On the Nature and Functions of the Amoebocytes of Ostrea edulis.

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With 12 Text-figures.

CONTENTS.

INTRODUCTION.

RECENT works by Yonge (1926, 1928) on the behaviour of the amoebocytes of the oyster have called attention to their great importance in the functioning of this animal. The present research was undertaken at the suggestion of Professor C. M. Yonge. A great deal of ground is covered in this paper, so that it has been impossible in the time available to investigate and discuss in detail every problem. It is hoped in future to carry out further investigations.

It is with great pleasure that I take this opportunity to express my indebtedness to Professor C. M. Yonge, for having first introduced me to the subject of these investigations, and for subsequent aid, criticisms, and encouragement throughout the work, and also for the preparation of this manuscript for the press.

I am also greatly indebted to Dr. E. J. Allen, F.R.S., the Director of the Laboratory of Marine Biological Association at Plymouth, for facilities for this research and to him and the other members of the Staff for their kindness and help.

I wish to express my thanks to Mr. A. V. Mitchell for his help in the preparation of this manuscript.

2. Morphology of the Amoebocytes.

A. Previous Work.

Many investigators have studied the blood-cells of Lamellibranchs. The first description of the blood-cells of Mollusca, so far as I am aware, is by Poli (1791) in Arca glycimeris. In 1848, Wharton gave a comprehensive account of the blood-cells in various groups of invertebrates including the molluscs, Buccinum and Mytilus, and subsequently Lieberkühn (1854), Hessling (1859), Keferstein (1862–6), Flemming (1878), Apathy (1888), Cattaneo (1889), Cuénot (1891), Griesbach (1891), Knoll (1893), De Bruyne (1895), Carazzi (1896), Kollmann (1906), Drew (1910), Goodrich (1919), Canegallo (1924), and others described the morphology of the cells of numerous molluscs. But the conclusions reached, especially with reference to classification and morphological features, were very varied. Wharton divided the blood-cells of Mytilus into three types, and stated that they are transitional stages in development. Cattaneo, Griesbach, and Knoll studied the blood-cells of a large number of Lamellibranchs and divided the cells into two types according to the presence or absence of the granules. De Bruyne gave a lengthy account of these cells in the Lamellibranchs, taking as his types Mytilus, Ostrea, Unio, Anodonta, and he recognized seven varieties of corpuscles.

Cuénot divided the cells into three types according to the

stage in development, coarsely and finely granular corpuscles, and a third variety consisting of a nucleus with very little surrounding protoplasm. He considered that these are all derived degenerative changes from the coarsely granular form. Drew described the blood-cells of Cardium, and also recognized three types which agree with Cuénot's classification. Canegallo recognizes three types in the blood-cells of Anodonta: lymphocytes, leucocytes, and granular leucocytes.

There are various descriptions of the blood-cells of the oyster. Ryder (1882) states that they are about $\frac{1}{300}$th of an inch in diameter, but vary somewhat; but he gives no description of the types of blood-cells. Herdman and Boyce (1899) in describing the blood-cells of the oyster state that 'The blood-cells are normally colourless leucocytes, measuring on an average 10μ in diameter, some, however, are granular, and the granules may be yellow, brown, black, or green in colour'. Carazzi (1896) in his work on the blood-cells of the green oyster states that there is one type, but that some contained many green granules. Griesbach (1891) and Lankester (1885) observed the green granules of amoebocytes of the oyster and their power of amoeboid movement, but they did not distinguish between different types. Orton (1924) mentioned that 'the blood-cells of the oyster are of two main kinds, namely, large granular ones, and large and small clear ones'. He states that the appearance of the large granular form taken fresh from the body is well shown by Herdman and Goodrich, but he gives no description or figures of the clear types.

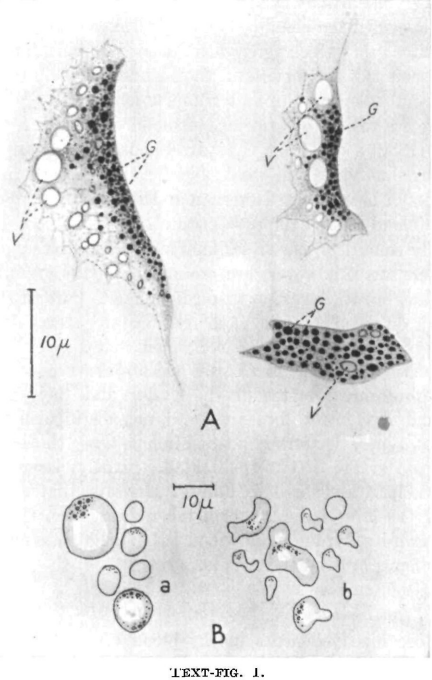
B. General Description.

The organized elements in the blood of Ostrea edulis consist of two main kinds of amoebocytes. One is the granular (granular leucocytes), the other more hyaline (lymphocytes). Both types can be easily distinguished in fresh unstained preparations of the blood from the heart.

(i) Granular leucocytes (Text-fig. 1 A).

These are relatively large and granular. The cytoplasm contains a number of granules, which, when fresh, are yellow or

yellowish green in colour, and vary in size and number. These leucocytes measure 9μ to 13μ in diameter when they are



TEXT-FIG. 1.

The two kinds of amoebocytes from the heart. Drawn from life. A. Granular amoebocytes in the expanded condition; *G.*, granules; *V.*, vacuoles. B. Hyaline amoebocytes. *a.*, spherical condition; *b.*, the change of form after about twenty minutes.

contracted. The nucleus is simple and compact, not lobed or divided, and 3μ to 4μ in diameter. (Carazzi stated that the

nucleus of the amoebocytes in the oyster is divided into fragments, but I never observed such a condition.) The leucocytes are amoeboid, varying in size and form, but the rate of progression is slow. The cell puts out delicate thin lamellae round the periphery forming the so-called 'pseudopodia' (see Section F). The fresh granular amoebocytes which are collected from the mantle cavity or gill surface readily unite with one another by means of bristle-like pseudopodia, but the boundaries of the cells can always be identified as the cells do not actually coalesce (Text-fig. 2 D).

(ii) Lymphocytes (Text-fig. 1 B).

These are never entirely hyaline; the cytoplasm sometimes contains a few granules. In fresh unstained preparations of the blood, they can easily be distinguished by their spherical shape and high power of refraction. They are pale in colour and very variable in size, 5μ to 15μ in diameter. Amoeboid movement is less marked in them than in the preceding type; they do not expand to the same extent or produce bristle-like pseudopodia.

In the course of examining these two kinds of amoebocytes, observations were made on fresh preparations in a moist chamber and examined at intervals. The granular leucocytes are actively amoeboid although the rate of progression is slow. The amoebocytes present an indefinite variety of forms when they move. After the lapse of about half an hour many become fully extended, and may then measure three or four times their length in the contracted state. In this condition they are very thin, sometimes transparent, and can be most conveniently observed under a narrow cone of illumination. Some of them link up together to form an open meshwork of chains of cells. Adjacent cells are joined up by cylindrical processes recalling the axons of a bipolar nerve-cell. The expanded amoebocytes show large and small vacuoles in the cytoplasm, and granules can easily be identified in this condition.

On the other hand, the amoeboid movement of the lymphocytes is very simple; they produce lobe-like pseudopodia and change their form, but not very actively.

C. Chromatic Properties of Granules.

There are several descriptions of the natural colour of the granules in the amoebocytes of the oyster; there are no detailed descriptions of their chromatic properties.

The granules of the amoebocytes are usually yellow or yellowish green when fresh, infrequently they are brown; but the black colour recognized by Herdman and Boyce was never seen. In the so-called 'green oyster' the colour is more greenish. The number of the granules varies in individual amoebocytes. Drew and Cuénot recognized two kinds of granules as above mentioned, but the granular contents of the amoebocytes of the oyster cannot be so divided. The following examination was undertaken with the hope of determining the affinity of the granules for dyes when in fresh condition.

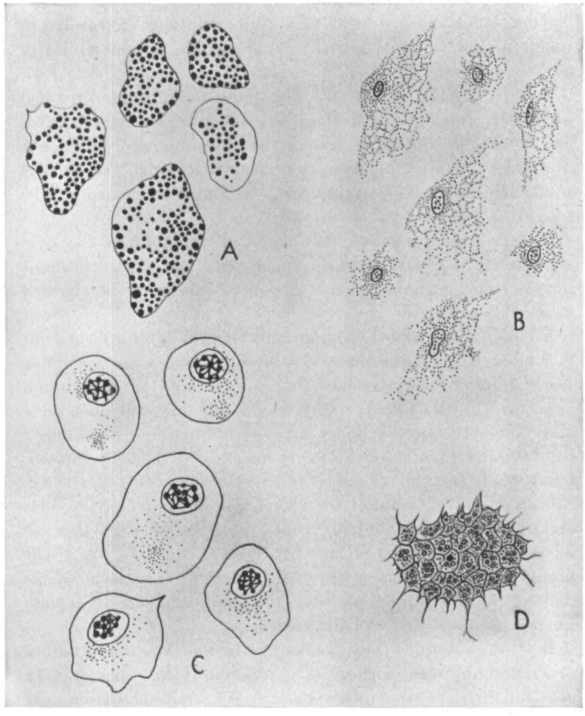
1. Methylene blue, Methyl violet B, and Neutral red were used. The granules are stained by these dyes when fresh, but the dilution of the dyes is a very important factor; certain concentrations stain only the nucleus, but not the granules.

(i) Methylene Blue.

0·1 per cent. methylene blue was first employed. The result was satisfactory for the nucleus, which always stained well, but never more than a small number of granules were stained. The amoebocytes are very sensitive to such a concentration; they change their form and become spherical, and the granules congregate eccentrically. When treated for about half an hour in the moist chamber with a more dilute solution, e.g. 1/10,000 or 1/100,000 strength, the granules stained vividly, but the nucleus only faintly.

(ii) Neutral Red.

This dye has a great affinity for the granules, which become red in dilute solution, and in certain concentrations a brownish red. Solutions of about 1/10,000 and 1/100,000 were used, and good results were always obtained. The stained granules varied in size, but they were never found in the delicate lamella. The



TEXT-FIG. 2.

A. Granules stained with neutral red intra vitam. ×450. B. Fixed and stained expanded amoebocytes in blood-film preparation. ×450. C. Fixed and stained amoebocytes found in vesicular tissue round the stomach. D. Aggregated amoebocytes. Drawn from life. ×250.

results are shown in Text-fig. 2 A. The nucleus never stained with this dye (Text-fig. 2 A).

