Thesis Submitted for the Degree of

Doctor of Philosophy

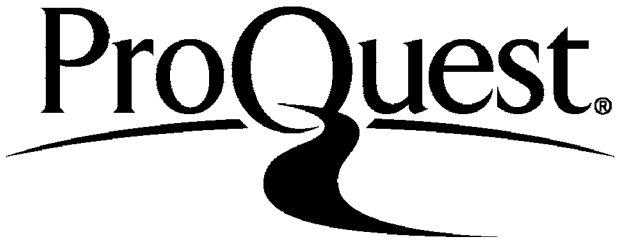
May 1964

STUDIES ON THE COELOMIC CELLS AND HAEMAL SYSTEM OF SOME ECHINOIDS

By

Margaret Pauline Mary Sargeant

Bedford College, University of London



STUDIES ON THE COELOMIC CELLS AND HAEMAL SYSTEM OF SOME ECHINOIDS

ABSTRACT

The investigation was based on a study of the fine structure, locomotion and function of the perivisceral coelomic cells of Diadema antillarum (Philippi) and Psammechinus miliaris (Gmelin).

The flagellated cells are probably not parasitic and may be unique amongst metazoan cells. The phagocytic bladder amoebocytes effect coagulation, first becoming filiform, then elongating to entangle cells in a clot. Inhibition of coagulation must be cautiously interpreted.

Vapour fixation and a new method of identifying fixed, stained cells were utilised with histochemical tests to investigate the composition of the inclusions of the spherule amoebocytes. It was concluded that the red spherules are not associated with appreciable amounts of protein or lipid, contrary to previous opinion. These spherules are however associated with carbohydrate, probably a sulphated mucopolysaccharide.

The often-quoted statement that the colourless spherules are 'albuminous' is now substantiated. Adam's (1957) test for indoles and chromatography of extraxts indicated that tryptophan might be an important constituent. There is an apparent correlation between reduction of the indole content of the colourless

spherules of Psammechinus miliaris and the onset of gonad maturation.

It was concluded from a re-examination of Stott's, (1955), work that absorption from the stomach lumen by amoebocytes has not been demonstrated, and probably does not occur.

The haemal system has been investigated. Existing accounts, in both original and text-book versions, are significantly different from what was observed in Echinus esculentus (Linnaeus) and Psammechinus miliaris, (Gmelin). In these species the radial strands could not be detected and the aboral haemal area was ill-defined. However, contrary to recent statements, there are contractile haemal vessels round the stomach, and there is considerable fluid movement within these vessels, which may comprise a circulatory system.

Preliminary experiments indicate that these vessels may be concerned in the initial absorption of material from the stomach lumen.

STUDIES ON THE COELOMIC CELLS AND HAEMAL SYSTEM OF SOME ECHINOIDS

CONTENTS

	PAGE
INTRODUCTION	6
THE COELOMIC CELLS	
1.	
Material and methods	10
The types of cell	16
The cytology and locomotion of the perivisceral coelomic cells	22
The distribution, incidence and origin of the coelomic cells	67
2. Functions of the coelomic cells	
Phagocytosis and coagulation	94
The spherule amoebocytes:	118
Composition of the inclusions,	
Pigments	120
Other constituents	132
Variation in the indolic content of the colourless inclusions of P.miliaris	173
Digestion and Transport	190

CONTENTS con.

	PAGE
THE HAEMAL SYSTEM	210
3.	
Introduction and historical	211
The anatomy of the haemal systems of P.miliaris and E.esculentus	218
Contractions of the haemal vessels, flow and circulation	227
The function of the haemal vessels, experiments on absorption	241
DISCUSSION	248
ACKNOWLEDGEMENTS	255
SUMMARY	256
APPENDIX	261
REFERENCES	263

INTRODUCTION

The coelomic cells of echinoids were first observed by Valentin, (I843) and Erdl, (I843), and were first described in detail by Geddes, (I880). Since that time these cells have been the subject of at least 37 papers, which have been principally confined to descriptions of the cell-types in various species, (with some confusion of nomenclature), and preliminary investigations of coagulation.

Quite a large proportion of the volume of a regular echinoid is occupied by coelomic spaces. The main coelomic spaces are the perivisceral and peripharyngeal coeloms, the water vascular and the perihaemal systems. The perivisceral coelom alone occupies 40-50% of the total test volume of a small echinoid of 2-3 cm., diameter, and about 70-80% in a large echinoid, of about I2 cm., diameter. All these coelomic spaces contain fluid in which the coelomic cells are suspended. Some of these cells are actively amoeboid and are therefore not confined to the coelom, but are found throughout the echinoid. Their ubiquity has aroused considerable interest, as has the possible function of these and the other coelomic cells.

Although various important functions, besides coagulation, and phagocytosis of 'foreign' material, which is known to occur, have been attributed to the

coelomic cells, none have been conclusively demonstrated.

Cuénot, (I89Ia), thought that some of the coelomic cells of the echinoids he studied were 'organes de reserve'; 'les produits de réserve sont evidemment accumulés à l'intérieur d'amibocytes'. This suggestion has been reiterated by many subsequent authors, and Liebman (I950) presented rather scanty evidence, rejected by Hyman (I955), that these cells transfer nutritive material to developing ova in Arbacia punctulata. The accompanying idea that the coelomic cells are involved in active transport of nutritive substances has been extended by the concept that these cells actually participate in intracellular digestion and absorption within the gut lumen, Stott (I955). Conversely it was tentatively suggested, Oomen (I926), that some of the coelomic cells might provide extracellular digestive enzymes, but this was refuted by Stott (I955), who stated that the site of extracellular enzyme secretion is the stomach epithelium.

Several authors have found that coelomic cells migrate to the exterior with effete material; Hyman (I955) believes that the coelomic cells may have a definite excretory function, with the axial organ a 'way-station' en route to the exterior via the stone-canal. Again there is no evidence that this is so.

Some of the coelomic cells are pigmented and are concerned in melanogenesis, Jacobson and Millott (I953), and may deposit pigment in the body wall, and there are

signs of disintegration in the axial organ, Millott (personal communication). It was early suggested, MacMunn (I885), that the pigments of the coelomic cells have a respiratory function, but this has never been substantiated for echinoids.

Finally, there is some evidence that besides coagulating at the site of injury, coelomic cells may be responsible for scleroblastic reconstitution, Kindred, (I924).

It was hoped that the present investigation would further indicate the functional significance of these cells, which have been the subject of so much interesting conjecture.

This investigation was necessarily based on a cytological study of the cells found in the perivisceral coelom of specific echinoids, principally Diadema antillarum (Philippi) and Psammechinus miliaris (Gmelin); Part I, pp. 10-67

The composition of the inclusions of the coelomic cells is obviously of considerable interest, and the histochemistry of these inclusions was therefore studied; Part 2, pp. 132-173. Previous work on the composition of the inclusions is both scant and confused, probably due to inaccurate differentiation of the different cell-types after fixation and staining. It was found possible to surmount this difficulty and to come to some conclusions as to the principle constituents of the inclusions of the coelomic amoebocytes.

The contents of the inclusions of one type of amoebocyte appears to become reduced at the breeding season of Psammechinus miliaris. Tests for gonad ripeness and the content of the inclusions were therefore performed on 280 animals at regular intervals over I2 months, to discover if these cells release materials to the ripening gonads, as originally suggested for echinoids by Liebman (I950), and as occurs with some polychaetes, Dales (I96I). Part 2, pp. 173-190.

Stott (I955) suggested that echinoid coelomic cells are directly responsible for absorption of nutrients, which they subsequently distribute in their passage through the haemal system. This prompted a re-investigation of the role of the coelomic cells in absorption and transport, Part 2, pp. 190-210. Psammechinus miliaris proved to be a good laboratory animal and was used for these particular studies.

The preliminary investigation into the anatomy and the function of the haemal system, Part 3, pp. 210-247, arose from the study of absorption and transport of nutrients, from which it became evident that existing accounts, in both original and text-books versions, were significantly different from what was observed in the haemal vessels associated with the gut.

MATERIAL AND METHODS

Species studied

Coelomic cells from the following species have been studied;

Regular echinoids

Psammechinus miliaris (Gmelin)
Echinus esculentus (Linnaeus)
Paracentrotus lividus (Lamarck)
Diadema antillarum (Philippi)
Arbacia lixula (Linnaeus)

Irregular echinoids

Spatangus purpureus (Müller)
Echinocardium cordatum (Pennant)
Brissopsis lyrifera (Forbes)

Cells for detailed studies were obtained initially from Diadema antillarum from the Zoological Society of London Aquarium, and later from Psammechinus miliaris. The latter animals were obtained from Millport Marine Laboratory, on the Firth of Clyde. They were kept in polythene or glass tanks, preferably at 15-17° C., and either supplied with circulating filtered sea water, or with fresh sea water every 2 or 3 days. The tanks were aerated, and the animals fed readily on brown algae, e.g. Fucus, stripping the fronds and leaving the bare stipe. They would not feed on animal protein such as fresh

mussels or minced ox heart, but would occasionally show cannibalism, attacking and apparently feeding on sickly companions, which were stripped of skin and internal organs in some cases, see also comments on cannibalism, Hancock, (I957).

Once settled in after arrival these animals were kept for up to 3 months and would probably have survived for much longer.

Method of obtaining coelomic cells

Coelomic fluid is readily obtained from regular echinoids by cutting the peristomium and allowing the fluid to drain into a suitable container, or by withdrawing it with a hypodermic syringe. The latter method is preferred because the urchins survive after such treatment. Fluid obtained by these methods is not from the perivisceral coelom alone; some will be from the peripharyngeal coelom. More selective withdrawal, by cutting through the test is likely to cause other contamination and destroys the animal. The contents of the peripharyngeal coelom can be selectively withdrawn from animals opened aborally, and examined separately.

Heart urchins do not have a soft peristomium and did not survive when any of the above methods were used. It is therefore best to open the test and remove directly all the coelomic fluid available.

FIXATION

Special methods of fixation were necessary to preserve the delicate structures of the cells.

Where it was necessary to use sections, in electron microscopy, freshly clotted cells were used, to give enough material for handling. It was considered essential however, to use fresh unclotted cells wherever possible, for the cytological and histochemical work, in case chemical change occurs during coagulation, and because the cells are easier to observe.

The cells are less numerous than vertebrate blood cells and smear techniques are unsatisfactory; moreover they cause distortion.

Liquid fixatives are not very satisfactory, principally because there is no penetration barrier and the sudden change causes marked distortion. In fact Bouin or Carnoy added to coelomic fluid causes cytolysis of at least one type of cell. It is also very difficult to attach sufficient cells to slides when using liquid fixatives, as also found by Endean, (1958), who found that many cells were lost when he poured fixatives onto a slide. He also observed that addition of Carnoy's fixative to a drop of coelomic fluid from Holothuria leucospilota cytolysed all the coelomic cells.

