experiments 2 and 3 merely showed the decrease in iron, as Chlorella was consumed.

It was desirable that a more sensitive technique should be used and an attempt was made to incorporate radioactive iron into Daphnia. Firstly into the gut and then into the blood and shell glands. Subsequently, when activated red animals were losing haemoglobin, the activity of the water was measured, (as counts per minute on a Geiger counter). If radioactive iron is excreted by Daphnia the activity of the water will increase as haemoglobin is lost from the body.

RADIOACTIVE IRON STUDY.

1. THE ASSIMILATION OF RADIOACTIVE IRON BY DAPHNIA.

Radioactive iron was obtained from the Atomic Energy Research Establishment, Harwell. Fe^{59} (half-life 47 days) was used in a ferric chloride solution (1.8 mg. Fe/ml. solution).* Measured volumes of this solution were added to water containing Daphnia, and the iron uptake was estimated by measuring the activity of individuals after they had been in the water for a period.

Estimation of radioactivity.

The activity of an individual was determined by placing it on a thin glass plate, which was held in place by means of a loop of filter paper and dropped on the window of a Geiger-Müller counter tube; the activity was recorded as counts per minute. Ten consecutive, one minute readings were taken for each individual, and the average of these gave an estimate of the activity. The background activity was estimated similarly and was taken before each experimental reading.

* This study was made possible through the kindness of Dr. Geoffrey Bourne, and was undertaken in the Histology Department of the London Hospital Medical School.

Details of method.

Thirty colourless, mature individuals of Daphnia were placed in a 300 ml. covered trough containing 150 ml. tap water. Similarly, 30 colourless animals were placed in a conical flask containing 150 ml. tap water. The water in the conical flask was kept poorly aerated (20 % air saturated) by bubbling a gas mixture through the water continuously. A small quantity of yeast, as food, was added to the water in the trough and the flask. Radioactive iron was then added to the water; 0.9 ml ferric chloride solution containing 1.62 mg. Fe^{59} to each dish. Animals were removed at intervals, dropped into tap water for a few seconds and their activity was then measured. Since it was probable that some of this activity would represent contamination on the surface of the body, the activity was measured again after animals had been in non-active tap water for an hour.

Results.

Animals in the trough remained colourless, those in the flask became pink after 4 days, and red after 7 days. All the animals tested showed a measurable activity over that of the background. The results are summarized in table 12a and b. After 1 day, colourless animals gave approximately 95 counts per minute, this increased to approximately 145 counts per minute after 4 days, and

Table 12.

The total activity of colourless animals and animals gaining haemoglobin from the active Fe^{59} solution.

A.

Time in trough, days.	Background counts/min.	Colourless individuals counts/min.
1	12.7	115.5
	12.3	83.9
4	13.7	137.3
	10.7	155.7
6	13.1	207.4
	11.2	154.3
	12.0	177.6

B.

Time in trough, days.	Background counts/min.	Animals gaining haemoglobin counts/min.
1	13.5	133.7
	13.8	101.3
4	12.1	122.3
	13.0	116.3
6	10.6	79.8
	11.5	75.3
	11.7	84.0

approximately 180 counts per minute after 6 days (table 12) Red animals gave approximately 115 counts per minute after 1 day; about the same activity after 4 days, and a lower activity, approximately 80 counts per minute after 6 days. Evidently the animals in the flask were getting less iron than those in the trough.

After being left in tap water for an hour all animals showed a loss in total activity and in some animals up to two-thirds of the activity was lost (table 13). Red animals defaecated soon after transference to the tap water, colourless animals did not do so. This could be a shock reaction and may account for the greater loss in activity shown by some red animals after washing, than was shown by colourless animals.

Evidently much of the activity counted initially, was that of contaminated areas such as e.g. the limbs and carapace, and it is not certain that iron has been absorbed into the body. For this reason radio-autographs were made in order to determine whether any internal structures contained iron.

Table 13.

The activity of animals before and after washing.