(iii) Methyl Violet B.

The granules also stained with dilute solutions of this dye in much the same manner as with methylene blue, but not so clearly as with neutral red.

2. Leishman's Stain. The granules are stained reddish purple by this dye, and there are no granular amoebocytes having such fine or coarse granules as those described by Cuénot and Drew. The cytoplasm is not stained clearly, except that the contracted amoebocytes appear a faint bluish purple. The nucleus is stained blue or purple.

3. Giemsa's Stain. As a result of staining with this dye, the granules appear reddish violet, and the nuclei brilliant purple. The granules in the amoebocytes cannot be divided into different types.

When stained with Leishman and Giemsa careful treatment is necessary or granules may not be found in the amoebocytes. Both staining reactions show that the granules are neutrophil.

4. Other Methods. The blood was smeared on a slide and fixed with Bouin's fluid, corrosive sublimate, or Flemming's solution and then stained with eosin and Delafield or Heidenhains haematoxylin or picro-indigo-carmine. The blood was collected from the heart, the two kinds of amoebocytes were identified by these methods; but it is very strange that no nucleus could be found in the hyaline amoebocytes. The granules do not stain with eosin, but the cytoplasm stains lightly. I have repeatedly tried to obtain evidence of eosinophilous granules, but without success.

In stained sections the amoebocytes in the organs and tissues are round or oval, with retracted pseudopodia, though very occasionally they are shown expanded. The nucleus is prominent and contains smaller dark bodies (Text-fig. 2 c).

The first description of the granules contained in the amoebocytes of Lamellibranchs is by Lebert and Robin (1846). Subsequently a number of investigators have worked on this subject because their affinity for dyes and their size are important in their classification.

According to Knoll (1893), the granules of amoebocytes of Lamellibranchs vary in the different species with respect to

the affinity for dyes; those of Unio, Anodonta, and Solen are acidophil, and Pectunclus is neutrophil. Kollmann (1908) gave a full account of the chromatic leucocyte granules in a number of invertebrates and sought to apply the classification of Ehrlich to the invertebrates. From the results obtained with various coloured substances, he thought that leucocytic granules of the Lamellibranchs are amphophil, with a preference for acid substances. He also divided the properties of the granules of the amoebocytes of Lamellibranchs into two groups according to their habitat; those of fresh-water molluscs are amphophil with a tendency to become stained by acidic dyes, and those of marine molluscs are acidophil. He experimented on the character of the affinity for dyes by making cultures of marine Lamellibranchs in fresh water and vice versa; but he failed to find any change in their affinity for the dyes, and stated, therefore, that these affinities are specific for the different species. If this is true, the granules of the amoebocytes of the oyster which I examined (Ostrea edulis) should be acidophil. However, with several kinds of dyes the granules are not acidophil, they are rather neutrophil like those of Pectunculus (Knoll).

D. Distribution and Origin of the Amoebocytes.

One of the striking features about the alimentary canal of the oyster is the universal presence of large aggregations of amoebocytes around the stomach, intestine, and also other parts. The presence of these amoebocytes is characteristic of most of the Lamallibranchs, and attention has been drawn to their presence by many investigators. Jenkins (1900) observed this to be the case in starved oysters, while Orton (1923) noted their great abundance and widespread distribution in the tissues and particularly round the alimentary canal. He stated that 'It has been noted that this kind of aggregation of blood-cells in a vertebrate would be taken as a sign of inflammation, and that the phenomena in the oyster may have contributed to Pettet's erroneous conclusions about the oyster; but there can be no doubt that in bivalves an aggregation of blood-cells around the

gut and especially the stomach is by no means an alarming symptom. The bathing of the gut in the oyster with a fluid containing blood-cells may, therefore, certainly be taken as a normal and necessary condition, and there can be little doubt that one of the functions of the blood-cells is to assist in absorption of food.'

In 1926 Yonge observed in detail the distribution and phagocytic function of the amoebocytes of the oyster, and recognized their great importance in the physiology of digestion in these animals.

Indeed, as shown in a later section, the distribution of the amoebocyte is of the greatest significance in nutrition and excretion in the oyster.

The origin and mode of production of amoebocytes of the oyster are not known; no definite organ is known to produce them. Cuénot (1891, 1914), when discussing conditions in various Lamellibranchs, stated that 'La glande lymphatique est très diffuse; elle est placée à la base de la branchie, mélangée avec les cordons musculaires, le tissu connectif, le nerf branchial, de telle sorte que le sang, en allant respirer, la traverse et entraîne les éléments mûrs qui s'y forment'. Croft (1929) stated that surrounding the whole of the digestive system of Haliotis there is a sheath of connective tissue. This is lymphoid tissue, and contains large cells with blood-cells in all stages of development.

In the oyster, tissue at the base of the gills and the connective tissue surrounding the digestive canal were examined after various methods of fixation and staining, but no evidence could be obtained of any production of amoebocytes under normal conditions.

E. The Functions and Properties of the Granules.

Various hypotheses have been advanced as to the function of the granules of the amoebocytes in invertebrates. They have been considered fat granules, reserve materials, ingested particles, excretory substances, or ferment granules, &c. Cuénot (1891) called the granules of the amoebocytes 'ferment albumino-

gène ou granules albuminogènes', and he considered that these albuminoid granules were produced by the transformation of the peptone in the digestive tube which poured into the blood after digestion. Kollmann considered them to be actually a reserve albuminoid substance. Canegallo (1923) states that in Anodonta he cannot exclude the idea that some of these granules may be excretory.

The following work was carried out with the view of testing their properties.

(i) Distilled Water.

There is no immediate effect on the granules, they remain intact at the end of thirty minutes.

(ii) Alcohol.

Alcohol (70 per cent., or absolute) does not destroy the granules immediately, but they seem to become gradually coagulated.

(iii) Millon's Reaction.

Lowit (1889) observed the positive reaction of Millon's reagent on the granules of decapod crustaceans, and he concluded that the granules are in the nature of globulins.

No positive results could be obtained with this reagent on the granules of the amoebocytes of the oyster.

(iv) Osmic Acid and Fat Solvent Reagents.

Kollmann studied the chemical nature of the granules of several kinds of amoebocytes in invertebrates and stated that these granules do not react to osmic acid and are also insoluble in ether and other fat solvents. Herdman and Boyce, however, stated that 'With osmic acid a black reaction is occasionally given by the granules'. In my observation, osmic acid (2 per cent.) does not produce any distinct black effect on the granules of the amoebocytes of the oyster, their margins sometimes appear blackish, but never the entire granules. I have repeatedly tried the effects of ether and other fat-solvent reagents on the granules, but they were not dissolved by any of these reagents.

(v) Physiological Diminution of Granules.

Kollmann considered that the granules are reserve material and therefore, when the animal is starved, they diminish gradually as they are utilized by the amoebocytes. He obtained positive results on the amoebocytes of Crustacea (Carcinus).

The number of granules in the amoebocytes of the oyster varies greatly in different cases. Even after ten days' starvation, the granules of the oyster do not decrease to any appreciable extent.

(vi) Sudan III, Scarlet Red.

Canegallo observed fatty globules in the corpuscles of Anodonta by staining with Sudan III; but no such positive results were ever obtained with the amoebocytes of the oyster.

(vii) Dilute Acid and Alkali Solutions.

The actions of dilute hydrochloric acid and acetic acid on the granules are gradual; after a few minutes the granules shrink and afterwards they are destroyed. Dilute sodium hydroxide $\left(\frac{N}{10,000}-\right)$ causes the granules to swell gradually and then to dissolve.

(viii) Bouin's Fluid, Corrosive Sublimate, and Chromic Acid.

Several kinds of fixing fluids for histological processes, such as Bouin's fluid and corrosive sublimate, affect the granules which are gradually destroyed. The cytoplasm which is evenly distributed in the living state becomes a dense network or sponge and sometimes a small number of granules can be found here. If fixation with these fluids is only for a short time, the granules can frequently be identified. Chromic acid (0·5 per cent.) has a moderate effect on the granules which are well fixed by it.

The amoebocyte granules are generally destroyed by these fixing fluids, but such granules as are not destroyed after treatment with them cannot be stained by ordinary dyes such

as eosin. This probably explains the absence of granules in the amoebocytes when blood-film preparations are fixed or the fixed sections stained.

(ix) Copper Reaction.

Herdman and Boyce found by histochemical investigation of the green American oyster with 'leucocytosis'[1] that the leucocytes which form the green patch contain a considerable quantity of copper.

Numerous histochemical tests were made with the normal amoebocytes and revealed in some instances faint traces of copper. During the present experiments, a number of oysters suffering from leucocytosis were obtained and then examined for copper. Positive reactions were only obtained with the plasma of the amoebocytes and not with the granules. No copper was ever found in the granules either in healthy amoebocytes or in those suffering from leucocytosis. The occurrence of copper in the plasma of amoebocytes is very interesting and is an important characteristic of the oyster blood.

(x) Temperature.

The effect of temperature on the granules was studied in different degrees of dampness. At about 62° C. the granules gradually coagulate and after a few minutes the cytoplasm which contains the granules assumes the appearance of a dense network.

The preceding results show that the granules of the amoebocytes of the oyster have a comparatively strong resistance to several kinds of reagents. The fat solvents, such as ether, do not dissolve them, and no positive reaction for fat was obtained with Sudan III; but sometimes the margins of the granules

[1] 'Leucocytosis.' A diseased condition of the oysters in which the heart becomes gorged with whitish green masses of blood-cells was described in some English and American oysters and named leucocytosis by Herdman and Boyce (1899) who could find no other explanation for this than the presence of large amounts of copper in the blood-cells. Subsequently Orton (1923) remarked that the blood-cells of the oysters suffering from the leucocytosis have highly granular and much vacuolated protoplasm, but that they do not appear to be unhealthy.

become slightly black after treatment with osmic acid. This fact seems to indicate the presence of fat or lipoid substance distributed in this region. The granules have a great affinity for neutral red as already shown. This property is similar to that of the brown cells of the pericardial glands (excretion is discussed later), though the granules of the former are smaller. The granules of the brown cells are, however, not destroyed by various fixations and stain with iron haematoxylin. The properties of these two kinds of granules are thus dissimilar in various respects, and there is no good reason for thinking that the granules represent excrement.