Vapour fixation overcomes these difficulties. Liebman, (1950), first realised this, and fixed cells with formalin vapour in a Petri dish. He then allowed the cells to dry onto the slide. This technique is very satisfactory, and can be usefully modified for particular purposes. Vapour fixation was good with formalin, osmic

acid, acetic acid, and Carnoy. Osmium fixation was obtained by inverting the slide over the mouth of the 2% osmium teroxide bottle for about a minute. Osmium fixed slides were washed overnight in running water. Acetic acid and Carnoy fixation was completed in about IO minutes, and formalin fixation in I0-I5 minutes. The latter fixative was usually used for routine work.

Staining

Slides for general study were stained with Leishman's and Wright's blood stains, Haidenhain's iron haematoxylin and eosin, or with Mallory's connective tissue stain.

Staining with certain of the techniques, e.g. the blood and connective tissue stains was extremely variable, even when a standard procedure was employed. This will be discussed later, it may account for some of the discrepancies in the literature, see p. 132 et seq.

MICROSCOPY

The light microscope

Living and fixed cells were best observed under the highest power of the light microscope; with the oil-immersion objective. The phase-contrast microscope does not facilitate observation of the coelomic cells with dense inclusions but is helpful in aiding observation of the fine pseudopodia and the transparent peri-nuclear region of the bladder and filiform amoebocytes.

The electron microscope

The coelomic cells are too dense for successful whole mounts, as are used for examination of smaller organisms like bacteria, although the flagella could be visualised after osmium fixation.

Ultra-thin sections were therefore prepared for examination. Freshly withdrawn coelomic fluid was used, either in the freshly clotted state, or with the suspended cells centrifuged down to a fairly concentrated mass. The cells were fixed by Palade's method, (1952), with 1% buffered osmium tetroxide, which was found to be satisfactory and had the added advantage of distinguishing the echinochrome containing spherules of the red spherule amoebocytes from the colourless spherule amoebocytes because osmium is heavily deposited on the former, due to the reducing effect of echinochrome, see p.160. Sometimes however, deposition of osmium on these spherules and on the nuclear and cell boundaries was too intense, and obscured these regions. Luft's (1956) potassium permanganate fixative, which has proved very useful for plant and some animal tissues, was tested to see whether it gave a more delicate fixation of both membranes and spherules. Although fixation of the nuclei was satisfactory, the cytoplasm was very poorly fixed.

The most consistently satisfactory fixation was given by 20-30 mins fixation in 1% osmium tetroxide, buffered to 7.6 with veronal.

Fixed material was dehydrated in a graded series of ethanol-water mixtures, as recommended in 'Servall' (1959), treated in 50:50 methacrylate-ethanol, pure methacrylate, and finally, methacrylate containing the catalyst -

2% Luperco C.D.B. The material was embedded in the methacrylate, either pure butyl, or butyl with up to 20% methyl methacrylate added. Polymerisation was effected in gelatine capsules, at 60° C. by the Luperco.

Sections were cut on a converted Cambridge rocker microtome, using glass knives, floated on 20% ethanol, spread with xylol vapour, and mounted on formvar coated Athene (Sjöstrand) copper grids, protected on examination by a brass ring.

THE TYPES OF COELOMIC CELL

The many papers, in French, German and English, describing coelomic cells, have caused some confusion in nomenclature. I therefore propose to describe the cells I have found, naiming them in the most unambiguous and suitable way, agreeing substantially with the most recent publications of Boolootian and Giese (1958) and Boolootian, (1962). I cannot agree, however, with the name 'eleocyte' these authors use for the cells described below as 'red spherule amoebocytes', because it implies that they contain fat, (eleos = oil), an unwarranted assumption.

Cells I have obtained from the perivisceral coelom of all the species listed on p.10 may be divided into 3 morphological types:-

I. Cells 20-25 micra in diameter, with a central mass of cytoplasm, containing the nucleus and a variable number of inclusions surrounded by thin bladder-like or filiform ectoplasmic processes. These ectoplasmic processes show continual changes in from and are usually called pseudopodia, for this reason the cells are usually termed amoebocytes, although they do not show clear locomotory progression. In irregular echinoids these cells may have dark red or purple inclusions in the endoplasm. For reasons which will be evident later, the types with filiform and bladder pseudopodia will be considered together, and will be referred to as <u>bladder</u> and/or <u>filiform amoebocytes</u>.

2. Active amoeboid cells, up to 35 micra long when extended. They contain numerous round inclusions, which

move freely in the cytoplasm, as does the nucleus. In the urchins studied there are 3 distinct types of cell, with colourless, red or yellowish-green inclusions. The cells with green inclusions are found in irregular echinoids, and a few cells with green inclusions also occur in the coelomic fluid of Arbacia lixula but are not found in the other regular echinoids examined, apart from the spasmodic occurrence of one or two very dark green cells in samples of the coelomic fluid of Diadema antillarum.

These cells will be referred to as red, colourless and green spherule amoebocytes.

3. Small flagellated cells, the 'body' being 5-10 micra in diameter. These cells contain colourless inclusions.

They will be referred to as flagellated cells.

Previous classification and nomenclature

As already observed, there have been numerous descriptions of coelomic cells, It would be tedious and repetitious to review each paper in detail, and points of particular interest are discussed later in the text. The cell-types described and the nomenclature used by previous authors are therefore summarised in Tables IA,IB and 2. It has been possible to arrange the descriptions of the previous workers in the same 3 categories as those outlined above, with only a few exceptions. Such cells are grouped in the column headed 'other cells'.

Table IA classifies the descriptions of cells from the perivisceral coelom of regular echinoids, whereas Table IB classifies previous descriptions from irregular echinoids. Table 2 lists the nomenclature of certain coelomic cells

used in more specialised publications.

TABLE 1A

REGULAR ECHINOIDS

AUTHOR	DATE	SPECIES	TYPES OF COELOMIC BLADDER or FILIFORM A/CYTES	CELL DESCRIBED FLAGELLATED CELLS	SPHERULE AMOEBOCYTES RED	COLOURLESS	GREEN	OTHER CELLS
GEDDES	1880	Echinus esculentus Paracentrotus lividus	White corpuscles with filiform pseudopodia or complete rings	Ciliated cells	Cells pigmented 'en brun acajou'	White corpuscles with large granules		
PROUHO	1887	Cidaris cidaris	Cells with long pseudopods	Ciliated 'globules'	'Globules amoeboides en brun acajou'	Muriform corpuscles		
CUENOT	1891, 97	Cidaris cidaris Paracentrotus lividus Echinus acutus Psammechinus microtuberculatus Sphaerechinus granularis Echinocardium cordatum	Hyaline, phagocytic a/cytes	Vibratile 'globules'	'Amibocytes brun acajou'	Muriform corpuscles		
HENRI	1906	Paracentrotus lividus	A/cytes 'avec prolongements'	Spherical 'elements'	'Amibocytes brun acajou'	Muriform corpuscles		
KINDRED	1921, 24, 26	Arbacia punctulata Strongylocentrotus drobachiensis	Leucocytes with hyaline vacuoles	Vibratile corpuscles	Red spherule a/cytes	A/cytes with colourless spherules		
BOLIEK	1935	Lytechinus variegatus	Leucocytes	Vibratile corpuscles	Brown a/cytes	A/cytes with colourless spherules		
OHUYE	1936	Temnopleurus hardwickii	Hyaline cells; Compartmental cells; vesicular cells		Brown a/cytes	Fine & coarsely granular a/cytes		
KUHL	1937	Psammechinus miliaris	Leucocytes; Bladder cells	Flagellated cells	Red-brown a/cytes	Colourless a/cytes		
LIEBMAN	1950	Arbacia punctulata	Phagocytes; fibroblasts	Flagellated phagocytes	Red trephocytes	Colourless trephocytes	Green t/ctes	
SCHINKE	1950	Psammechinus miliaris	Leucocytes	Flagellated cells	Red a/cytes	White a/cytes		
KAWAGUTI & YAMASU	1954	Anthocidaris crassispina Pseudocentrotus depressus Hemicentrotus pulcherrimus Echinometra mathei Tripneustes gratilla Diadema setosum Echinothrix calamaris Temnopleurus toreumaticus Mespilia globulus Toxopneustes pileolus	Hyaline a/cytes Vesicular a/cytes Compartmental a/cytes Large vesicular cells		Red granular a/cytes	Granular a/cytes		
BOOLOOTIAN & GIESE	1958	Allocentrotus fragilis Strongylocentrotus purpuratus S.franciscanus	Bladder a/cytes Filiform a/cytes	Vibratile corpuscles	Eleocytes	Colourless spherule a/cytes		Hyaline haemocytes
BOOLOOTIAN	1962	Echinometra mathei E.oblonga E.vanbrunti Echinthrix diadema E.calamaris Echinostrephis aciculatus Heterocentrotus trigonarius H.mammillatus Diadema savignyi	Bladder a/cytes Filiform a/cytes	Vibratile corpuscles	Eleocytes	Colourless spherule a/cytes		Hyaline haemocytes Reniform cells in Echinometra mathei & E.oblonga only

TABLE IA REGULAR ECHINOIDS

TABLE 1B.

AUTHOR	DATE	SPECIES	TYPES OF COELOMIC	CELL DESCRIBED								
			BLADDER & FILIFORM A/CYTES	FLAGELLATED CELLS	SPHERULE AMOEBOCYTES							OTHER CELLS
					RED	COLOURLESSS*	GREEN	YG'	BG'	P'	YB'	
BEHRE	1932	Mellita quinquiesperforata	Compartment cells Cells with filiform pseudopodia	2 kinds, one small, one with bristle-like flagella	+	colourless berry-like amoeboid cells	+					
BOOKHAUT & GREENBURG	1940	Mellita quinquiesperforata	Cells with hyaline ectoplasm Cells with vesicular compartments		+	colourless spherule a/cytes					+	A/cytes with scattered brown particles
KAWAGUTI & YAMASU	1954	Echinocardium cordatum	Hyaline cells		+	COLOURLESS granular	+	-	+	-	-	
		Echinarachnius mirabilis	Vesicular cells		+	+	-	-	+	+	-	
		Peronella japonica	Compartmental cells		-	+	+	+	+	-	-	
		Clypeaster japonicus	Large vesicular cells		-	+	+	+	+	-	-	
		Astriclypeus manni			+	-	+	+	+	-	-	
		Lowenia elongata			+	-	-	-	+	-	-	
BOOLOOTIAN & GIESE	1958	Dendraster excentricus	Bladder a/cytes									Osmiophils Red corpuscles Large spherical corpuscles
BOOLOOTIAN	1962	Rhissus sp. Rhinobrissus sp.	Bladder a/cytes Filiform a/cytes	Flagellated cells								Red corpuscles