A. Colourless animals

Activity before washing.	Background	Activity after washing.	Background.
counts/min.	counts/min.	counts/min.	counts/min.
111.5	12.7	79.2	12.3
137.3	13.7	108.1	10.7
155.7	10.7	118.9	13.9
207.4	13.1	95.1	11.7
154.3	11.2	50.2	13.1
177.6	12.0	69.8	11.3

B. Animals gaining haemoglobin.

Activity before washing	Background	Activity after washing.	Background.
counts/min.	counts/min.	counts/min.	counts/min.
133.7	13.5	106.6	13.8
122.3	12.1	66.1	13.0
116.3	13.0	42.8	13.4
79.8	11.3	43.4	11.7
75.3	10.6	41.3	11.6
84.0	11.5	28.6	13.0

The method of autoradiography as applied to Daphnia.

Methods in autoradiography have been reviewed by Bourne (1952). Using Daphnia, radioautographs were made in the following way. Animals to be used were washed for 24 hours in tap water and their approximate activity was estimated. They were then fixed in neutral 10% formalin for 24 hours, washed in 70% alcohol, dehydrated to absolute alcohol, and placed on a clean glass plate to dry. Since it was essential to the success of the method that no moisture should remain, the majority of radioautographs have been made using animals which were placed in an oven, at 35°C. for 20 minutes to dry thoroughly. After this period the animals were quite dry and brittle. Animals were then placed on a photographic plate (Ilford thin film, half tone N59), flattened with a clean glass plate and kept in contact* with the emulsion by placing both plates between wooden sheets which were screwed firmly together. The length of exposure varied from 4 to 11 days, the whole apparatus was kept in a cupboard in the dark room.

Radioautographs were made of colourless animals (figure 7), which had been living in troughs of well

* Original contact method was that of Hamilton, Soley & Eichorn (1940).

aerated water for 9 days. (150 ml. tap water. 0.9 ml. active ferric chloride solution); animals gaining haemoglobin (figure 8), which had been in poorly aerated water for 9 days. (150 ml. tap water. 0.9 ml. active ferric chloride solution); animals losing haemoglobin (figure 9), which had been removed from the active ferric chloride solution when red, and left in aerated water for 5 days in order that they might lose haemoglobin.

Results of autoradiographic procedure in Daphnia.

Colourless animals (activity $\simeq$ 30 counts/min.) exposed for 4 days, showed iron in the region of the gut lumen, it was not possible to be sure if iron was also in the gut wall. A general slight darkening of the plate suggested that a surface contamination may have been present (figure 7). Animals gaining haemoglobin (activity $\simeq$ 20 counts/min.) exposed for 4 days showed a weak reaction in the region of the gut lumen and in the eggs. The outline of the body was indicated in the plate (figure 8) this could have been on the surface of the body or perhaps just under it i.e. in the blood. Since there is no apparent reason why red animals should be more contaminated than the colourless animals, this might well be in the blood. Two other observations substantiate this. After fixation blood accumulates in the bracts of the thoracic limbs in

RADIOAUTOGRAPHS, SHOWING RADIOACTIVE IRON IN DAPHNIA.

Plates exposed to radio-active animal for 4 days.

Figure 7. Colourless animal.

Figure 8. Animal gaining haemoglobin.

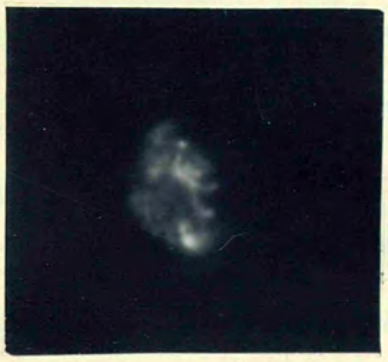


Figure 9. Animal losing haemoglobin.

some animals; these could be clearly seen on some radioautographs. The second observation is recorded below (page 116).