There have been several suggestions as to the coloration of the amoebocytes of the oyster. Ryder (1882) has demonstrated that the green colouring matter of the American oyster is taken up by the amoebocytes and may be phycocyanin. Subsequently Lankester (1886) considered that the green pigments in the gills of the green oyster is concentrated in secretion cells (leucocytes, 1893) and there localized in granules. He studied the solubility of this green pigment, but was unable to dissolve it. MacMunn (1900) thought that the presence of colouring matter in amoebocytes was the result of the decomposition of the ingested chlorophyll which was named by him 'Enterochlorophyll'. Recently Galtsoff (1930) stated that the green pigment of the oyster is readily soluble in methyl alcohol, less so in ethyl alcohol, and insoluble in buthyl or amyl alcohol. It is also insoluble in such fat solvents as chloroform, ether, acetone, or benzene, but soluble in pyridine. He concluded that the green pigment of the body exists in a highly dissociated state.

F. So-called 'Pseudopodia'.

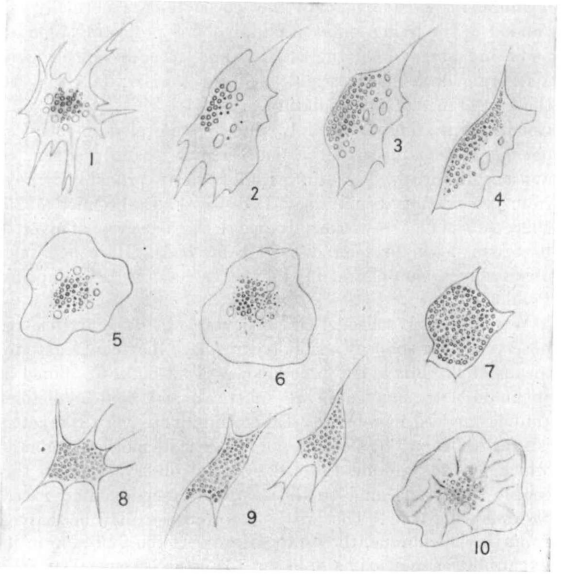
The pseudopodia of the blood-cells of Lamellibranchs have been investigated by many authors, and it is stated that when they come in contact with air or are exposed to changes in the physico-chemical environment they put out pseudopodia. Ryder remarked that the amoebocytes of the oyster put out pseudopodia which may be branched, when at rest. According to Goodrich (1919), the freely projecting pseudopodia of the leucocytes of invertebrates usually described are either figured

from optical sections of folded membranes, or due to the changes taking place under abnormal conditions. He studied the leucocytes in many invertebrates, including Ostrea, and concluded that they are provided with more or less extensive membranous processes of cytoplasm, and that in most cases the membranous pseudopodia of the leucocytes are normally expanded in the living animals, but that fine projecting pseudopodia are absent. On the other hand, leucocytes floating in a hanging drop may resemble delicate Heliozoa owing to the appearance of fine radiating processes. This, according to Goodrich, is almost certainly due to the physico-chemical changes taking place in the fluid, and is possibly a sign of approaching death. These fine pseudopodia are probably derived from pre-existing membranes. Recently Fauré-Fremiet (1925, 1928, &c.) and his co-workers studied the amoebocytes of invertebrates in detail by microdissection methods. He called such membranous cytoplasm in the invertebrates 'Pseudopodes Petaloïdes'.

When freshly collected amoebocytes from the oyster are observed, their shape is generally irregular; they congregate by means of the bristle-like pseudopodia (Text-fig. 2 D). Some of them do not congregate, are spherical, and have a distinct ectoplasmic cell membrane. This membrane is very characteristic as recognized by Goodrich; it is a delicate motile membrane which extends around the cell. Careful observations on this motile membrane, and on the so-called 'pseudopodia', reveal the following facts. The bristle-like processes may be derived from this membrane, these processes being the first step in its expansion, or they may also be due to physico-chemical stimulation. Shortly after the amoebocytes have been removed from the animals, the hyaline ectoplasmic membrane begins normally to stretch out in various directions, and the bristle-like pseudopodia become supporting folds in this membrane (Text-fig. 3, 9).

At this stage some pseudopodia-like processes can be observed under a low power; but more careful examination reveals that these 'pseudopodia' are in reality but optical sections of membranous expansions of ectoplasm thinning out peripherally to a very delicate and almost invisible film. Here and there the

membranous ectoplasm is strengthened by a rib or folds, which appear as an outstanding process under low power (Text-fig. 3, 10). Sometimes short filamentous pseudopodia are produced accompanied by an expansion of the membranous processes,



TEXT-FIG. 3.
Various forms which may be assumed by the amoebocytes and their pseudopodia. Drawn from life.

or the bristle-like pseudopodia gradually expand, being really formed by folding of a single layer of cytoplasm around the cell (Text-fig. 3, 9).

This ectoplasmic membrane can be identified by rapidly fixing the amoebocytes in their expanded condition by means of

Bouin's fluid or corrosive sublimate. It is sometimes very smooth, and sometimes has many folds or ribs, which produce the deceptive appearance of delicate pseudopodia in some fresh material as noted by Goodrich (Text-fig. 3, 10). Generally, the granules are absent in the membranous ectoplasm.

The margin of this membrane is irregular and sometimes shows pseudopodia-like processes which are occasionally branched. This may be related to changes of surface tension. Whenever the membranous cytoplasm comes into contact with a foreign object, as, for instance, a glass slide or a cover-slip, the membranes tend to cling to it and spread over its surface as a thin film (Text-fig. 3, 1 and 2).

The amoebocytes of the oyster are frequently provided with fine filamentous pseudopodia (as figured by Herdman and Boyce). They are probably abnormal, the result of environmental conditions.

In fresh condition the membranous cytoplasm consists of a delicate transparent plasma sheet, and exhibits a plastic rather than viscous flow. Sometimes there are vacuoles, some of which are due to abnormal expansion or may be concerned with intracellular digestion. In fixed stained material it appears reticular or vacuolated. My observations indicate that the delicate membranous sheet is not so expanded in the body as it is outside.

3. Physiology of the Amoebocytes.

A. Amoeboid Movement.

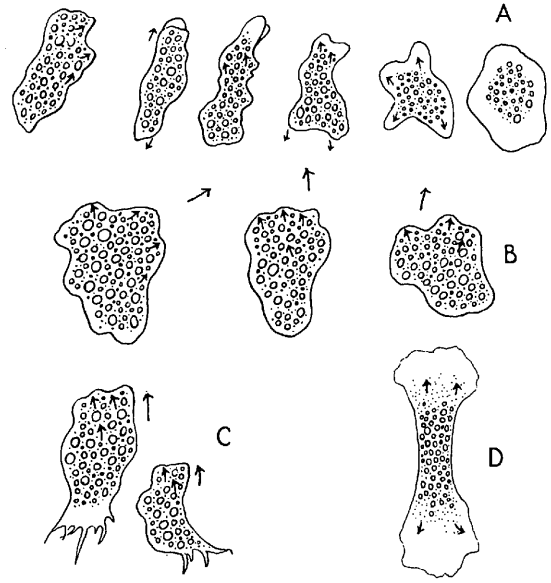
Many workers have been content to apply the general designation 'amoeboid' to the movements of the blood-cells of invertebrates after they have been taken from the body. Observation shows, however, that the forms of movement manifested by the blood-cells of different invertebrates are not identical. Cattaneo's (1889) observations appear to throw some doubt on the amoeboid characteristics of the ordinary blood-cells of invertebrates. He found that the pseudopodia put out by cells when under observation on a glass slide are not withdrawn. Griesbach (1891) was evidently prepared to admit that there are differences between the movements of molluscan corpuscles and the movements of Amoeba.

The amoeboid movement of the blood-cells of the oyster has been recognized by several authors; Ryder (1882), Lankester (1886), Orton (1923), Yonge (1926), &c. They do not give a detailed description of these movements, but simply refer to them as 'amoeboid' or 'phagocytic'.

The amoeboid movement of the blood-cells of the oyster may be easily seen, although the rate of progression is very slow; unlike those of other invertebrates, such as crustacea, they have a relatively stable constitution. When removed from the body they gradually change in form and, having a relatively high specific gravity, they sink through the plasma or sea-water. The method of movement is variable; the variation may depend upon a number of different factors, such as physico-chemical changes in the environment. The amoeboid movement is due to the so-called 'pseudopodia', which are new processes of clear ectoplasm. Sometimes these are needle-shaped or bristle-like, but more frequently a thin lamella of clear ectoplasm may be seen to flow outwards from all regions along the margin of the amoebocytes. This thin lamella slowly extends when in contact with the surface of the slide. At this stage the cell might almost be likened to a snail emerging from its shell. Loeb (1921) and Tait (1920) consider that this movement is probably due to capillary forces, but it is apparently not entirely so, as the amoeboid behaviour of the cell also seems to have its influence. Now, the direction in which the cell will move depends on the expansion of this external lamella. The part of the external lamella which is in the direction of the forward movement consists of a greater or lesser amount of clear ectoplasm, the granules being usually checked in their forward flow just behind this clear area, although they are carried forward continuously by the endoplasmic stream to the region of the advancing ectoplasm. The main mass of the cell advances to the area previously occupied by the ectoplasm, then a further fringe of ectoplasm extends in the same, or it may be in a new, direction, guiding it still further onwards (Text-fig. 4 A). The glass surface, however, so impedes the movement that active locomotion is difficult.

The expansion of the lamella is not regular. Sometimes two

separate lamellae of ectoplasm extend from the opposite poles of the amoebocyte. In this case, each lamella continues to advance, the granular endoplasm becoming extended into a



TEXT-FIG. 4.

Movement of amoebocytes. Drawn from life. A. Progression with so-called pseudopodia. Left to right. B. Progression by flowing of the granules in different directions. C. Amoebocytes showing tail-pieces. D. The expansion of two separate lamellae of ectoplasm from the opposite poles of an amoebocyte.

long leech-like shape as observed by Tait in the blood-cells of the cockroach. The fringe of the ectoplasmic lamella is not similar to the pseudopodia of Amoeba limax. The advancing part

spreads outwards like a fan and is probably due to surface tension or thigmotactic action (Text-fig. 4 D).

Pantin (1923) states that in Amoeba limax 'At the hind end was a rugose tail-piece, which with careful observation could be seen to bear a number of fine clear processes, very different from the anterior advancing pseudopodia'. When in motion the hind end of the amoebocytes of the oyster is clear and has a tail-piece less rugose than that of Amoeba. This is probably entirely due to physical differences of retraction of the ectoplasm (Text-fig. 4 C).

This amoeboid movement does not last long. The external lamellae of the cell extend all around the margin of the amoebocytes, but the glass surface so ties them down that free locomotion is impeded. Subsequently the amoebocytes gradually degenerate (Text-fig. 4 A).