TABLE IB IRREGULAR ECHINOIDS

TABLE 2

NOMENCLATURE USED IN PUBLICATIONS NOT INCLUDING A REVIEW OF ALL CELL-TYPES

AUTHOR	DATE	SPECIES	TYPE OF CELL DESCRIBED, with equivalent in terminology, p. 16-17
FAURE-FREMIET	1927	Paracentrotus lividus	'amoebocytes vesiculeux' = bladder amoebocytes
DONNELLON	1938	Arbacia punctulata	'coelomic cells' = bladder & filiform a/ctes
JACOBSON & MILLOTT	1953	Diadema antillarum	'amoebocytes with spheroidal inclusions' = spherule amoebocytes
STOTT	1955	Echinus esculentus	'agranulocytes' = bladder amoebocytes. 'granulocytes' = spherule amoebocytes

THE CYTOLOGY AND LOCOMOTION OF THE PERIVISCERAL COELOMIC CELLS

THE BLADDER AMOEBOCYTES

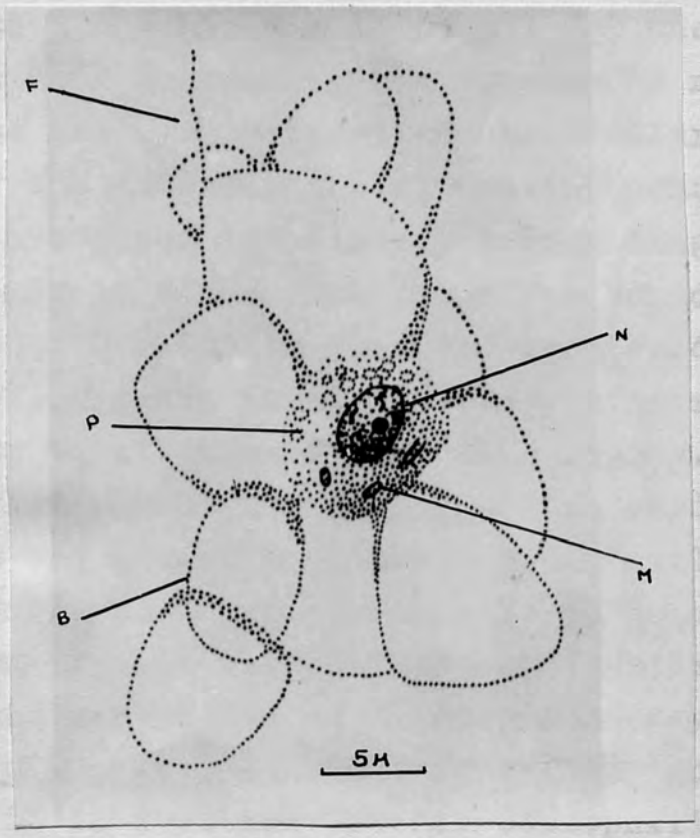
As will be evident from Tables I and 2, coelomic cells with bladder-like or filiform pseudopodia have been described previously in all the echinoids studied. Such cells also occur in holothuroids, asteroids and at least some crinoids and ophiuroids. Similar cells have been described from crustacea and some annelids; Goodrich, (I9I9); Fauré-Fremiet, (I927).

These cells were first described in echinoids by Geddes, (I880), who examined the perivisceral fluid of Toxopneustes (= Paracentrotus) lividus and Echinus sphaera. He stated that these cells have filiform pseudopodia, and thought that the ring-like extensions, now called bladders, were formed by the filiform pseudopodia uniting at the tips. He realised that these cells were concerned in coagulation, and although this view has since gained general acceptance there has been some continued confusion about the relationship of the 'bladders' with the filiform pseudopodia, and their relative roles in coagulation. For the moment we shall consider the cells as they are found in fresh coelomic fluid, and coagulation will be considered separately, p. 100 . Bladder amoebocytes are only found in fresh coelomic fluid and it is considered that this is the condition of the cells in the intact animal.

Structure of the bladder amoebocytes

These cells consist of a central mass of endoplasm, surrounded by transparent flap-like pseudopodia, usually termed petaloid or bladder-like. The endoplasm contains a nucleus and a variable number of inclusions, Figs. I and 2,

FIGURE 1



PARACENTROTUS LIVIDUS

LIVING BLADDER AMOEBOCYTE - DRAWN UNDER PHASE CONTRAST

B - Bladder pseudopodium F - Filiform pseudopodium

M - Mitochondrion (?) N - Nucleus

P - Perinuclear cytoplasm

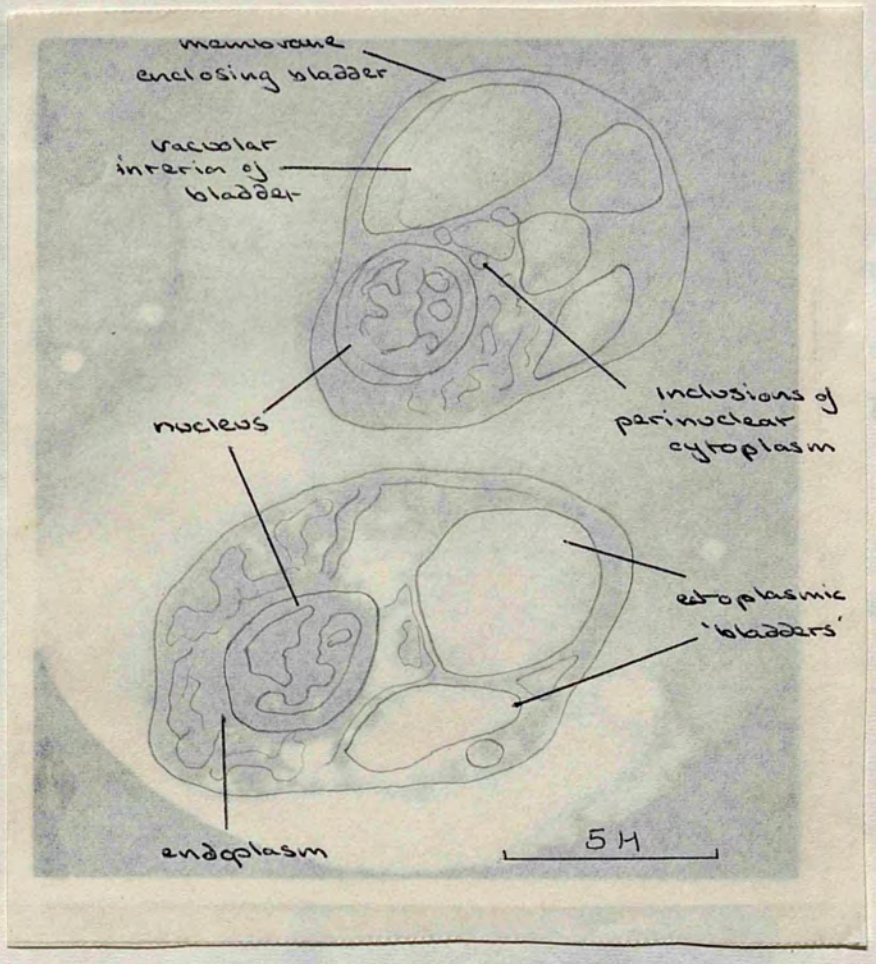
which are described below.

The nucleus is not conspicuous in the living cells; as far as can be determined there is no endoplasmic streaming, except perhaps in phagocytosis, and the nucleus remains static, by contrast with the nucleus of the spherule amoebocytes. The nucleus is round, 2-3 micra in diameter, and contains a nucleolus, (as previously noted by Liebman, I950). Its staining properties are similar to those of the nuclei of the coelomic cells, showing granular chromatin.

The endoplasm immediately surrounding the nucleus is granular and it often contains a few rounded inclusions. It is about IO micra in diameter as viewed from above in living cells. Janus green vital staining shows what appears to be mitochondria in this region. The Regaud's haematoxylin method for mitochondria, used as described by Bhanadwaj and Love (I959) shows round inclusions in the perinuclear area of some cells. It is interesting to note that Fauré-Fremiet (I927) found osmiophilic structures in the perinuclear region of bladder amoebocytes of Paracentrotus lividus, which he called 'chondriome', although it is doubtful whether osmiophilia could be considered in any way specific for mitochondria. Fig. 2A shows an electron micrograph of a section through a group of bladder amoebocytes at the adge of an osmium-fixed clot, before they became involved in its formation. Some fine detail is obvious in the perinuclear region. It is not possible to distinguish mitochondria with certainty, although they may be represented by the structures labelled M, which show some features, e.g. faint 'cristae', reminiscent of mitochondria.

The peripheral ectoplasmic region of these cells is both transparent and labile, which impedes accurate