Animals losing haemoglobin (activity ≃ 20 counts/min.) show variable amounts of iron in the gut lumen, in the limbs and in the shell gland. This was indicated on the plate exposed for 4 days, but was more clearly seen after exposure for both 7 and 11 days (figure 10).

No darkening of the photographic plate in the region of the shell glands was seen when colourless animals and animals gaining haemoglobin were exposed for 7 and 11 days.

Futher evidence of the presence of radioactive iron in the blood of red animals.

The total activities of 3 activated red animals which had been washed in non-active tap water for 24 hours, were counted. The carapaces were then pierced near the postero-dorsal spine and red blood could be seen to leave the animals. Drops of this blood were collected on filter paper and the activity measured as counts per minute. In order to make conditions comparable during these counts animals were placed directly on the filter paper when their activity was being counted, instead of on the glass plate as before.

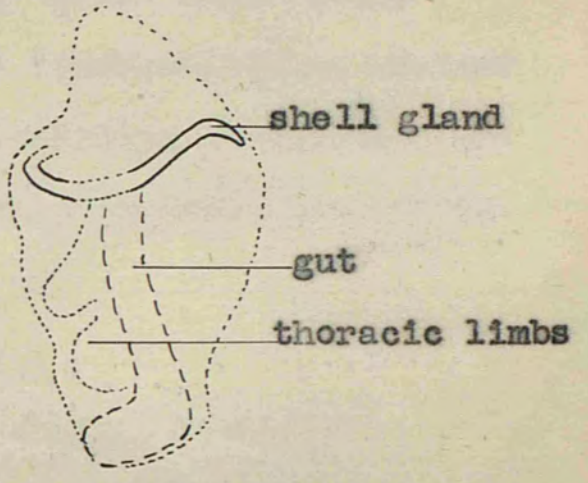
The results (table 14) show that in the 3 animals

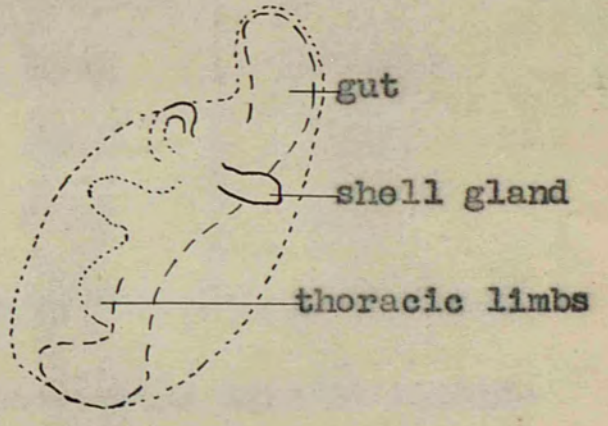
RADIOAUTOGRAPHS, SHOWING RADIOACTIVE IRON IN DAPHNIA.

Plates exposed to radio-active animals for 11 days.

Figure 10.

Animals losing haemoglobin.





tested much of the activity was in the blood, since the count obtained from the blood alone, was almost equal to that for the whole animal. It is known that self absorption of activity occurs so that the count given by the blood might be disproportionately large. Since much of the radioactivity from Fe^{59} is derived from γ radiation, however it is probable that little self absorption occurs at these thicknesses, and much of the activity is therefore in the blood.

Table 14.

Activity of whole animal and blood only, compared.

Activity of red animal.	Background.	Activity of red blood.	Background.
26.4	12.7	21.5	12.7
42.7	14.5	37.0	11.7
39.1	13.0	35.2	11.8

Thus it is probable that radio-active iron is assimilated by animals gaining haemoglobin. Further, iron is apparently present in the blood at this time and in the shell gland during haemoglobin loss. It is not certain whether colourless animals absorb radioactive iron, it has only been identified with certainty in the gut lumen.

When colourless animals and animals gaining haemoglobin are placed in identically active solutions, the colourless animals acquire the greater activity and less of this is removable by washing probably because red animals defaecate and so lose activity when placed in aerated water.