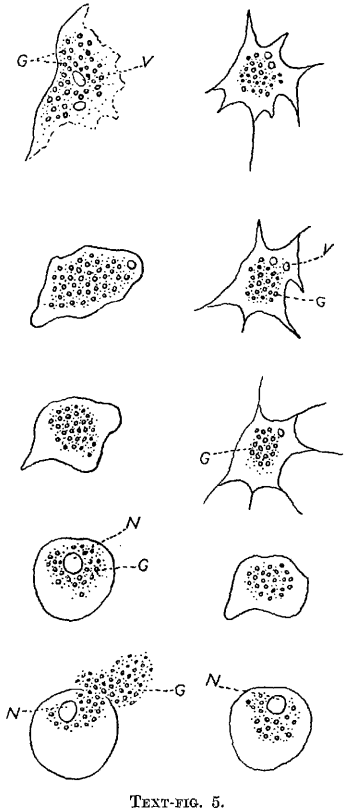
Pantin (1923) considers that the ectoplasm is more acid in the neighbourhood of an active pseudopodium than is the case elsewhere, and concludes that the normal contraction of ectoplasm is due to the production of an acid, leading to an imbibition of water with local swelling. Unfortunately, as previously mentioned, the thin lamella of amoebocytes of the oyster could not be stained with several kinds of intravitam dyes, therefore I could not confirm this.

Sometimes movement appears to consist only of the flowing of the granules in the cell in different directions, and so changing the form of that part of the amoebocyte towards which the granules are flowing. In such cases only a small ectoplasmic lamella is present. I have frequently observed this type of movement in living animals (Text-fig. 4 B).

These two kinds of movement are not distinct from each other. The former type is probably much more affected by environmental conditions than the latter which is probably more normal.

B. Effect of Reagents (Text-fig. 5).

The amoebocytes of the oyster are very sensitive to external conditions. If they are stimulated either mechanically or chemically, the expanded lamellae are retracted and the amoebo-



TEXT-FIG. 5.

Effect of distilled water on amoebocytes. Showing the change of form in two different types. *G.*, granule; *N.*, nucleus; *V.*, vacuole

cytes gradually become spherical. The following experiments were carried out.

(i) Hypotonic Solution; Distilled Water.

The effect of distilled water on the amoebocytes is remarkable; the form gradually changes as shown in Text-fig. 5. They may not immediately undergo cytolysis, but they swell till they are almost spherical. Sometimes the cells, while swollen, undergo a number of violent contractions, ending in cytolysis. The normal process is as follows: the expanded amoebocytes at first become spherical and granules accumulate at the centre of the cell, and then the periphery becomes transparent. At this time the granules show very active Brownian movement, the interior cytoplasm becomes fluid and, when the amoebocytes are becoming spherical, tongue-like processes are produced. This was described by Loeb (1921) as a 'Circus movement' in the amoebocytes of Limulus; it was also observed by Pantin in Amoeba. It is of a purely physical character.

Secondly, the form of the cell becomes much more spherical, and sometimes fine filamentous processes are seen which are the remnants of folds or ribs of the previously existing membrane; they are not processes actually put out from the cell. Thirdly, the cell becomes almost spherical by absorption of water and the granules become distributed eccentrically, the nucleus may be easily identified at this stage, the cell membrane is clearly distinguishable and the cell resembles an ovum with a fertilization membrane. Lastly, in some of them part of the cell breaks down and the granules escape. (The granules are not soluble in distilled water.)

Sea-water diluted with an equal bulk of distilled water produces essentially the same effects, although action is slower.

(ii) Hypertonic Solutions.

In hypertonic solutions water is abstracted from the amoebocytes. Considerable adjustment to the medium is made if the osmotic pressure is changed slowly. In an osmotic pressure twice that of sea-water the amoebocytes gradually change their form, and sometimes large thread-like or filamentous pseudopodia are observed. This is probably due to the physical difference of retraction of the ectoplasmic lamella or possibly to surface tension. The granules move about considerably

within the cell which becomes spherical owing to loss of water. Afterwards great shrinkage occurs, though cytolysis does not take place immediately. The cells sometimes resemble the glia cells of the cerebrum because of the thread-like pseudopodia around the margins of the shrunken cell as noted by Loeb.

(iii) Acids, Alkalies, and Fat Solvents.

Under the action of dilute acids, e.g. acetic acid and hydrochloric acid, amoeboid movement is diminished and the nucleus shrinks and becomes more distinct. In certain concentrations a granular precipitate is formed in the cytoplasm around the nucleus. Together with these changes a part of the protoplasm gradually swells out, forming a clear rounded expansion. On the other hand, under the action of alkalies, e.g. by the addition of a very small quantity of caustic soda to the solution containing the amoebocytes, the granules become swollen, and frequently the physical amoeboid movement occurs in the manner demonstrated by Rhumbler and others.

Alcohol and chloroform vapours also arrest amoeboid movement, but it recommences after a time if the action of the reagent is not too prolonged. When gradually warmed the amoebocytes become more and more active up to a certain point (about 35° C.). Above this point they become spherical, and the protoplasm is finally coagulated and killed at about 62° C. At lower temperatures amoeboid movement is also arrested, but although their movement is stopped at about 0° C. they are not killed, and when the temperature is raised movement begins again.

C. Coagulation.

The fact that the blood of Lamellibranchs does not coagulate has long been known. But the amoebocytes usually gather together in irregular clumps and strands. These are the so-called 'plasmodia' described by Geddes (1879), who thought that the cells completely fused and formed a mass comparable to the plasmodia of Myxomycetes. Drew (1910) experimented on the coagulation of the amoebocytes in Cardium blood, and stated that when a wound is made the blood first escapes from

it, then the corpuscles begin to agglutinate at the sides of the wound, and connecting strands of protoplasm may be formed between masses of corpuscles and haemorrhage stopped. He also suggested that some change takes place in the corpuscles of the blood which has been withdrawn from the animal, the haemorrhage possibly conferring the power of agglutination on the corpuscles as a result of the liberation of some enzymes from them. He made no attempt, however, to confirm this suggestion. Cuénot (1891), Couvreur (1908), and Camus (1900) commented on the absence of fibrinogen. Nolf (1909) also recognized that there is neither fibrinogen nor thrombine in the blood of certain molluscs.

The amoebocytes of the oyster were allowed to stand after being drawn from the body; the mass of amoebocytes did not become jelly-like by coagulation as in the case of crustacean and vertebrate blood,[1] and no fibrin appears in the blood-plasma which remains fluid on standing in the air and even after being heated. The amoebocytes characteristically become entangled by means of bristle-like psuedopodia or hyaline ectoplasm. When they are accumulated in masses the amoebocytes do not readily fuse together. Indeed those on the edge slowly creep away. The majority of agglutinated amoebocytes do not unite at all, except at the centre where the boundaries of the cells seem gradually to disappear and become more or less structureless, resembling a mass of protoplasm. The amoebocytes which wander away assume various forms; some of them resemble a blood-cell in the act of dividing and become almost separate, until connected by only an extremely fine strand of protoplasm which becomes so fine as to be almost invisible. In certain conditions the amoebocytes will form chains united by long protoplasmic threads. These strands may serve as an entanglement for other corpuscles; they closely resemble strands of fibrin. These structures may act as a plug and so with the contraction of the tissue itself check haemorrhage. The agglutination as described above seems entirely a function of the amoebo-

[1] Orton (1922) observed that the blood-cells (leucocytes) of the oyster can live for three or four days in sea-water in dishes, and he suggested that it might be possible to cultivate them under appropriate conditions.

cytes and not of the blood-plasma. Unfortunately, time has not permitted me to examine the causes of this agglutination.

D. Phagocytosis.

Phagocytosis[1] by the amoebocytes of Lamellibranchs has attracted the attention of many investigators, and has been experimentally demonstrated by injection or feeding methods (Cuénot, Canegallo, &c.). More detailed experiments were carried out by Yonge (1926) during the course of work on digestion.

The process of phagocytosis has been followed in a variety of ways.

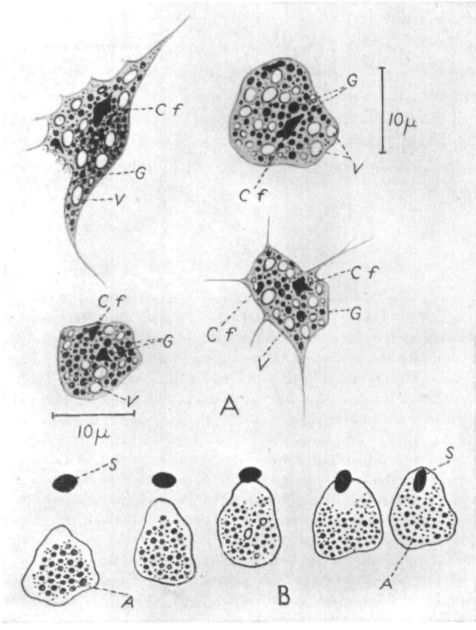
(i) On Glass Slides.

These experiments were carried out with a view to observing

[1] Recently the theory of phagocytosis has received a great deal of attention. Tait (1918) stated that the phagocytic behaviour of the amoebocytes of Ligia may be due to physical causes; accidental contact of particles with the surface of the cell would lead to adhesion. Amoeboid movement is not an essential property of the phagocytic cell. The hyaline thigmocytes of Ligia are not amoeboid, but they engulf particles. Phagocytosis and amoeboid movement are considered different processes by de Haan (1922). The former is 'static' a state of equilibrium produced in the leucocyte under the combined influence of the medium and the object ingested, the leucocyte itself being a non-variable factor; amoeboid movement is a reaction between the leucocyte and the medium, in which the leucocyte exhibits continual variation. Fenn (1920, &c.) made an elaborate investigation of phagocytosis. His fundamental concept was that the taking up of the foreign particles by leucocytes can be explained on purely physical grounds. MacKendrick (1914) considered phagocytosis from a purely mathematical standpoint.

On the other hand, many cases of vertebrate leucocytes taking up foreign organic particles are very probably due to the existence in the blood of an opsonin, a substance which apparently causes the leucocytes to attract particles. Humberger (1919) found blood-serum necessary for the phagocytosis of starch but not of carbon particles. Porges (1909) found that starch is taken up from isotonic solutions without blood-serum as well as with it, but the accelerating effect of blood-serum becomes evident in hypertonic solution. Fenn (1921) stated that there is practically no phagocytosis of carbon particles in the absence of serum or in heated serum. He considered that part of the effect of serum is due to its calcium, since the same amount of Ringers solution also increases the phagocytosis, though to a lesser degree.

the actual process of ingestion (although the environmental conditions were somewhat abnormal). Substances, such as



TEXT-FIG. 6.