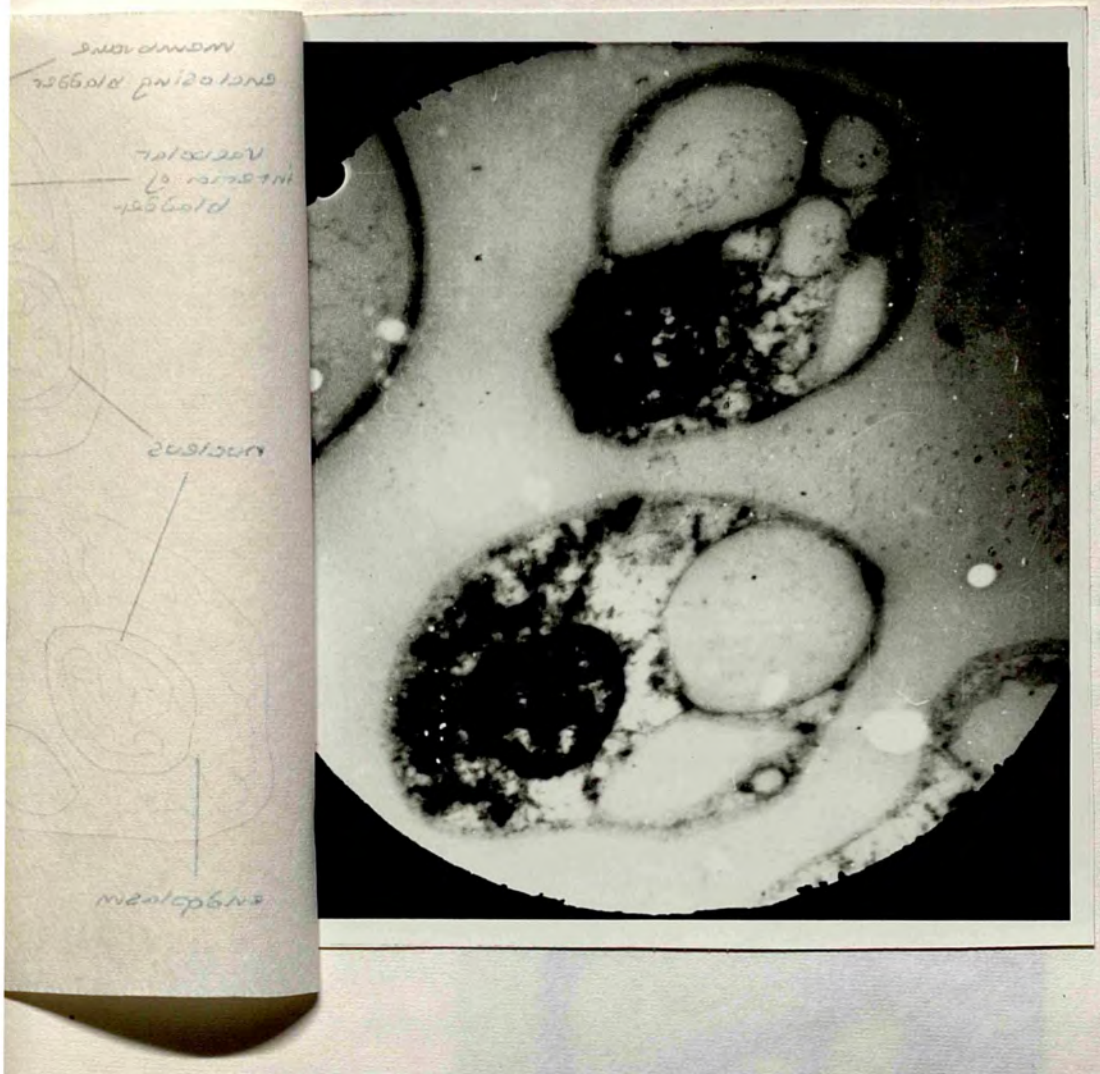
FIGURE 2



DIADEMA ANTILLARUM

LOW POWER ELECTRON PHOTOMICROGRAPH OF AN ULTRATHIN SECTION THROUGH TWO BLADDER AMOEBOCYTES: PALADE FIXATION

FIGURE 2



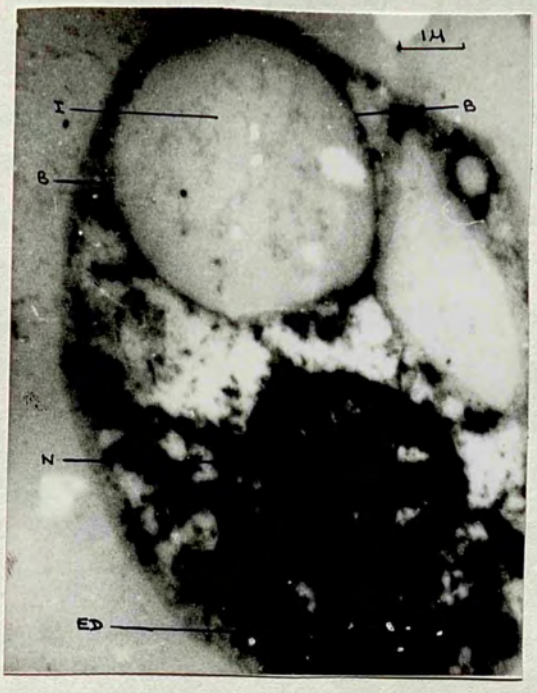
DIADEMA ANTILLARUM

LOW POWER ELECTRON PHOTOMICROGRAPH OF AN ULTRATHIN SECTION THROUGH TWO BLADDER AMOEBOCYTES: PALADE FIXATION

FIGURE 2 A,B

MAGNIFIED VIEW OF PART OF FIG. 2

A



B = Membraneous wall of 'bladder'

ED= Endoplasm

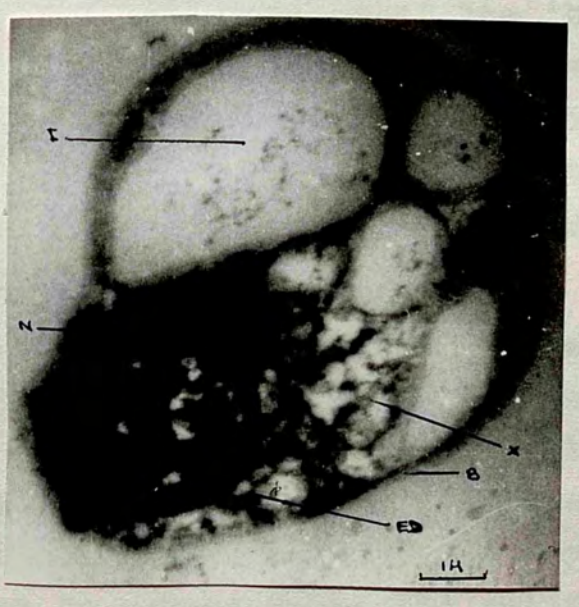
I = Interior of 'bladder'

M = Mitochondrion (?)

N = Nucleus

X = Unidentified cytoplasmic structure

B



observation. There has therefore been some sharp disagreement as to its nature. Geddes (I880) described these cells as having pseudopodia, either filiform or united to form rings; 'ils s'unissent l'un avec l'autre pour former des anneaux complets'. This was a misconception, in that the 'filiform' pseudopodia are always formed from the bladders and have never been observed to reform bladders, see the discussion on the irreversible change associated with coagulation, p.103. Goodrich (I9I9) criticised Geddes' description on the basis that no long branching pseudopodia occur in fresh corpuscles; 'they, like the ring-like arches, are merely representations of optical sections of an extensive membrane, folded with rounded surfaces.' He described the filiform processes as being derived on removal of the cells from the animal when the 'membrane begins to stretch out wing-like films in various directions, and these soon acquire a jagged edge with sharp points.' However although Goodrich's descriptions of the derivation of the filiform pseudopodia is fairly accurate, see p.103, his concept of the 'bladder' pseudopodia was less so. Boolootian and Giese (I958) found that when examined in 3 dimensions the bladder-like nature of the ectoplasmic extrusions was readily observable, though they appeared petaloid rather than vesicular in fixed preparations.

It is certainly true that the ectoplasmic extrusions are not flat and may be aptly termed bladder-like. When floating in the perivisceral fluid on a slide, slight movement will often cause them to be carried across the field of view, rotating and rolling as they move, and the bladders are seen to extend round the cells like elliptical balloons. The bladders vary in size and number in each cell,

and they are labile; although they change shape very slowly, unless they are about to undergo the coagulation change. Usually the bladders vary from 5-20 micra, but occasionally large ones, up to about 40 micra long, are observed, Fig. 3.

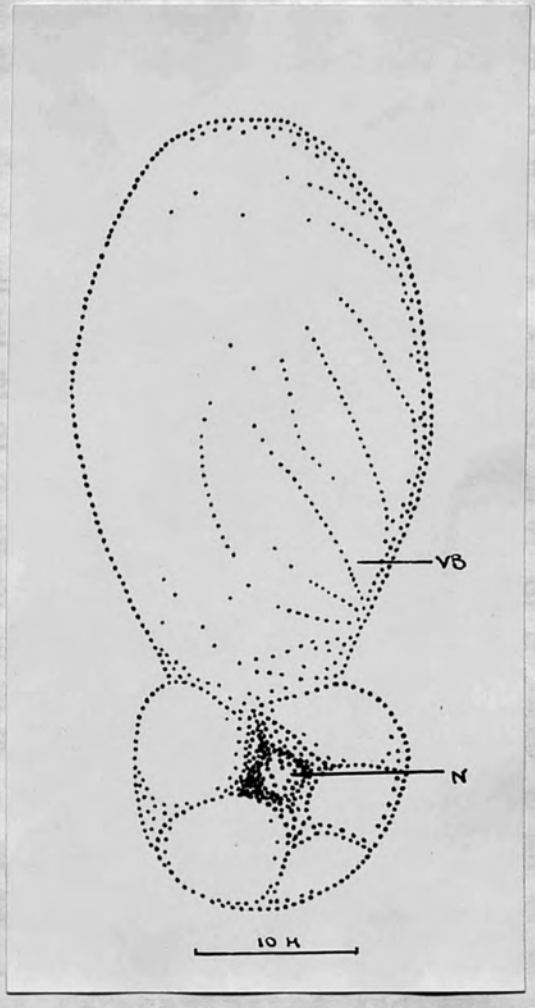
Only the narrow, peripheral region of the bladder stains, and even the electron microscope reveals little internal structure, Fig. 2. According to Ohuye (1936) who studied cells from Temnopleurus hardwickii, the bladders are filled with a colourless fluid, which stains with vital dyes, including neutral red and methylene blue. However I have not been able to stain the interior of the bladder, even with vital stains.

Electron photomicrographs, Figs. 2, and 2A, 2B, show that the bladders are bounded by a layer with little obvious structure but containing a denser 'membrane' which may be seen to be double in some places.

Movement

As already noted, the pseudopodia show a continual change of form, but no active progression has been observed. Liebman's (1950) idea, that, in vivo, these cells have 2 phases, the filiform or locomotory phase, attained on contact with a substrate, and the bladder stage, formed on detachment for flotation, appears to be a misconception. The filiform amoebocyte is indeed usually formed on contact with a glass slide, but this, see p.65, is a pre-coagulation change, which has never been observed to be reversible.

FIGURE 3



DIADEMA ANTILLARUM: LIVING AMOEBOCYTE WITH LARGE BLADDER

N = nucleus VB = very large bladder

THE FLAGELLATED CELLS

OCCURRENCE

This type of cell is found in the perivisceral fluid of most echinoids that have been previously examined.

The first description was given by Geddes (I880), who studied the coelomic fluid of Echinus sphaera and Toxopneustes (=Paracentrotus) lividus. These cells have since been described from the following species; Dorocidaris papillata (=Cidaris cidaris), Prouho (I887); Dorocidaris papillata (=Cidaris cidaris), Paracentrotus lividus, Echinus acutus, Echinus (=Psammechinus) microtuberculatus, Sphaerechinus granularis, Echinocardium cordatum, Cuénot (I89I,97); Arbacia punctulata, Strongylocentrotus drobachiensis, Kindred (I924); Mellita quinquieperforata, Behre (I932); Lytechinus variegatus, Boliek (I935); Psammechinus miliaris, Schinke (I950); and Strongylocentrotus purpuratus, S.franciscanus and S.fragilis, Boolootian and Giese (I958). Boolootian (I962) has recently described these cells as occurring in a number of other echinoids; Echometra mathei, E.oblonga, E.vanbrunti, Echinothrix diadema, E.calamaris, Echinostrephis aciculatus, Heterocentrotus trigonarius, H.mammillatus, Diadema savignyi, Brissus latecarinatus and Rhinobrissus spp.

Flagellated cells were not found in the following species; Temnopleurus hardwickii, Ohuye (I936); IO regular urchins including Diadema setosum, and 6 irregular echinoids including Echinocardium cordatum, Kawaguti and Yamasu (I954); Dendraster excentricus, Boolootian and Giese (I958).

Flagellated cells have been found in the coelomic fluid of every animal that has been examined in the present study.