2. THE ESTIMATION OF RADIOACTIVE IRON IN WATER IN WHICH DAPHNIA IS LOSING HAEMOGLOBIN.

Activated, colourless and red animals were obtained as described on page 107. All animals were then left in tap water for 24 hours in order to remove surface contamination. The red animals were then left to lose haemoglobin in 4 covered troughs. Each trough contained 30 ml. tap water, 2 individuals and a little yeast as food. Colourless animals as controls, were set up in a similar manner. Samples of water were removed from each flask after the fourth and seventh day and the activity of the water was estimated. This was done by placing the water (about 8 ml.) in a Geiger-Müller liquid counter, and recording its activity as counts per minute. The average of ten consecutive readings was taken to be the activity of the water. The background activity was counted before each experimental count. The water was replaced in the troughs after the estimations.

Results.

The activity of the water in the control and experimental troughs, and that of the background, is recorded in table 15.

TABLE 15.

ACTIVITY OF WATER CONTAINING COLOURLESS ANIMALS AND ANIMALS LOSING HAEMOGLOBIN after 4 days, in counts per minute.

COLOURLESS	BACKGROUND	LOSING HAEMOGLOBIN	BACKGROUND.
24.5	29.0	21.8	22.8
22.5	22.2	20.4	22.0
22.3	18.1	22.1	21.0
22.3	20.3	24.6	24.9

ACTIVITY OF WATER CONTAINING COLOURLESS ANIMALS AND ANIMALS LOSING HAEMOGLOBIN after 7 days, in counts per minute.

COLOURLESS	BACKGROUND	LOSING HAEMOGLOBIN	BACKGROUND
23.6	19.6	30.5	24.4
21.6	20.4	28.4	25.9
23.6	23.9	26.9	27.1
25.7	23.1	25.4	21.3

After 4 days, the average activity of the four controls and experimental troughs, and the background, was as follows:-

Colourless (Control)	22.9	counts per minute
Background	22.4	" " "
Losing haemoglobin	22.2	" " "
Background	22.6	" " "

After 4 days, the activity of the water in which Daphnia was losing haemoglobin was therefore not above that of the background or that of the control. After 7 days the average activity of the four control and experimental troughs and the background, was as follows:-

Colourless (Control)	23.6	counts per minute
Background	21.7	" " "
Losing haemoglobin	27.8	" " "
Background	24.6	" " "

After 7 days, the average activity of the water, in which Daphnia was losing haemoglobin, exceeded that of the colourless controls by 4.2 counts per minute. The average background count had also increased, by 3.1 counts per minute. Though a difference is apparent between the control and experimental animals it is extremely small.

In view of the low activity counts the experiment was repeated with twice the number of individuals i.e. 4 animals in 30 ml. water in each trough. Two troughs

instead of four were used however. The water was tested after 11 days, when the red animals had lost almost all their blood haemoglobin. The results are recorded in table 16.

TABLE 16

ACTIVITY OF WATER CONTAINING COLOURLESS ANIMALS AND ANIMALS LOSING HAEMOGLOBIN, after 11 days, in counts per minute.

COLOURLESS	BACKGROUND	LOSING HAEMOGLOBIN	BACKGROUND.
19.4	19.2	25.2	20.4
20.0	19.4	21.0	17.2

After 11 days, the average activity of the water, in which Daphnia was losing haemoglobin, exceeded that of the colourless controls by 3.4 counts per minute. The average background activity had not increased, but had decreased slightly.

Summary of radioactive study.

It is thus evident that when individuals of Daphnia are losing haemoglobin, with radioactive iron in the shell glands, a slight increase of activity can be recorded in the water, after a period of 11 days. It is also evident that an accurate, quantitative estimate of the increase can only be determined if more data are obtained. This might be possible if more troughs were used, and the number of consecutive one minute readings increased from 10 to perhaps 30. This might remove the effect of the occassional high background count.