A. Amoebocytes from mantle cavity ingesting carmine fibrin. Drawn from life. *c.f.*, carmine fibrin piece; *G.*, granules of amoebocyte; *V.*, vacuole. B. Amoebocyte attracting a starch grain, and showing the process of ingestion. Drawn from life. *A.*, amoebocyte; *S.*, starch grain.

carmine particles, starch grains, and silver sand, were added to drops of blood on a glass slide, the cover-glass being supported by a small quantity of wax at each corner. After a few minutes

in a moist chamber the slides were examined under the microscope. Such an examination is rendered much easier by the fact that the blood as a whole does not coagulate and the amoebocytes move actively in this medium. Examination shows that the amoebocytes certainly ingest particles, although the different particles call forth different reactions. The amoebocytes spread out towards starch grains, to such an extent that they appear to be attracted to them. Sometimes ingestion was assisted by 'pseudopodia'. Ingestion by amoebocytes is a comparatively slow process. The formation of a 'food-cup' or vacuoles preceding ingestion was never seen (Text-fig. 6 B).

Several investigators have observed similar directive movements of the amoebocytes; Drew observed the actual process of phagocytosis of the amoebocytes of Cardium, and stated that the amoebocytes can be seen to send out pseudopodia in the direction of bacteria and engulf them. Agglutinated corpuscles do not appear to possess this power, although motile bacteria in the course of their movements may touch and adhere to them; this is probably a purely passive action on the part of the corpuscles. Commandon (1919) observed that leucocytes of vertebrates are attracted by starch grains. Fenn (1920) found that carbon particles are ingested by leucocytes three or four times more readily than quartz particles of the same size. He also found directive movements of leucocytes towards manganese dioxide particles and stated that the ingestion of such a substance is not due to chance collision. In fact a leucocyte seemed obviously to be attracted, pseudopodia were put out, and one particle ingested.

On the other hand, the amoebocytes are not attracted to silver sand, although after a few minutes this is certainly ingested. This is probably due to chance collision.

When in contact with a glass surface the amoebocytes tend to spread out into a thin film, after which they degenerate. Tait and Ponder (1925) consider that the force which binds the cells to the glass is essentially the same as that which binds the cell to a particle of glass during the preliminary phases of ingestion. Such a force has probably no real connexion with their phagocytic powers as stated by Lison (1929) and Gray (1930).

(ii) In the Test-tube.

Suspensions of amoebocytes and particles of various kinds were kept in a small test-tube for some time, after which a sample was removed and examined on a glass slide. Carmine particles, starch grains, or carmine fibrin pieces are entirely ingested by amoebocytes. In this case the actual process of ingestion by amoebocytes cannot be directly observed. The chance of collision, and so ingestion, in such experiments must be considered in addition to the attraction of particles by amoebocytes.

The results of Yonge's experiments show that amoebocytes digest blood-corpuscles of fish within a comparatively short period after having ingested them. I followed for a long time the process of digestion in the amoebocytes which had ingested carmine fibrin or starch grains, but satisfactory results could not be obtained. The amoebocytes after ingesting particles tend to round up and move away (Text-fig. 6 A).

(iii) Injection.

Several kinds of material, such as carmine particles, Indian ink, starch grains, and olive-oil emulsions, were injected in the body and the oysters then left in an aquarium tank for a given length of time. In this case the amoebocytes become congregated at the place of injection and the particles are ingested. It is probable that the amoebocytes are attracted when foreign substances enter the body in the same way as phagocytes aggregate at foci of infection.

Cuénot observed phagocytosis in the blood-cells of several Lamellibranchs after the injection of Chinese ink. Canegallo (1924) by injecting olive oil stained with Sudan III into Unio, found that this was quickly taken in by the leucocytes. This method admirably demonstrates the ingestion power of the amoebocytes; some of them ingested so many carmine particles that they became red.

(iv) Injection in the Mantle Cavity.

The shells of the oyster, into the mantle cavity of which

carmine particles, olive-oil emulsions, &c., were injected, were clamped together to prevent the particles from floating away. After a certain number of hours the amoebocytes were collected from the mantle cavity and examined for signs of phagocytic action. The amoebocytes which wander into this cavity ingested particles, as was frequently observed in natural conditions. Some amoebocytes then passed into the epithelium.

The power of amoebocytes to ingest and digest solid particles is an essential part of the digestion process in the case of Lamellibranchs; ingestion also results in the elimination of waste or foreign particles from the body of the animal. As mentioned in previous experiments, the amoebocytes of the oyster appear to ingest anything that is captured, and there is no evidence of any selection, although ingestion may vary when experiments are made with several kinds of particles. These differences have previously been observed.

Some authors state that when particles are ingested by vertebrate leucocytes they are engulfed in vacuoles from the suspending medium. However, although I have followed the phagocytosis of a great number of particles of different kinds, in all cases they took place by the direct extension of the surface of the amoebocytes (membranous cytoplasm) over the particle. I have never observed ingestion in vacuoles. The membranous cytoplasm of the amoebocytes was in direct contact with the surface of the particle undergoing phagocytosis. Later the ingested particle was entirely enclosed in the endoplasm.

E. Intracellular Digestive Enzymes.

Several investigators have studied the enzyme in molluscan blood. Kobert (1903) found an amylase in the blood of *Octopus* and a zymase in the blood of *Aplysia*. Sellier (1906, 1901) recognized a lipase in the blood of *Helix*, and also in *Octopus* and *Sepia*. He also remarked that there is an enzyme which prevents the coagulation of milk. Heymann (1914) found what he called a blood pepsin in the oyster. Recently Sawano (1929) recorded the presence of amylase in the blood of oysters after this had been freed from amoebocytes. None of these

workers, however, investigated the digestive power of the phagocytes.

On the other hand, Cuénot (1891) named the granules of amoebocytes of invertebrates 'ferment albuminogène ou granules albuminogènes'. He used the word ferment because it was convenient and on the whole a sufficiently elastic term; he stated that these granules do not form an organized ferment like bacteria or yeast, nor a soluble ferment like a diastase or pepsin; it is only a ferment in a figurative sense. Finally, Yonge (1926) suggested, on the basis of his experiments on the phagocytic action of amoebocytes in the oyster, that 'the phagocytes must also possess powerful digestive enzymes of various kinds'. He identified the lipolytic enzymes in amoebocytes by feeding the oyster with olive-oil emulsions and observing the actual digestion of this and of blood-corpuscles of fish. Graham (1931) recently investigated the digestive enzymes of Ensis, and stated that experiments proved the presence of lipolytic and of amylotic enzymes in the mid-gut and rectum, but they are probably due to the phagocytes which are so numerous in these parts.

The following experiments were undertaken in the hope of determining the nature of the digestive enzymes in the amoebocytes.

The amoebocytes were collected in several ways; when oysters have been heated to about 32° C. in sea-water for some five hours, the amoebocytes may be extruded in numbers on the gill surface or mantle cavity (this phenomenon named 'bleeding' was first described by Orton (1921)). Sometimes another method is employed which was introduced by Yonge (1928) to induce bleeding; the mouths of oysters being plugged by covering the palps with wax held in place with plasticine, this in turn being secured by string tied tightly round the oyster. The oysters were prepared for 'bleeding' by leaving them in the aquarium tanks for one or two weeks, at the end of which period the mantle cavity often contained a great mass of leucocytes. The amoebocytes were collected and preserved in an ice box. After some days the large quantity of amoebocytes accumulated was examined for enzymes. Amoebocytes were also

collected directly from the mantle cavity where they are often present in considerable numbers.

In all experiments a water extract of the amoebocytes was used as a source of enzymes; the enzymes were obtained by grinding up the amoebocytes with silver sand, and then extracting with sea-water or distilled water. Throughout these experiments toluol was used as an antiseptic, and rigorous controls consisting of boiled extracts were set up.

Amoebocytes collected in the above-mentioned manner always contain some mucus. According to Gorka (1916) the gill mucus of Anodonta and Unio contains digestive enzymes capable of digesting polysaccharides, glucosides, and fat; and in the mucus of palps he also found a protease. But recently Yonge (1926) tested the mucus of the oyster and was unable to confirm this, although sometimes there was slight enzymatic action, on proteins and fats, which Yonge concluded was probably due to enzymes from the phagocytes.

(i) Action of Extracts of Amoebocytes on Carbohydrates.

The following table gives the results of the principal experi-

TABLE 1. ACTION OF EXTRACT OF AMOEBOCYTES ON CARBOHYDRATES.
Experiment. 2 c.cm. substrate solution + 2 c.cm. extract of amoebocytes.
Control. Ditto boiled.
Temperature. 32° C.

Substrate.	*Time.*	*Result.*	
		Experiment.	*Control.*
1. 1 per cent. starch	2 days	Strong reduction	Very slight reduction.
2. 0·5 per cent. starch	8 hours	Reduction	No reduction.
3. 1 per cent. glycogen	2 days	,,	,,
4. 0·5 per cent. glycogen	8 hours	,,	,,
5. 1 per cent. sucrose	2 days	Slight reduction	,,
6. ,, ,,	20 hours	,, ,,	,,
7. 1 per cent. salicine	2 days	Reduction	,,
8. ,, ,,	20 hours	Slight reduction	,,
9. 1 per cent. maltose	2 days	,, ,,	,,
10. 1 per cent. lactose	,,	,, ,,	,,
11. 1 per cent amygdaline	,,	Reduction	,,
12. ,, ,,	20 hours	Slight reduction	,,

ments on carbohydrates. Reduction was determined qualitatively by means of Fehling's solution except for the disacharides where Barfoed's solution was used.

Table 1 shows the presence of enzymes which can reduce starch, glycogen, maltose, lactose, and sucrose amongst carbohydrates, and also amygdaline and salicine. Action on starch and glycogen was particularly well-marked. A similar amylotic enzyme has been recognized by several authors in other parts of the body of the oyster such as the crystalline style and digestive diverticula.

It is interesting to note that the glucosides, amygdaline, and salicine are reduced by the extract of amoebocytes, though action is not so strong as on starch and glycogen. It is generally known that enzymes which split these glucosides are widely distributed in the plant kingdom, but do not occur in higher animals. But they have been identified in some invertebrates such as molluscs and crustacea; Yonge found such an enzyme in the digestive diverticula of the oyster. Giaja (1907) demonstrated the presence of one in the hepatopancreas of various decapod crustacea, and Yonge (1924) also found weak enzymes of this type in the hepatopancreas of Nephrops.