Kindred (I924) also records these flagellated cells as occurring in the ophiuroid Ophiopholis aculeata and the holothuroid Stichopus californicus, but there is no record of similar free-swimming cells in any other metazoan group outside the echinoderms. There has therefore been some doubt as to whether these cells might in fact be parasitic protozoans. This view was advanced by Cattaneo (I89I), and argued in some detail (I900, I9I2), by Cuénot. This controversy will be discussed later, p. 82

STRUCTURE

Although these cells have been noted by several observers they have not been adequately described.

A typical cell from Psammechinus miliaris consists of a small rounded body, with an average diameter of 7-8 micra, containing the nucleus and a number of small inclusions, with a flagellum about 50 micra long. In each individual the flagellated cells vary slightly in size, the cell-body being from 5-I0 micra in diameter, Table 3. The flagella vary in length between 25-70 micra, but it is not easy to measure them in normal active cells. The cells with large bodies usually have short flagella, and a large number of inclusions so that the nucleus is obscured, Fig.4A. The smaller cells are often egg-shaped and have fewer inclusions, Fig. 4B. The body becomes distorted if the cell is caught in a clot.

The nucleus is rounded and is 3-4 micra in diameter, and contains a nucleolus. It is situated at the anterior end of the cell.

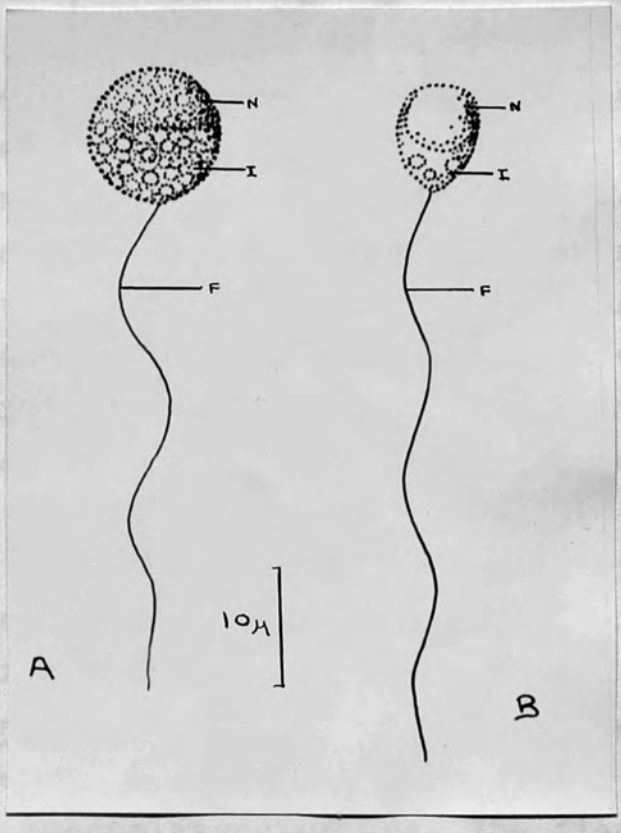
TABLE 3

VARIATION OF THE DIAMETER OF FLAGELLATED CELLS IN THREE INDIVIDUALS - PSAMMECHINUS MILIARIS

In each case living cells were measured when suspended in coelomic fluid.

Diameter of animal	Size distribution per 50 cells						Average
	10mu	9mu	8mu	7mu	6mu	5mu	
23mm	1	2	13	20	11	3	7mu
26mm	6	7	17	15	6	-	8mu
35mm	2	10	25	12	-	1	8mu

FIGURE 4 A,B



MEDIUM (A), AND SMALL (B) FLAGELLATED CELLS: LIVING, DRAWN FROM FRESHLY WITHDRAWN COELOMIC CELLS OF PSAMMECHINUS MILIARIS

N = nucleus I = inclusions F = flagellum

The inclusions are spherical and are usually slightly smaller than I micron in diameter, but may reach 2 micra in cells from Arbacia lixula. They are colourless or faintly greenish, resembling the inclusions of the colourless spherule amoebocytes. Liebman (I950) suggested that the presence of these inclusions in the flagellated cells indicates phagocytosis but I have never seen any signs of it and there is nother evidence for this idea. The inclusions show variable reactions to staining; they do not stain with Mallory's trichrome, Leishman's stain or haematoxylin and eosin, and indeed they are only evident in such preparations as faint outlines in the coloured cytoplasm. They have not shown any consistent positive reaction with the histochemical techniques used in investigating the spherules of the amoebocytes, although there has often been a tendency to stain with the DMAB-nitrite test for indoles, see p.144, particularly with cells from Psammechinus miliaris, and there is some evidence of reactions with alcian blue but not with stains for sulphated mucopolysaccharides. The flagellated cells from Echinocardium cordatum are exceptional and stain in alcian blue and Sudan black B. These spasmodic staining reactions, which recall the more positive and consistent reactions of the inclusions of the spherule amoebocytes, see pp.138-165, are of some interest in reflecting the possible origin of the latter, see later, p. 90 et seq.

Although the majority of the cells are packed with inclusions of uniform size, some of the smaller cells may contain only one or two. One cell from Diadema antillarum contained only 2 large inclusions and another from Psammechinus miliaris enclosed a few colourless spherules and 2 pink ones.

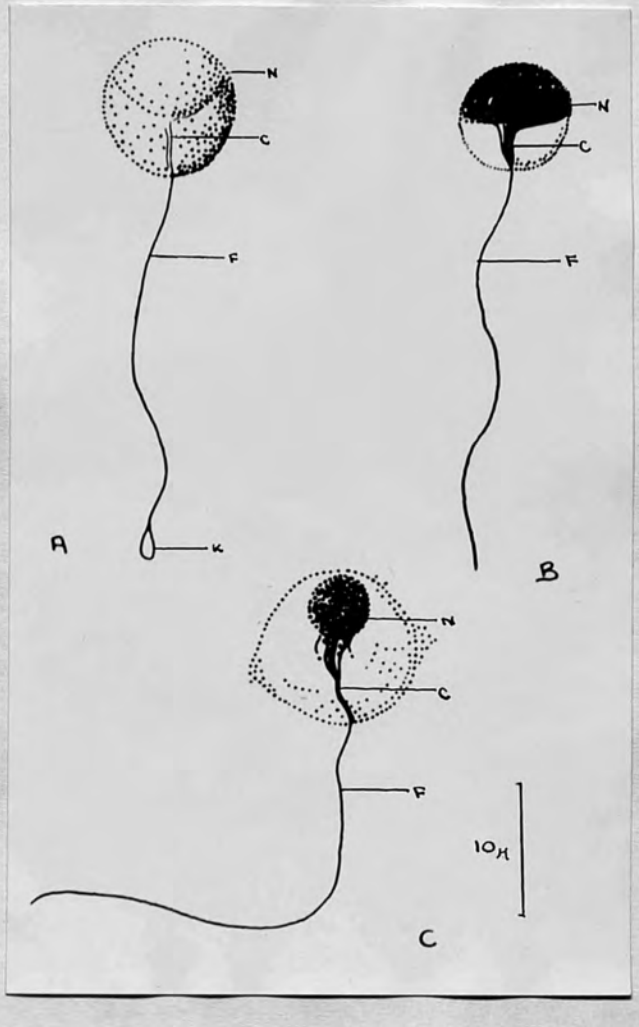
Liebman (I950) has described some cells from Arbacia punctulata as having two flagella. This condition has never been observed in the present study.

The flagellum emerges diametrically opposite the nucleus, at the posterior end of the cell. It appears to be a fine thread-like structure, of uniform thickness, with neither terminal or lateral mastigonemes. However, Boliek (I935) states that, with dark-field illumination, the flagellum appears blade-like in cells of Lytechinus variegatus. This recalls the optical artifact, produced with regular wave-motion in these lighting conditions, which has become known as the optical envelope, Gray (I955). Gray has utilised this phenomonen, by analysing the shape of the envelope, in his studies of sperm movement.

There is considerable variation in flagellum length, as already noted; the larger cells usually have short flagella and these short flagella often have a terminal knob-like structure, Fig. 5A. In fixed preparations it is often evident that there is a thick thread apparently linking the flagellum with the nucleus, Fig. 5B, 5C, 6A, 6B. This thread-like structure is not usually visible in life, due presumably to the masking inclusions, but a highly refractile extension of the flagellum into the cytoplasm has been observed in some living cells, Fig. 5A.

It has not proved possible to locate, under the electron microscope, a satisfactory transverse section of the flagellum from ultra-thin sections of partially clotted cells. Oblique sections were however obtained, and photographed, Fig. 7. Fig. 7A shows a cell cut obliquely through the base of the flagellum. Similarly, Fig. 7B shows a cell which is probably a flagellocyte with what, judging by its size and appearance, may be a transversely

FIGURE 5 A,B,C



FLAGELLATED CELLS FROM DIADEMA ANTILLARUM

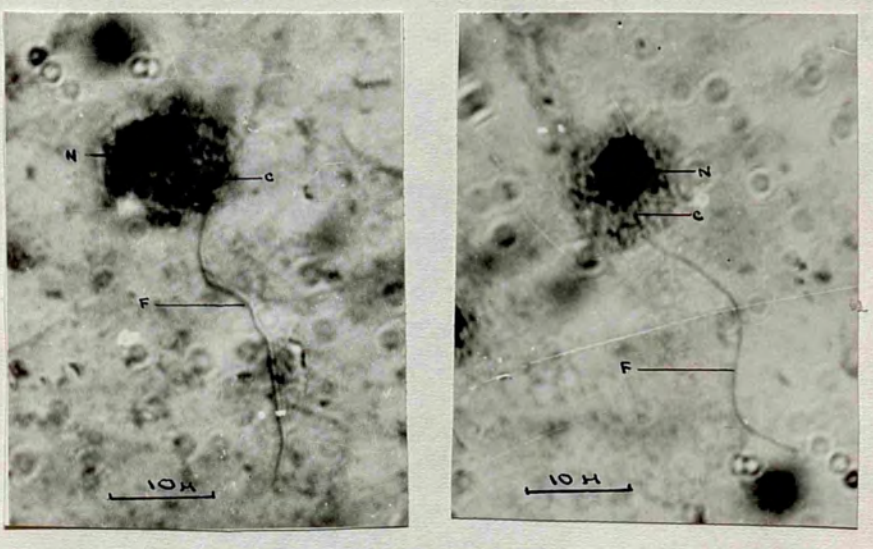
A: LIVING CELL B: CELL FIXED WITH OSMIUM VAPOUR? AND STAINED WITH HAIDENHAIN'S HAEMATOXYLIN.

C: CELL FIXED WITH FORMALIN VAPOUR AND STAINED WITH LEISHMAN'S 'BLOOD' STAIN.