Radioautographs have confirmed the presence of iron in the blood and eggs of animals which have haemoglobin. Colourless animals can become radioactive, but it has not been possible to determine the iron distribution in these animals by this method. Animals which are losing the blood pigment show radioactive iron in the shell glands.

The study has therefore confirmed the inorganic iron technique, in that iron has been shown in the excretory organs when the haemoglobin of the blood is decreasing. Preliminary work on the tracing of this iron into the water has suggested that the iron is excreted and so leaves the body.

SECTION 9

DISCUSSION.

DISCUSSION.

The studies made have suggested more than one possibility for (a) the site of absorption of iron, b) the valency of the iron when absorbed, c) the site of synthesis of haemoglobin and d) the site of breakdown of haemoglobin. The possibilities are discussed below.

The fore and hind gut walls contain no detectable iron, and thus may be assumed to play no part in the absorption of iron. The mid gut walls contain both inorganic and organic iron; absorption clearly takes place here. The particulate gut contents are mostly found toward the hind region of the mid gut, the anterior region of the mid gut contains no solid contents after fixation and no detectable iron. Thus, iron which is absorbed by the anterior mid-gut walls must pass forwards from the solid contents behind. This parallels the course taken by the food, which is digested posteriorly while absorption occurs in the anterior mid gut alone (von Dehn 1930). Some of the iron present in the mid gut walls, adjacent to large quantities of iron in the gut lumen, may have diffused from the lumen into the gut wall during the treatment; this could not have occurred in the anterior mid gut.

The valency of the iron in some parts of the gut walls is apparently determined by the aeration of the outside water.

When animals are in poorly aerated water, the iron in the mid gut walls (except caeca), is in the ferrous form. When animals are in well aerated water the iron in the mid gut walls (except caeca), is partly ferric iron but also, in part ferrous iron. The valency of the iron in the gut caeca however, is not related to the aeration of the water; ferric iron may occur here when the water is either well or poorly aerated. Large quantities of ferric iron occur when the haemoglobin content of the blood is changing i.e. during gain and loss of haemoglobin; but not when the haemoglobin content is steady or negligible. At these times the quantity may be small or negligible and may be ferrous or ferric iron.

It is not known in what form iron is absorbed by Daphnia. In man, divalent iron is more readily absorbed than the trivalent form (Pirrie 1950) and it is not certain why this should be so. It may be that ferric salts must be changed to the ferrous state before absorption; or both forms may be absorbed to an unequal degree; or ferric iron may be less available for absorption because it more readily forms insoluble compounds in the gastro-intestinal tract (Moore et al., 1944). Apparently other mammals such as the dog and the rat absorb both iron forms equally well, and this could be so in Daphnia.

The mechanism of iron absorption in mammals has been envisaged by Granick (1946) in the following way: iron can be absorbed only when the protein to which it becomes attached in the gut wall i.e. apoferritin, is not fully saturated with iron. An internal call for iron is met by release of iron from the saturated ferritin in the gut wall so that more iron can then be absorbed. Evidently the mechanism is different in Daphnia, since no ferritin can be detected.

The gut of Daphnia is everywhere bathed by blood, so that iron must pass through the blood space in order to reach other organs. Inorganic iron is found in the blood when animals live in poorly aerated water, this is ferrous iron; no inorganic ferric iron is found in the blood. This could be because ferric iron more readily forms complexes with other compounds (Heubner 1926), and so occurs in the blood as organic iron alone. The organic iron study did not give a satisfactory answer to this, since probably owing to variable diffusion of iron, the blood reacted inconsistently.

By analogy with vertebrates, synthesis of haemoglobin might occur in Daphnia at a site containing iron, either as stores in the form of ferritin or newly absorbed inorganic, diffusible iron. Similarly, haemoglobin would be expected to be present though perhaps in small quantities. No ferritin has been found in Daphnia however and the variable

reaction of animals collected from the field, suggests that constant iron stores are not maintained, but that iron in tissues is related to the quantity of iron available in the food and water. The organ of synthesis of haemoglobin may therefore show inorganic iron and haem iron. This condition is fulfilled by three structures only, the anterior mid gut walls, the fat cells and the ovaries. Though the appendage muscles sometimes contain both inorganic and haem iron, the inorganic iron is in the sarcoplasm while the haem iron is in the myofibrils.