Finally, maltose, sucrose, and lactose are slightly reduced by extract of amoebocytes.

(ii) Optimum pH of Sucroclastic Enzymes.

The effect of a change of pH upon the activity of these enzymes is shown in Tables 2 and 3 and Text-fig. 7. The results obtained were uniform, but in all cases the optimum was not so sharply defined as in the case of the enzyme of the style; the efficacy of the enzyme does not decrease so rapidly on either side of the optimum point. As a substrate, a solution of 0·5 per cent. starch or glycogen free from reducing sugar was used. The sugar resulting from the digestion of the substrate was estimated by a modification of the Hagedorn and Jensen method devised by Boyland (1928). This method is satisfactory, as the final titration gives a sharp end-point and so allows accurate results to be obtained. The following mixtures were employed as buffer solutions.

1. Phthalate—NaOH . . . pH 4·0 5·6
2. KH_2PO_4—NaOH . . . pH 6·0 8·0
3. H_3BO_3—KCl . . . . pH 8·6 10·0

TABLE 2. THE EFFECT OF pH ON THE ACTION OF THE AMYLASE OF AMOEBOCYTES.

Experiment. 3 c.cm. buffer solution + 3 c.cm. 0·5 per cent. starch solution + 1 c.cm. extract of amoebocytes.
Duration. 30 hours.
Temperature. 28° C.

No.	*Initial* pH.	*Glucose in mg. in* 1 *c.cm.*
1	4·0	0·15
2	4·6	0·25
3	5·0	0·73
4	5·6	0·73
5	6·0	0·73
6	6·4	0·76
7	7·0	0·86
8	7·6	0·71
9	8·0	0·66

TABLE 3. THE EFFECT OF pH ON THE ACTION OF THE GLYCOGENASE OF AMOEBOCYTES.

Experiment. 3 c.cm. buffer solution + 3 c.cm. 0·5 per cent. glycogen solution + 1 c.cm. extract of amoebocytes.
Duration. 30 hours.
Temperature. 28° C.

No.	*Initial* pH.	*Glucose in mg. in* 1 *c.cm.*
1	4	0·18
2	5	0·21
3	6	0·58
4	7	0·60
5	8	0·53
6	9	0·28

The optima of the amylase and glycogenase (which may be the same enzyme) lie at about pH 7·0.

The optimum hydrogen ion concentration for an enzyme reaction often depends upon the type of buffer solution used. According to Michaelis (1912) the optimum reaction for diastase

from saliva lies at pH 6·1 to 6·2 in phosphate acetate or sulphate mixtures, but at pH 6·9 in chloride and nitrate mixtures. These differences may be due to the stimulating or inhibiting action of the salts of which the buffer solution is composed.

According to Yonge (1926), starch and glycogen are the only substances acted upon by the enzyme of the crystalline style. There is no action on amygdaline, salicine, maltose, lactose, and sucrose. The optimum pH of the amylase is sharply defined and lies at about 5·9; the efficacy of the enzyme is rapidly reduced, particularly on the acid side. On the other hand, in the enzymes of the digestive diverticula of the oyster he recognized the presence of amylase, glycogenase, maltase, lactase, sucrase, and enzymes which act on amygdaline and salicine. These enzymes are very similar to the enzymes of amoebocytes; but appear to act in a more acid medium. (Optimum pH of the amylase of the digestive diverticula is about pH 5·5.) It appears, therefore, that the amylolytic and glycogenolytic enzymes of the amoebocytes are distinct from those of the crystalline style and digestive diverticula, the optimum pH being about 7·0, and so more on the alkaline side than the other amylases (Text-fig. 7).

(iii) Graham (1931) studied the relation between the optimum pH and the duration of the experiment in the amylolytic enzyme of the crystalline style of Pecten; he stated that 'there is no variations in the pH optimum with variation in the time of the experiment'. Similar experiments were carried out with the amylolytic enzymes from the amoebocytes as shown in Table 4, and similar results were obtained.

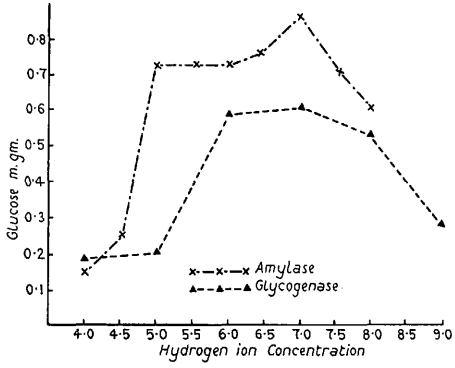
(iv) Lipoclastic Enzymes.

Lipoclastic enzymes of the oyster have been recognized by several investigators; Yonge (1926) found traces in the stomach contents of the oyster, but considered that this originates in the phagocytes, of which many are always present in the stomach. He also recognized the presence of this enzyme in the digestive diverticula of the oyster and stated that 'Since fat is taken in freely by the phagocytes and there digested, very little appearing in the digestive diverticula, there is probably no

TABLE 4.

Experiment. 3 c.cm. buffer solution + 3 c.cm. 0·5 per cent. starch solution + 1 c.cm. extract of amoebocytes.
Temperature. 28° C.

		Glucose in mg. in 1 *c.cm.*			
No.	*Initial* pH.	*Duration:*	24 *hours.*	30 *hours.*	54 *hours.*
1	4·0	..	0·03	0·15	0·18
2	4·6	..	0·12	0·24	0·89
3	5·0	..	0·25	0·72	2·00
4	5·6	..	0·42	0·73	2·01
5	6·0	..	0·43	0·73	2·20
6	6·4	..	0·44	0·76	2·30
7	7·0	..	0·49	0·86	2·49
8	7·6	..	0·45	0·71	2·02
9	8·0	..	0·43	0·66	1·96



TEXT-FIG. 7.

Based on Tables 3 and 4, the ordinates indicating the glucose in mg. 1 c.cm., the abscissae the pH.

necessity for the presence of a powerful lipase in the digestive diverticula. Indeed, the slight lipoclastic action of the extract may be due, in part at any rate, to the phagocytes in the tissue extracted and not to enzymes from the actual absorptive tubules.' He actually demonstrated the presence of a lipoclastic

enzyme in the amoebocytes by feeding the oyster with an emulsion of olive oil stained red with Nile blue sulphate.

Several methods were employed for the detection of lipoclastic enzymes. At first an emulsion of olive oil stained with phenol red was used, but as the results were not satisfactory even after three weeks, methyl acetate was used in the later experiments, being finally titrated with sodium hydrate in the presence of phenolphthalein.

The results of typical experiments on the enzymes acting upon fat and ester in the amoebocytes are shown in Table 5.

TABLE 5.

No.	*Duration.*	*Result.*
1. 2 c.cm. extract of amoebocytes; 2 c.cm. olive-oil emulsion	7 days	Slightly yellow.
2. Ditto boiled	,,	Pink.
3. 3 c.cm. extract of amoebocytes; 2 c.cm. olive-oil emulsion	14 days	Slightly yellow.
4. Ditto boiled	,,	Pink.
5. 2 c.cm. extract of amoebocytes; 2 c.cm. 5 per cent. methyl acetate	7 days	Acidity in term of 0·01 N. NaOH 1·7 c.cm.
6. Ditto boiled	,,	0·8 c.cm.
7. 2 c.cm. extract of amoebocytes; 3 c.cm. 5 per cent. methyl acetate	5 days	1·5 c.cm.
8. Ditto boiled	,,	0·5 c.cm.

The results indicate a lipase and an esterase which are probably the same enzyme. This enzyme is not so strong in its action on olive oil. The action of the enzyme is slow and it probably occurs in very small quantities. On the other hand, as mentioned in the feeding experiment, the colour change of the olive-oil emulsion stained with Nile blue sulphate is very distinct.

(v) Proteoclastic Enzymes.

According to Yonge (1926), there are two optima for the

proteoclastic enzymes in digestive diverticula; one at a pH of about 3·7 and the other at or above pH 9·0; there is no action at the normal pH of the tissue extract (pH 5·4–6·0), though action takes place at different degrees of alkalinity. He suggested from the above results that one enzyme may come from the digestive diverticula and the other from the phagocytes.

Several experiments were carried out in order to determine the presence of proteoclastic enzymes in the amoebocytes, though on account of the weakness of the enzymes this was not easy.

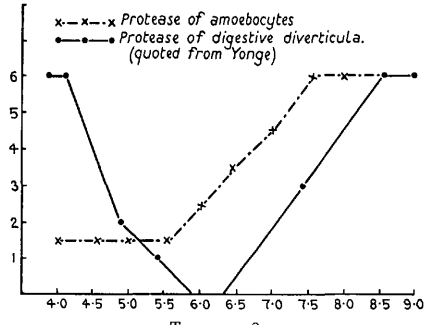
(*a*) Dernby's methods.

The method is briefly as follows: a 10 per cent. solution of gelatine was prepared and a series of experiments performed with extract of amoebocytes at different pH. At stated intervals measured portions (about 5 c.cm.) of the digests were removed. The progress of digestion was determined by placing the digests in an ice box for fifteen minutes, and then observing the degree of liquefaction. Dernby expresses the approximate degree of digestion by means of the following scale of seven numbers. 0 = Completely solid. 1 = Solid, but small pieces may be torn off by strong shaking. 2 = Solid, but the surface moves somewhat when tubes are shaken. 3 = Soft. 4 = Half liquid. 5 = Almost liquid. 6 = Entirely liquid.

TABLE 6. RECORDS THE RESULTS OF A TYPICAL SERIES OF SUCH EXPERIMENTS.

			Degree of Liquefaction at 32° C. for a given Number of Days.				
No.	*Initial* pH.	*Control.*	1.	2.	3.	4.	5.
1	4·0	0	1–2	1–2	1–2	1–2	1–2
2	4·6	0	1–2	1–2	1–2	1–2	1–2
3	5·0	0	0–1	0–1	0–1	0–1	1–2
4	5·6	0	0–1	0–1	0–1	0–1	1–2
5	6·0	0	1–2	2–3	2–3	2–3	2–3
6	6·4	0	1–2	3–4	3–4	3–4	3–4
7	7·0	0	2	3–4	3–4	3–4	4–5
8	7·6	0	2	4	4–5	5	6
9	8·0	0	2	5	5–6	6	6
10	9·0	0	5–6	6	6	6	6

It will be observed that digestion occurred only in alkaline media. It is interesting to note that, unlike proteoclastic enzymes of the digestive diverticula, there is only one optimum. This nearly corresponds with one of the optima of the digestive diverticula and so supports the suggestion of Yonge already mentioned (Text-fig. 8).