N= nucleus F = flagellum K = 'knob' at end of flagellum

C = possible connection between nucleus and flagellum

FIGURE 6 A,B

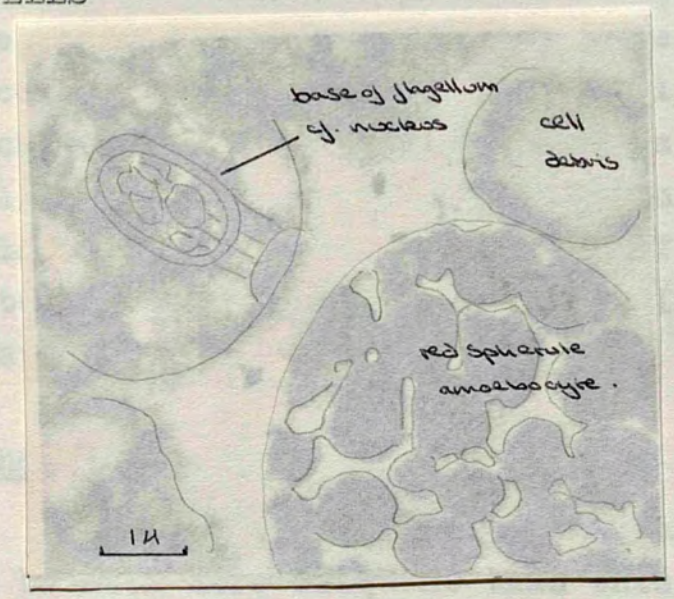


DIADEMA ANTILLARUM: FLAGELLATED CELLS OSMIUM FIXED, STAINED WITH LEISHMAN'S STAIN TO SHOW APPARENT NUCLEAR CONNECTION OF THE FLAGELLUM

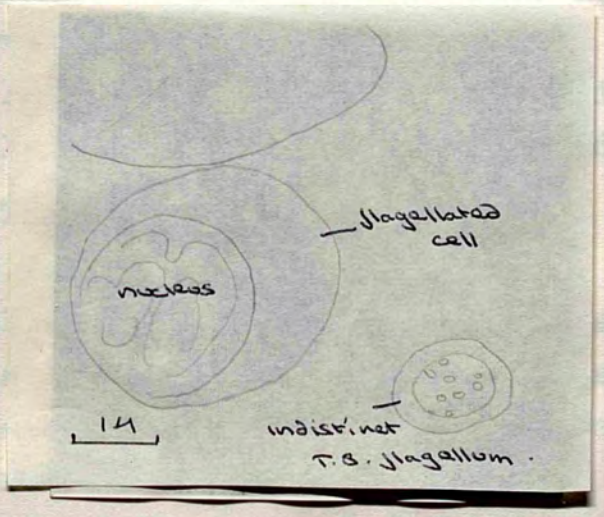
N = nucleus F = flagellum
C = extension of flagellum apparently connected with the nucleus

FIGURE 7 A,B

DIADEMA ANTILLARUM ELECTRON PHOTOMICROGRAPHS OF ULTRATHIN SECTIONS THROUGH PALADE FIXED FLAGELLATED CELLS



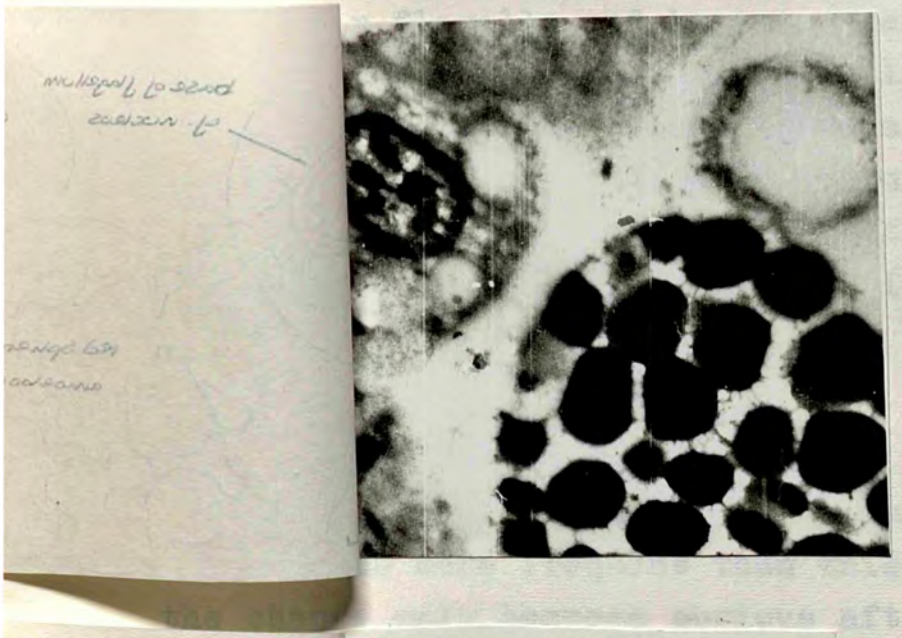
A: OBLIQUE SECTION THROUGH THE BASE OF THE FLAGELLUM



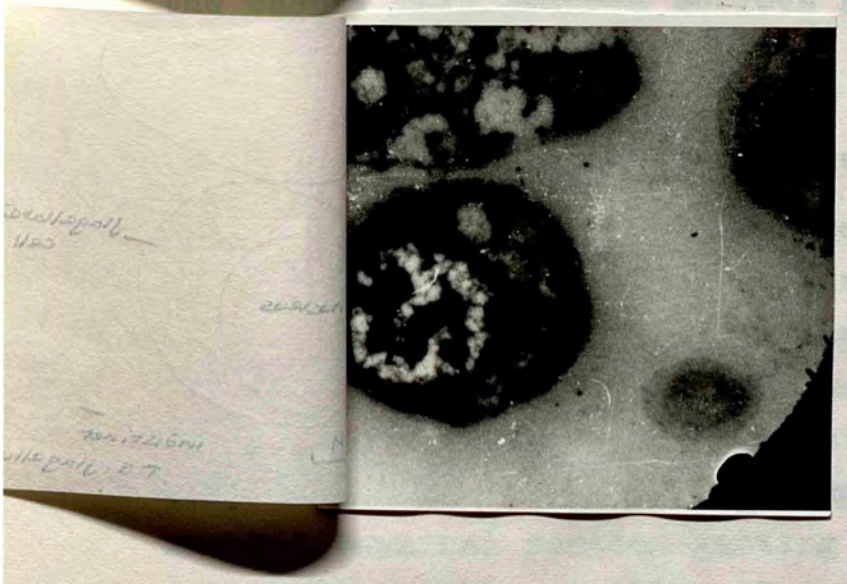
B: FLAGELLATED CELL AND ASSOCIATED INDISTINCT TRANSVERSE SECTION OF A FLAGELLUM

FIGURE 7 A,B

DIADEMA ANTILLARUM ELECTRON PHOTOMICROGRAPHS OF ULTRATHIN SECTIONS THROUGH PALADE FIXED FLAGELLATED CELLS



A: OBLIQUE SECTION THROUGH THE BASE OF THE FLAGELLUM



B: FLAGELLATED CELL AND ASSOCIATED INDISTINCT TRANSVERSE SECTION OF A FLAGELLUM

cut flagellum lying near it. There is no doubt that these flagella have a fibrillar ultra-structure, although it is not possible to be sure that this is of the almost universal 9+2 configuration, see Sleigh (I962).

Liebman (I950) has stated that flagellated cells may lose their flagella and become amoeboid, but he has not described the process and gives only inadequate figures, which do not show how 'loss' occurs, but merely show a cell before and after 'loss'. During my studies of coelomic cells I have observed withdrawal of living flagella once or twice and have also found 'casting' of flagella on fixation.

Withdrawal of the flagellum

I have only observed withdrawal occasionally, but it may be much more frequent than this would indicate, as the change only becomes obvious after some minutes of observation.

In view of the considerable interest of this change details were observed and recorded;

The most striking occurrence was seen in the coelomic cells from Diadema antillarum. In one such cell, a flagellum, initially about 5½ times the length of the body, with a slightly thickened end, was seen to shorten and was completely 'resorbed' in I5 minutes, at laboratory temperature, c. 20° C. Shortening was progressive, the knobbed terminal portion getting nearer and nearer the cell-body, until, finally, it reached the body and there was no longer any projection, although a thick refractile thread was still visible within the cell-body. The cells

was not observed to become amoeboid, but remained as shown in Fig. 8 until the slide dried. Shortening of the flagellum has also been observed in cells from Echinocardium cordatum, the flagella involved having the same knobbed terminal structure. Shortening was only observed on quiescent knobbed flagella, but similar flagella have been observed in active locomotion.*

'Casting'

On two occasions, fixed cells from Diadema antillarum had apparently 'cast' their flagella, which were all found to have varying sizes of 'knobs' at one ende.

* Transformation from the flagellate to the amoeboid form of Naegleria gruberi has been recently described, Pittam (1963), as being accomplished with a flagellar resorption in 10-20 minutes.

FIGURE 8

Living cell from DIADEMA ANTILLARUM drawn after resorbtion of the flagellum

N = nucleus

F = remains of flagellar structure

I = inclusions

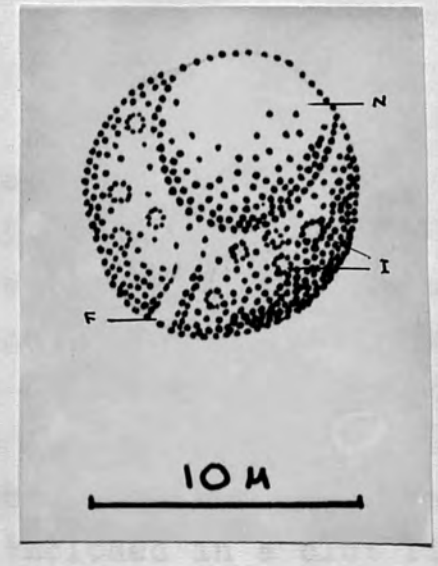
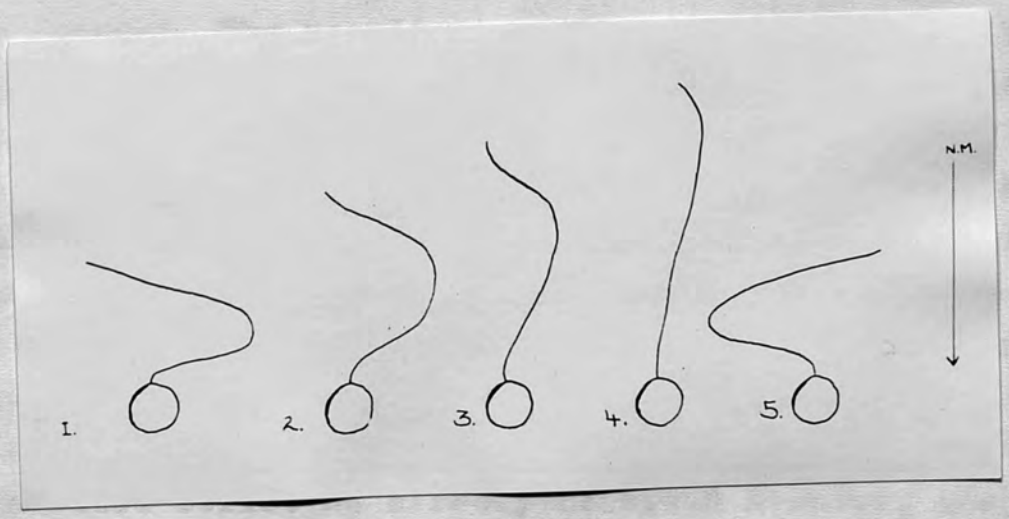


FIGURE 9

Living cell from DIADEMA ANTILLARUM, restricted by clot formation: cell not motile but showing regular bending movements of the flagellum

NM = normal direction of movement



MOVEMENT

Methods of examination

It is not easy to analyse the flagellar movement as the flagellum is not visible under low-power when the cells are moving unimpeded, even when the stroboflash is used, whereas it is very difficult to see the movement under higher power because the cell only remains in view for a second or two. The cells may be slowed down under a cover-slip, especially when clot formation takes place, but if the cells become included in a clot flagellar movement may become markedly abnormal; either spasmodic or jerky.