The synthesis of blood haemoglobin may occur in the anterior gut walls. It is likely however that the haem here is not haemoglobin but a haemochromogen, which has now been found in the gut walls (Phear, personnal communication) as well as in the lumen (Fox 1948). The blue reaction to cyanol could have been given by this. No oxyhaemoglobin bands have been identified in the anterior gut walls and in the absence of other evidence it is simpler to assume that the inorganic iron in the anterior caeca is newly absorbed iron which will be used for synthesis elsewhere.

The fat cells and ovaries could be sites of haemoglobin synthesis. Both have inorganic and haem iron during the synthesis of blood haemoglobin. On four separate occassions, oxyhaemoglobin has been identified spectroscopically in the

fat cells, which have appeared quite red. The normal haem reaction in the fat cells is slight, but this could be because the rate of transference of haem into the blood is high. The ovaries are known to contain haemoglobin. Since both these organs are constantly bathed by blood they could be the sites of synthesis of blood haemoglobin. Before completing the discussion as to the site of synthesis of haemoglobin, it is interesting to recall that a synthesis of other haem compounds accompanies haemoglobin synthesis. At the present moment it is impossible to say whether this occurs in situ in each organ, or in one structure alone. It is first necessary to confirm a quantitative increase in haem. It has however been outside the scope of this work to confirm the general increase in haem at low oxygen, indicated by the cyanol method. It is therefore proposed that a synthesis of haemoglobin occurs in the fat cells and perhaps also in the ovary.

The most interesting and surprising results have been shown from the study of animals which are losing haemoglobin. About three to four days after haemoglobin loss has been initiated, large quantities of inorganic iron are present in three organs: the gut caeca, the fat cells and the shell glands. The Prussian blue reaction given by the gut caeca and fat cells is more intense at this time than at

any other and the reaction in the shell gland occurs at no other time. This release of inorganic iron accompanies the loss of haemoglobin and the iron may well be derived from it. A general decrease in haem compounds at this time is indicated by the cyanol technique, so that some of the iron could have originated from the breakdown of other haem compounds. In which of these organs does haemoglobin breakdown take place? Results have indicated that the gut caeca are excreting iron at this time, since in the absence of iron in the gut lumen, iron is still found in the gut caeca. The iron in this part of the gut may therefore be released from haemoglobin breakdown elsewhere, to be subsequently excreted by the gut caeca.

It is probable that a breakdown of haemoglobin takes place in the fat cells. More iron is shown in these cells during haemoglobin loss than at any other time, some of this could be iron which has been converted to the trivalent from the bivalent form, following transference to aerated water. The results of a short experiment, indicate however, that some of this iron has come from another source, probably haemoglobin. Red animals which have no inorganic iron in the fat cells, can be obtained by feeding them on Chlorella alone. If these red animals are then transferred to aerated water, iron once again appears in quantity in the

ferric form in the fat cells, when the haemoglobin content of the blood falls. This could not have been a result of the direct oxidation of inorganic iron already present, nor could it have been absorbed from the food since no food was added when the animals were transferred to aerated water. This suggests therefore that the ferric iron is released from another source, probably from the breakdown of haemoglobin. One further point supports this hypothesis. The reaction of the fat cells to cyanol, is stronger when haemoglobin is being lost from the blood, than at any other time. This could be haemoglobin awaiting breakdown.

The shell glands also contain iron when haemoglobin is being lost from the body. The presence of iron in the excretory organs suggests that iron might be excreted. Further, the iron is present in the lumen, as well as in the walls of the shell glands, and in that part of the tubule nearest the orifice, namely, the vesicle. Some iron must escape from this region and it is probable that all the iron leaves the body by this route since iron disappears from the shell gland when haemoglobin has left the body.