TEXT-FIG. 8.

Based on Table 6, the ordinates indicating the degree of liquefaction, the abscissae the pH.

(*b*) Digestion of peptone.

In order to determine whether the same condition prevailed, when peptone was used as a substrate an experiment was carried out to determine the production of amino acid at various pH. A 1 per cent. peptone solution was used as a substrate, and for the detection of amino acid Sörensens formaldehyde titration method was employed. The results are shown in Table 7.

As it will be seen in Text-fig. 9 essentially similar results to those already recorded were obtained by this method (Text-fig. 8).

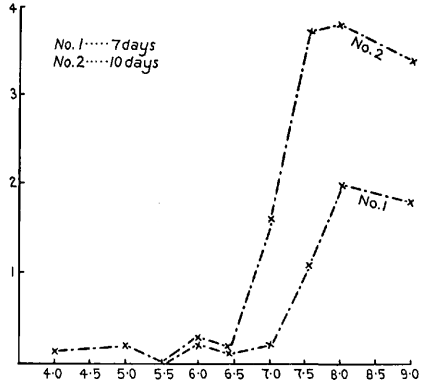
(*c*) Digestion of casein.

As shown in Table 8, the action of the enzymes on casein is comparatively slight, the pH optimum being obtained on the alkaline side.

TABLE 7.

Experiment. 1 c.cm. extract of amoebocytes + 3 c.cm. peptone (1 per cent.) + 3 c.cm. buffer solution.
Control. Ditto boiled. Temperature. 32° C.

		$\frac{N}{100}$ *c.cm. of* NaOH *titration:*					
		After 7 days.			*After 10 days.*		
No. of tubes.	*Initial* pH.	*Cont.*	*Exp.*	*Diff.*	*Cont.*	*Exp.*	*Diff.*
1	4·0	5·4	5·4	0	7·0	7·1	0·1
2	5·0	4·5	4·5	0	5·8	6·0	0·2
3	5·6	3·9	3·9	0	5·2	5·2	0
4	6·0	5·2	5·4	0·2	7·2	7·5	0·3
5	6·4	5·2	5·3	0·1	7·0	7·1	0·1
6	7·0	4·8	5·0	0·2	4·5	6·1	1·6
7	7·6	3·9	5·0	1·1	2·0	5·7	3·7
8	8·0	2·7	4·7	2·0	1·9	5·7	3·8
9	9·0	3·2	5·0	1·8	2·0	5·4	3·4



TEXT-FIG. 9.
Based on Table 7, the ordinates indicating the c.cm. of $\frac{N}{100}$ NaOH, the abscissae the pH.

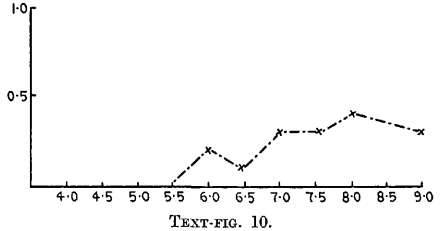
F. Presence of Oxidases.

Jatzenko (1928) observed the reduction of indigo carmine by

TABLE 8.

Experiment. 1 c.cm. extract of amoebocytes + 3 c.cm. buffer solution + 3 c.cm. casein (1 per cent.).
Control. Ditto boiled.

No. of Tube.	*Initial* pH.	$\frac{N}{100}$ *c.cm. of* NaOH: *after 7 days at 32° C.*		
		Cont.	*Exp.*	*Diff.*
1	4·0	7·8	7·8	0
2	5·0	4·6	4·6	0
3	5·6	1·4	1·4	0
4	6·0	3·5	3·7	0·2
5	6·4	5·9	6·0	0·1
6	7·0	4·7	5·0	0·3
7	7·6	3·0	3·3	0·3
8	8·0	2·6	3·0	0·4
9	9·0	2·2	2·5	0·3



TEXT-FIG. 10.

Based on Table 8, the ordinates indicating the c.cm. of $\frac{N}{100}$ NaOH, the abscissae the pH.

the amoebocytes of Anodonta, and concluded that they have the power of taking up oxygen. According to Koch (1917), the blood of Anodonta contains haemocyanin which Jatzenko concluded from his own experiments must be contained in the amoebocytes and not in the plasma.

(i) Indigo Carmine Reduction.

The reduction of indigo carmine by the amoebocytes of the oyster was not rapid and varied considerably.

Experimental solutions, made with 1 c.cm. of pure indigo

carmine (saturated) mixed with 10 c.cm. of clean filtered sea-water. Oxygen was first removed as much as possible from these solutions by a vacuum pump, and then an extract of amoebocytes or minced living amoebocytes was added. Then a thick layer of liquid paraffin was added.

The blue colour of indigo carmine gradually became green and then faintly yellow, finally becoming colourless. These reactions took place very gradually, the process being complete in about twenty-four hours at ordinary room temperature (20° C.). Controls were run either without amoebocytes or with boiled amoebocytes. These solutions did not reduce the indigo carmine within twenty-four hours. (The boiled amoebocyte extracts showed a slight reduction.) The minced tissues of the mantle showed no distinguishable reduction.

This reduced indigo carmine did not become oxidized when air was admitted to this solution. The reduction was therefore irreversible. On the other hand, a similar experiment using haemocyanin from crabs was readily reversible, oxidation occurring when air was admitted. The reduction of indigo carmine by haemocyanin took place even after the blood containing it had been coagulated by heat.

(ii) Indophenol Reaction.

The indophenol reaction was employed to test for oxidases in the amoebocytes, and positive results in the absence of H_2O_2 were obtained. In a few minutes the solutions containing the extract of amoebocytes become deep purple, other tissues such as minced mantle or labial palps become lighter purple in about twenty minutes. Minced gills gave as rapid reaction as did the extract of amoebocytes. This is probably due to the great number of amoebocytes in the gills. The reaction did not seem to be affected by high temperatures, for activity did not cease after the extract was boiled.

(iii) Guaiacum Reaction.

The reaction of the amoebocytes on guaiacum was very slow and after a day very slight traces of activity were found in the absence of H_2O_2. In the presence of H_2O_2 the reaction was

more vigorous, but not so well marked as with the indophenol reaction.

There has been much discussion on the existence of oxidase or peroxidase in the blood of molluscs. Portier and Pieri (1897) found that the reaction of guaiacum on the blood of certain Lamellibranchs produces a blue colour. They also found that an extract of the gills of the oyster was not acted upon by guaiacum with absence of H_2O_2. Subsequently Alsberg (1908) obtained the same results, with the body fluid of the oyster; the reaction was slight, and the results varied in different individuals. He considered that this variation was influenced by the copper content in different individuals. Yonge (1926) also demonstrated that the guaiacum reaction took place very slowly in certain tissues of the oyster.

The results of these experiments on the oyster do not confirm the conclusions of Jatzenko. There is no evidence that haemocyanin is present in the amoebocytes, for the reduction of indigo carmine is apparently due to an oxidase and not to a blood pigment.

G. Absorption.

Absorption in the alimentary canal of the oyster has been followed by Vonk (1924) and Yonge (1926) in detail. Soluble matter such as iron saccharate is absorbed exclusively in the cells of the digestive diverticula (Yonge). Fine particles such as carmine or Indian ink are also taken in phagocytically by the cells of the digestive diverticula (Vonk and Yonge in Ostrea, List in Mytilus), but all larger particles such as droplets of oil, blood-corpuscles, or even such small diatoms as Nitzschia, according to Yonge, are ingested by phagocytes. These are everywhere abundant in the mantle cavity and the gut, but particularly in the stomach, ducts of the digestive diverticula, and in the mid-gut. Particles of any size very rarely enter the tubules of the digestive diverticula, those that enter the ducts being there seized by phagocytes, part of the products of digestion being passed into the cells of the epithelium, and the remainder carried to the vesicular connective tissue. Yonge states emphatically from his experimental results that 'No

evidence of any absorption in the epithelium of the gut or of any free surface in the mantle cavity, other than by the agency of phagocytes, was found'.

1. Absorption in Alimentary Canal.

The actual process of absorption in the alimentary canal (including the digestive diverticula) was observed after animals had been fed with various substances such as Indian ink, carmine particles, and olive-oil emulsions, the animals having been previously starved. Various regions of the body were later fixed with Bouin's fluid or 5 per cent. formalin, paraffin or frozen sections being later prepared.

(i) Stomach and Mid-gut.

In oysters opened within one day of feeding, the stomach and mid-gut were full of these substances. In the lumen of the stomach there were a great number of amoebocytes, most of them with ingested particles. Portions of the epithelium of the stomach cleared in glycerine, or after being fixed and sectioned, showed that amoebocytes laden with particles were passing through it. Sometimes a small number of particles not contained in amoebocytes were found in this region. These particles are probably carried in by amoebocytes and not directly absorbed by the epithelium of the stomach itself.

It is impossible, as Yonge has noted, for carmine or Indian ink to be absorbed by the epithelium of the stomach and mid-gut directly, other than by the agency of amoebocytes.

Olive-oil droplets stained red with Nile blue sulphate and ingested by amoebocytes turned a blue colour, but all oil droplets lying free in the lumen in the stomach and mid-gut retained the original red colour. The former is due to the influence of the lipoclastic enzymes in the amoebocytes. The latter provides evidence of the absence of free lipoclastic enzymes in the lumen of the stomach as noted by Yonge in the oyster.

No actual absorption of olive-oil emulsions was found in the ciliated epithelium of stomach or mid-gut other than by the agency of amoebocytes.

(ii) Digestive Diverticula.

Carmine particles and Indian ink are ingested phagocytically by the cells of digestive diverticula. The structure and function of this organ was investigated by Yonge in detail; in brief, the digestive diverticula consist of a brownish mass of blind tubules which surround the stomach and communicates with it by means of two large ducts. These ducts possess cilia and the lumen is irregular owing to variation in height of the epithelium. When examined in sections, cilia are never seen in the tubules. The outline of the cells is indefinite, and their free surface is irregular. They possess phagocytic powers. This organ has been called 'liver', 'hepatopancreas', or 'digestive gland'; but recently Yonge (1926) showed that it is an organ of assimilation and of intracellular digestion with none of the functions of a true liver or pancreas. He therefore suggested that it would be more suitably termed 'digestive diverticula'.