Observations

The flagellated cells invariably move with the 'cell-body' anterior to the flagellum, and the normal speed, determined from freshly withdrawn cells, is 60-80 micra/second. The resultant pathway is usually a slight curve. However, the actual course that is traced has the appearance of a zig-zag as the cell-body apparently moves from side to side as the cell progresses. The cell-body also rotates about the longitudinal axis. Rolling of the 'head' of spermatozoa is thought to be due to the terminal part of the flagellum beating in a slightly different plane, Gray (I958), Bishop (I958). A similar irregularity may account for the rotation which occurs with these flagellated cells. As already observed however, adequate analysis of movement of the flagellum has not proved possible.

In one particular case however the regular flagellar movement associated with rapid swimming was seen to persist when a cell was resticted by clot formation. The movement of the flagellum as then observed was a series of undulations. These passed down from the cell-body to the tip of the flagellum in regular succession, but only one wave occupied the flagellum at any one time; it is possible however that in fast swimming several waves may pass back at any one time. The consistent rhythmical movements of the flagellum showed the sequence depicted in Fig. 9. The wave passed up one side of the flagellum; I-4. The next wave passed up the other side; position 5, the pattern being that of a regular symmetrical beat.

The movement of the echinoid flagellated cells compared with that of other flagellated organisms.

The alternate bending waves of the echinoid cells' flagella, depicted in Fig.9 and described above, are characteristic of flagellar movement, Sleigh (I962). Other features of general comparison; speed, rotation etc., are shown in Table 4.

However movement of these cells is effected by a flagellum held posterior to the cell-body. As Lowndes, (I94I) says, of protozoa; 'it is really very exceptional to have a flagellum attached to the hinder end of the organism and pushing it through the water.' As far as can be ascertained the movement of these cells, in fact, closely resembles that of spermatozoa, in which the cell-bodies also precede the flagella.

Gray, (I955, I958) has made detailed studies of the movement of sea-urchin and bull sperm. He found that, with

TABLE 4

COMPARATIVE DATA OF FLAGELLAR MOVEMENT

SPECIES & CELL	AUTHOR Date	SPEED of cell μ/sec.	FREQ. BEAT ~/sec.	ROTATION of cell no/sec.	LENGTH OF FLAGELLUM μ
Euglena species	Lowndes 1941	168	12	1	128
Psammechinus miliaris: sperm	Gray 1955	120-190	30-40	0.5-3	
Polytoma species	Lowndes 1941	127	7-11		17.5
Psammechinus miliaris: 'flagellated' coelomic cell	Self	60-80		2	20-60
Chlamydomonas species	Lowndes 1941	65.4	8+		35
Menoidium incurvum	Lowndes 1941	50	12	1	25
Peranema tricophora	Lowndes 1941	20			20-70

the former, all parts of the flagellum show similar lateral movements, like the movements of an eel or snake, whereas with the latter the anterior part of the flagellum acts as a fulcrum against which the posterior part of the flagellum exerts a propulsive force.

On the available information it would seem that the movement of the echinoid (coelomic) flagellated cells is similar to the former, 'sea-urchin sperm' type of movement.

SPHERULE AMOEBOCYTES

These cells show amoeboid progression and contain numerous rounded inclusions. The staining properties, and histochemistry of these inclusions, and the pigments associated with these cells will be discussed later.

Colourless spherule amoebocytes

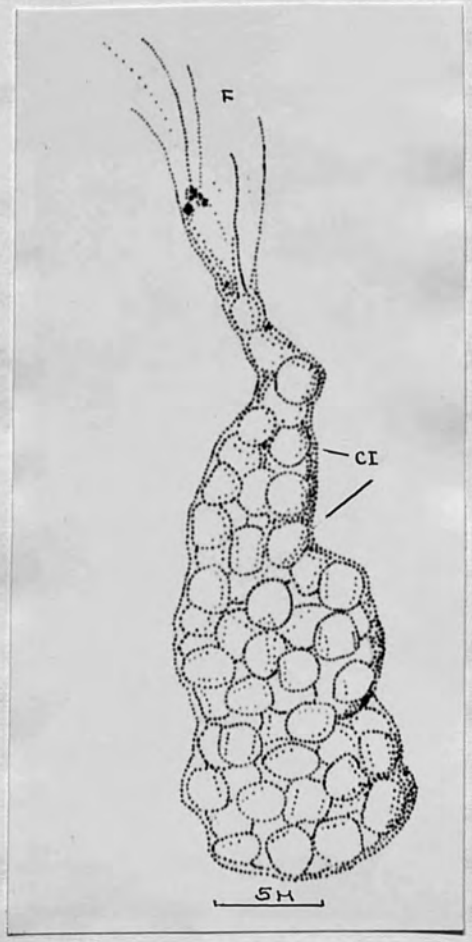
These have been described from almost every echinoid investigated since the time of Geddes (I880). Similar cells are found in holothuroids, and in some asteroids and ophiuroids. Amoeboid cells packed with colourless inclusions have also been described from many other invertebrates; notable in this context are descriptions of cells from annelids, Fauré-Fremiet (I927), Dales (I957, 6I); molluscs, Takatsuki (I934); and ascidians, Endean (I960).

Colourless spherule amoebocytes occur in the coelomic fluid of all species examined in the present study.

In the species examined here the cells may be 35 micra long, and 5-I0 micra in width, when extended. The nucleus is obscured by the inclusions in the living cells but is seen as an oval structure 3-4 micra long, in fixed preparations. As Liebman (I950) observed, there does not seem to be a nucleolus.

The tightly packed inclusions are spherical with a diameter of I-3 micra, and some present a somewhat irregular appearance, possibly due to compression, Fig. I0. These inclusions are colourless but may appear slightly green in transmitted light and have therefore been termed

FIGURE 10



LIVING COLOURLESS SPHERULE AMOEBOCYTE IN LOCOMOTION: FROM COELOMIC FLUID OF DIADEMA ANTILLARUM

CI = colourless inclusions

F = flexible filiform 'tail' processes

refringent. The spherules have never been observed to cytolyse, unlike the red spherules described later. Observations with the light microscope on stained and on living cells, and with the electron microscope show that these inclusions are more homogeneous than the red spherules and do not appear to have a separable envelope,

A low-power electron micrograph, Fig. I2B, showing a section through some of these inclusions, reveals very faint striations on the inclusions. This may indicate that the spherule contents are laid down in a laminated fashion, similar to the structure of the inclusions of vanadocytes of Phallusia mammillata, Endean (I960).

Red spherule amoebocytes

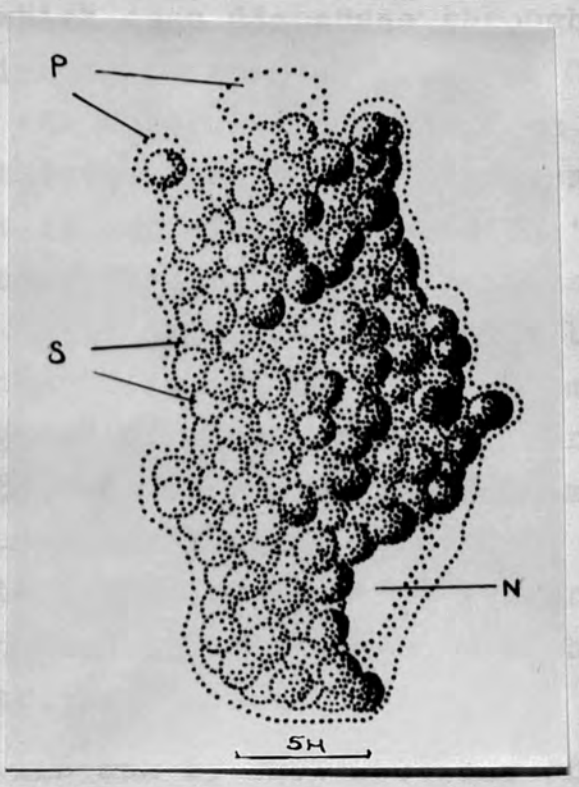
These are found in all echinoids that have been previously investigated, with the exception of certain irregular echinoids, Table IB, and also Echinocyamus pusillus, Cuenot (I89I). They are present in every echinoid examined in the present study. Although pigmented cells occur in other echinoderms they are not similar in structure, neither do they contain the same kinds of pigments, see later, p. 120.

These cells are similar to the colourless spherule amoebocytes but the inclusions are pigmented, Fig. II, Fig. 24, p. 130.

The nucleus is often evident as a disc-like or bean-shaped structure, 3-4 micra long, moving freely in the cytoplasm. It is without a nucleolus.

The inclusions are smaller than the inclusions of the colourless spherule amoebocytes, being I-2 micra in diameter. Some small colourless inclusions may also occur

FIGURE 11



LIVING RED SPHERULE AMOEBOCYTE:
FROM COELOMIC FLUID OF PSAMMECHINUS MILIARIS

N = nucleus P = pseudopodia S = spherules

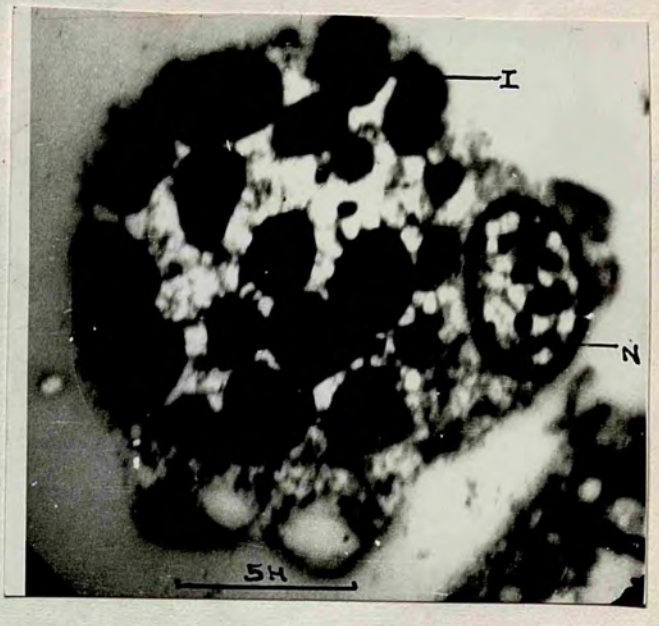
and have been seen at the posterior end of the moving cell, in the 'tail' region. Normally pigmentation is confined to the inclusions, but the spherules readily cytolyse under osmotic stress, such as is given by adding distilled water, and discharge the pigment as a slightly viscous fluid, which soon disperses through the surrounding fluid. Liquid fixatives, such as Bouin or Carnoy have a similar effect, the spherules 'popping' as the fixative reaches them. Observations on this reaction indicates that the pigment is contained in definite 'envelopes', which rupture under stress, and the remains of which are left within the cell; as faint 'ghosts' - barely visible at the highest magnification of the light microscope.