It has been possible, by ashing animals, to show a decrease in the total iron in the body after haemoglobin has gone from the blood. It is known that some iron is lost when haemoglobin passes into the eggs so this knowledge

of a decrease in total iron, does not confirm a loss of iron, other than that in eggs. An attempt was made to use males for the ashing analysis, when there would be no complication of the loss of iron through eggs. The maximum number of male Daphnia available at any one time however, was 120, insufficient for analysis.

It has been exceedingly difficult to prove an excretion of iron, using quantitative methods. A thiocyanate method was not sufficiently sensitive. The radioactive iron study however, yielded results which support the hypothesis that iron is excreted, but it has not been possible to obtain an accurate assessment of the quantity excreted.

Is haemoglobin broken down in the shell gland? The presence of large deposits of iron makes the excretory organs as likely sites of haemoglobin breakdown as the fat cells, especially since inorganic iron is found here at no other time. The shell glands seem frequently to contain haem compounds, but these may be oxidative haem enzymes and not haemoglobin awaiting breakdown.

The iron in the wall of the labyrinth of the shell glands could be iron awaiting excretion or iron reabsorbed from the lumen of the gland. If the latter were so Daphnia would be conserving iron, as does the mammal. If reabsorption of iron occurs, the labyrinth with iron in its wall is the

only part of the gland which can reabsorb the iron. Similarly, the labyrinth is the only part of the gland which excretes iron into the lumen, since the saccule contains no iron and the vesicle contains no iron in the walls. It is difficult to conceive, however, that the iron in the lumen of the vesicle is merely an accidental escape of iron which could not be absorbed in the labyrinth. All the evidence available suggests that some iron is excreted and all the iron in the gland may well have the same fate. The results therefore indicate that haemoglobin is broken down and iron is released, in the fat cells and perhaps in the shell glands. Iron is passed into the lumen of the labyrinth, through the vesicle, to the orifice and some, perhaps all is excreted.

When the haemoglobin content of the blood of Daphnia has reached a steady high concentration, the iron distribution resembles that of animals which are synthesizing haemoglobin. There are slight differences however in the quantity of iron in the gut caeca, fat cells and ovaries which are difficult to understand. No inorganic iron is present in the shell glands at this time, so that excretion of iron through the shell glands is not taking place. By analogy with vertebrates the haemoglobin molecule would be expected to have a fixed length of life and after a period to break

down. No release of iron to the shell glands can be detected at this time however, but perhaps a turnover is restricted to the fat cells. This might account for the increased iron content of the fat cells in animals with a steady haemoglobin concentration.

It is not known how the "steady level" is maintained. The rate of synthesis of haemoglobin may be constant but after a time an increase of haemoglobin may cease because more haemoglobin is passed into the eggs; this could be tested quantitatively by estimating the haem content of embryos. Perhaps, however the rate of synthesis of haemoglobin varies and decreases during the "steady level", because less iron is absorbed. Certainly, the gut caeca have less iron at this time than when a gain in haemoglobin content is evident. If a turnover occurs after a period, it must be limited to the fat cells since no release of iron into the shell glands occurs while animals remain in poorly aerated water up to 30 days. It would be interesting to see whether iron appears in the shell glands if animals are reared and kept in poorly aerated water for their duration of life. When more data, on the "steady level" become available, the iron distribution may be more accurately interpreted.

It is thus apparent that the metabolism of iron compounds in Daphnia is unlike that in vertebrates. The processes of economical retention of iron, regulation of iron intake, storage of iron as ferritin and formation of bile pigments, such as occur in mammals, are not practiced by Daphnia. Daphnia seems not to have bile pigments or ferritin nor to keep iron stores. Instead Daphnia loses its iron and obtains more for further haemoglobin synthesis from the food.

ACKNOWLEDGEMENTS.