Carmine particles and Indian ink are taken in as round masses in the cells of the tubules and not in a diffuse condition which, according to Yonge, occurs in animals, such as Arthropods and Annelids, in which digestion is extracellular.

After feeding, a small number of droplets of olive oil were observed in the cells of the digestive diverticula, the colour of the Nile blue sulphate in some cases having turned blue as a result of digestion and of the consequent formation of fatty acids.

(iii) Rectum.

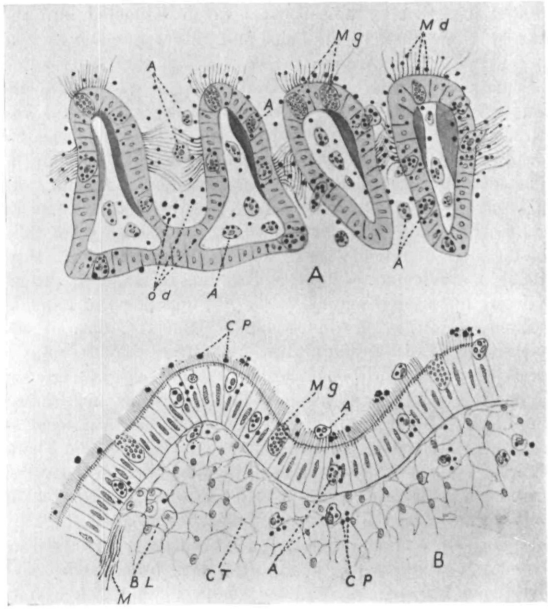
In the lumen of the rectum there were also many amoebocytes, some of which contained ingested particles, but none of these was seen in the epithelium.

According to my observations, the principal centre of phagocytic activity of the amoebocytes in the gut of the oyster is situated in the stomach and ducts of the digestive diverticula. This conclusion agrees with the finding of Yonge in various Lamellibranchs.

2. Absorption of Substances from the Body Surface (Text-fig. 11 A, B).

The amoebocytes in the mantle cavity ingested particles of carmine or Indian ink, but the degree of ingestion is not great

even after long periods (twenty hours to about one month). Those amoebocytes which did ingest particles passed into the



TEXT-FIG. 11.

A. Absorption of olive-oil emulsion stained red with Nile blue sulphate by amoebocytes on the surface of the gills. ×450. Freezing method. B. Absorption of carmine particles by amoebocytes on the surface of the labial palps (inner face). ×450. *A.*, amoebocytes; *B.L.*, blood lacunae; *C.T.*, connective tissue; *C.P.*, carmine particle; *M.*, muscle; *M.g.*, mucus gland; *o.d.*, olive-oil droplet.

tissues. After these had been fixed with Bouin's fluid and then sectioned, wandering amoebocytes with ingested particles were found in various tissues of the mantle cavity. These amoebocytes

probably come directly from the mantle cavity where they have ingested the particles. The experiments with olive-oil emulsion stained red with Nile blue sulphate or Sudan III also showed ingestion by amoebocytes which wandered into the tissues. Some parts of the palps and gills were coloured blue as noted already by Yonge.

Sometimes a little carmine, Indian ink, or oil droplets undoubtedly appeared in the epithelium of the gills, palps, &c., after animals had been in a suspension of these particles for several weeks or more. According to Churchill, this may be effected by phagocytic action of the cells. List and Vonk state that the appearance of these particles in the epithelia may be due to the action of leucocytes which had carried them from the digestive diverticula where they are freely absorbed. Hatt (1926), however, states that Indian ink is taken in phagocytically by the epithelium of the gills and palps of Lamellibranchs. This is very questionable; the structure of the epithelial cells of the palps, gills, and mantle provides no evidence of phagocytic behaviour as do the cells of digestive diverticula. The particles appear in these tissues in a diffuse condition, and not accumulated in a round or oval form as seen in the cells of the digestive diverticula. Everything I have observed agrees with the statement of Yonge that in the oyster there is no evidence of direct ingestion of these particles by the body surface and that they are probably deposited there by the phagocytes which either had absorbed them directly from the mantle cavity, or had transported them from the digestive diverticula. An experiment was conducted in which oysters were kept in sea-water containing 0·5 per cent. glucose for five days. The amoebocytes were collected from the mantle cavity and analysed for the presence of sugar by Boyland's method.

Quantity of Sugar in Amoebocytes.

	per cent.	
Experiment . .	4·4–5·5	3 individuals
Control . . .	2·2–3·5	,,

It appears from the above experiments that the amoebocytes

readily absorb glucose. This must be greatly facilitated by the very large surface in proportion to the volume.

H. Role of Amoebocytes in Excretion.

The amoebocytes of the oyster take part in excretion as well as in assimilation. The final destination of carmine particles was observed after having been injected into the body and the animal subsequently left in an aquarium tank for a given length of time. The carmine particles were ingested by the amoebocytes and carried to various regions of the body. The final destination of these particles is either in the excretory tubules or in the lumen of the rectum, but the presence of amoebocytes containing ingested carmine was observed in the pericardial epithelium, mantle cavity, and gono-ducts.

(i) In the Excretory Organ (Text-fig. 12 A).

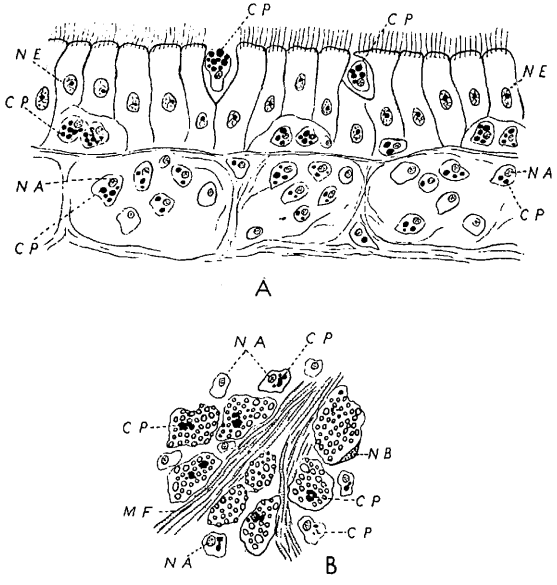
The amoebocytes containing ingested carmine were observed at several levels in the ciliated epithelium of the excretory tubes about three days after carmine had been injected, but free particles were never observed in the cells of this epithelium. The actual discharge of the carmine particles of the amoebocytes takes place directly, and not by way of the epithelial cells of the excretory organ which never received the particles from the amoebocytes.

(ii) Pericardial Epithelium.

The pericardial epithelium plays an important part in the elimination of waste matter. Many amoebocytes containing carmine particles were found in this region. Some particles are actually taken in by the brownish granular cells. Probably the amoebocytes directly discharge their particles into the pericardial cavity from this region, but some particles are discharged indirectly by way of the granular cells which received the particles from the amoebocytes. The brownish granular cells are amoeboid and behave phagocytically. The same mechanism was observed in the surface of the auricles of the heart (Text-fig. 11 B).

About two weeks after the particles are injected into the body

they are found in the pericardial cavity. These particles may, perhaps, be discharged from the pericardial epithelium and



TEXT-FIG. 12.

A. Amoebocytes in the epithelium of nephridial tube ingesting carmine particles about three days after these were injected. ×650. *N.A.*, nucleus of amoebocytes; *C.P.*, carmine particles in amoebocytes; *N.E.*, nucleus of epithelial cell. B. Amoebocytes and brown cells in the auricle ingesting carmine particles about three days after these were injected. ×650. *N.A.*, nucleus of amoebocyte; *N.B.*, nucleus of brown cell; *C.P.*, carmine particles; *M.F.*, muscle-fibre.

auricle surface, either directly from the amoebocytes or, together with other concretions, by way of the brownish granular cells. No amoebocytes were found in this cavity.

(iii) In the Gonoducts.

Many amoebocytes which had ingested carmine were found in the gono ducts near the excretory tubules. The reason for the occurrence of the particles in this region is not yet known.

The opening of the excretory organ of the oyster is in the same region as the genital pore, but actually it is situated a little behind this. True communication does not exist between these two openings (Hoek). The particles ingested by amoebocytes found in the gono ducts are directly discharged through these ducts.

(iv) In the Rectum.

About one week after injection free carmine particles were found in the lumen of the rectum. In the epithelium there were many amoebocytes which contained ingested carmine particles, and it is reasonable to assume that the particles in the lumen had been discharged from such amoebocytes.

(v) In the Mantle Cavity and the Blood-vessels.

Amoebocytes with ingested carmine particles were found in the mantle cavity, providing evidence that the ingested particles may be discharged here.

Amoebocytes with ingested carmine are, of course, numerous in the blood-stream before migrating to the regions described above.

4. Summary.

1. There are two kinds of corpuscles in the blood of the oyster; one consists of granular, the other of hyaline amoebocytes.

2. The granular amoebocytes are amoeboid, though the speed of their movement is slow.

3. The granules are yellow or yellowish green in the fresh condition.

4. The granules are neutrophil with a tendency to become stained by the basic dyes intra vitam.

5. Granules can never be distinguished in fixed and stained amoebocytes.

6. The amoebocytes are distributed everywhere throughout

the body, an especially large number being normally present around the gut.

7. The so-called 'pseudopodia' of the amoebocytes are described and their nature discussed.

8. The effects of several kinds of reagents on amoeboid movement and on the amoebocytes are described.

9. The amoebocytes become entangled with one another by bristle-like 'pseudopodia', or by elongated strands of hyaline ectoplasm outside the body. There is no true coagulation of the blood, and no fibrin production.

10. The mechanism of phagocytosis is described and discussed.

11. Sucroclastic, lipoclastic, and proteoclastic enzymes are present in the amoebocytes and enable the amoebocytes to digest intracelluarly.

12. The optimum action of the amylase is about pH 7·0 and of the proteoclastic enzymes about pH 8·0. These optima are not very well marked.

13. A complete oxidase system is present, revealed by the indophenol reaction, by the power of reducing indigo carmine, a process which is irreversible, and by a slight reaction with guaiacum.

14. The amoebocytes can absorb glucose both in the gut and the mantle cavity.

15. There is no evidence of absorption of soluble matter nor of solid substances by the epithelium of the mantle cavity, e.g. gills, labial palps, &c., other than by the agency of amoebocytes.

16. The amoebocytes play a prominent part in excretion. They reject directly foreign or indigestible matter by way of the epithelium of the excretory organ, pericardium, surface of the auricle, rectum, and mantle cavity.

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