My thanks are due to my colleagues in the Department of Zoology, Bedford College for their interest and criticism, to Dr. Geoffrey Bourne of the London Hospital Medical School through whose kindness the radioactive study was made possible. My special thanks are due to Professor H. Munro Fox for his generosity in allowing this work to be carried out in his department and for his constant guidance and constructive criticism.

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Ph. D. 1955 Subsidiary

(*Reprinted from Nature, Vol.* 173, *p.* 782, *April* 24, 1954)

Iron Excretion by *Daphnia* during Hæmoglobin Loss

Daphnia contains hæmoglobin in solution in the blood. The concentration of the hæmoglobin is variable and depends upon the quantity of oxygen dissolved in the outside water : the less oxygen, the redder is the blood[1]. I have made a histological study of the distribution of iron in the tissues of *Daphnia magna* Straus when gaining or losing hæmoglobin in response to a changed oxygen content of the water, and have found that when hæmoglobin is being lost from the blood, inorganic iron accumulates in the excretory organs.

The paired maxillary glands, or shell glands, of *Daphnia* are the excretory organs. Each consists of a closed internal cœlomic end-sac, the saccule, a long winding tube, the labyrinth, and a terminal loop, the vesicle, opening to the exterior[2]. These shell glands lie in the hæmocœle and so are bathed by circulating blood. The presence of inorganic iron was shown by Bunting's modification[3] of the Prussian blue histochemical technique. This does not reveal the iron in hæmoglobin.

Iron was found in the shell glands only at the time when the hæmoglobin concentration in the blood was decreasing, which suggests that the iron is derived from the breakdown of hæmoglobin and that it is being excreted. The distribution of iron within the shell gland supports this view, for iron was found both in the walls and in the lumen of the middle section or labyrinth, but only in the lumen of the distal section or vesicle. No iron was found in the inner portion or saccule. In the labyrinth walls the iron stains as blue granules, but in the lumen it stains as a diffuse blue coloration. It is thus apparent that iron is excreted from the walls into the lumen of the labyrinth and thence passes through the vesicle lumen out of the body.

At the same time as iron appears in the shell glands of *Daphnia* which is losing hæmoglobin, it is found in the walls of the paired anterior gut cæca and in the cells of the fat body. One cannot be certain that the iron in the gut cæca is being excreted, since, unlike the shell glands, the cæca also have iron when the blood is gaining hæmoglobin. But more iron is present in the cæca of animals when losing hæmoglobin than at other times, which points to excretion. In the fat body, too, there is most iron when *Daphnia* is losing hæmoglobin, and this suggests that here is a

site of hæmoglobin breakdown, with the liberation of iron to be excreted by the shell glands and perhaps by the gut cæca.

The expulsion from the body of iron derived from hæmoglobin breakdown is in direct contrast to the state of affairs in mammals, where the iron from the continuous destruction of red blood cells is conserved and used again for further hæmoglobin synthesis. In some mammals the iron derived from hæmoglobin breakdown is temporarily stored as ferritin[4], an iron-protein complex found in liver, spleen and bone marrow. I have been unable to obtain crystals of ferritin from *Daphnia* with the aid of cadmium sulphate, but it must be remembered that no ferritin has been found in oxen, sheep or deer.

Another striking difference between *Daphnia* and vertebrate animals is that in the latter, which continuously break down the hæmoglobin of old red blood corpuscles, the hæm portion of hæmoglobin, after losing its iron, is excreted as bile pigment, whereas in *Daphnia* no bile pigment has been detected, either in extracts of large numbers of individuals[5] or histologically. For the latter purpose I have used the method of Stein[6]. Since not only has no bile pigment been found in *Daphnia* losing hæmoglobin, but also no porphyrin either[5], it appears that the hæm is broken down beyond the bile pigment or porphyrin stage to simpler colourless compounds.

The work reported here will be described in full elsewhere.

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Feb. 11.

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Printed in Great Britain by Fisher, Knight & Co., Ltd., St. Albans.