The red pigment of these cells was first described by MacMunn (1885), who named it echinochrome. The pigment has aroused considerable interest, and where it has been adequately characterised it appears to be a hydroxy-naphthoquinone. Pigmentation will be discussed in more detail later, p.120.

Figs. 12A, 12B and 13 show sections of these cells photographed in the electron microscope at low magnification. Osmium tetroxide is reduced by echinochrome and the red spherules are therefore electron dense after osmium fixation, and appear black in these electron photomicrographs. 'Connections' between the inclusions are possibly artifacts as the spherules move freely in the living cell. The artifact might be caused by fixation at the onset of cytolysis, or crenulations due to osmotic stress, or, possibly, fixation and artificial alignment of other cellular constituents.

FIGURE 12 A

RED SPHERULE AMOEBOCYTE
Electron photomicrograph of a Palade fixed ultrathin section; N = nucleus
I = inclusions
DIADEMA ANTILLARUM



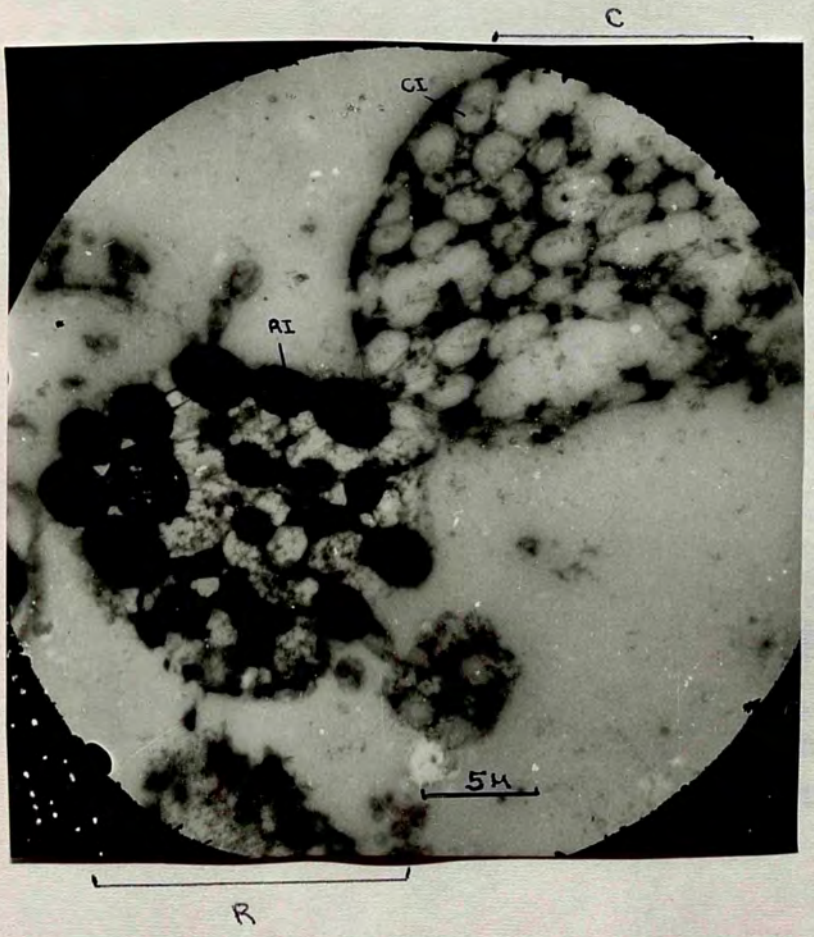
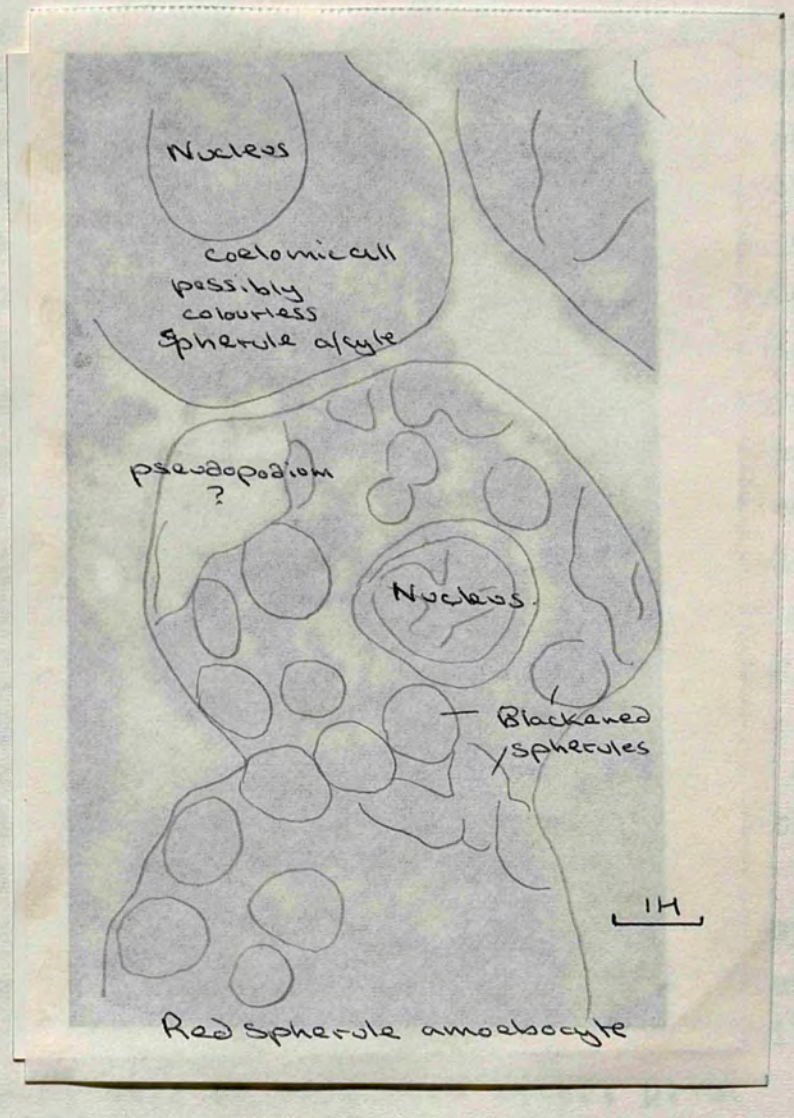


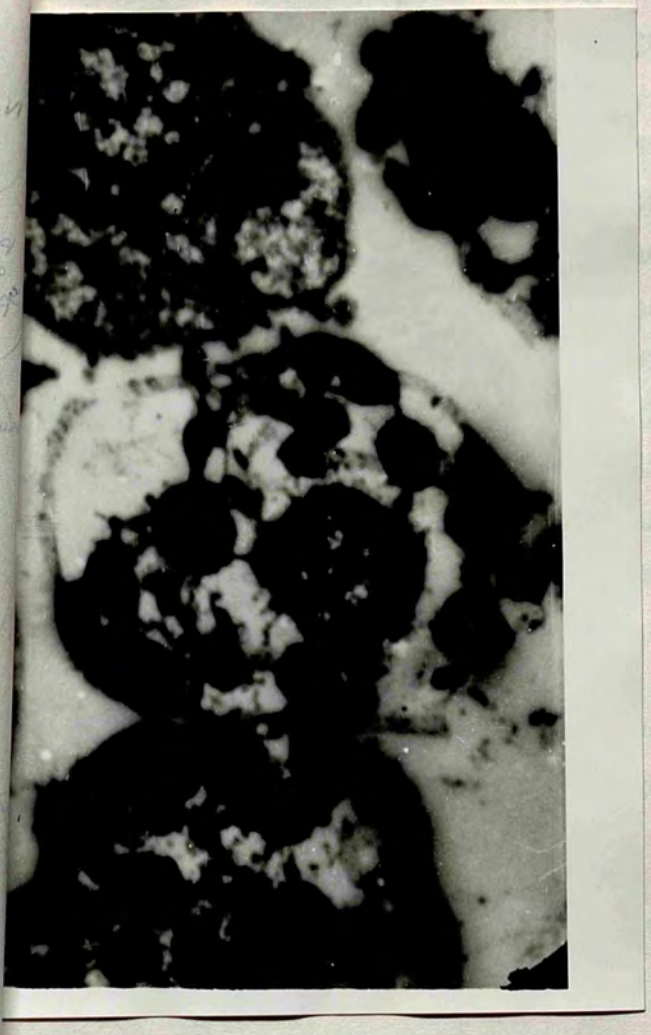
FIGURE 12 B
Electron photomicrograph of a Palade fixed ultrathin section through part of a RED and a COLOURLESS SPHERULE AMOEBOCYTE of DIADEMA ANTILLARUM
RI = red inclusion
CI = colourless inclusion
R = red spherule a/cyte
C = colourless spherule amoebocyte

FIGURE 13



Electron photomicrograph of an ultrathin section of part of a red spherule amoebocyte of DIADEMA ANTILLARUM Palade fixation Note nucleus and what is apparently an early stage of pseudopodium formation

FIGURE 13



Electron micrograph of an ultrathin section of part of a red spherule amoebocyte of DIADEMA ANTILLARUM Palade fixation Note nucleus and what is apparently an early stage of pseudopodium formation

Green spherule amoebocytes

Table IB, p.20 , shows the wide range of pigmented spherule cells recorded from irregular echinoids, principally from Japanese species, by Kawaguti and Yamasu, (1954). I have only observed two types of pigmented cell, in addition to the red cells described above, in the species studied, and propose to restrict discussion to these. The cells containing dark purple inclusions, which occur in the heart urchins examined appeared to be bladder amoebocytes. No further observations were possible, due to lack of material. The other type of cell observed may be described as the green spherule amoebocyte. These cells were found to occur in the heart urchins studied; Echinocardium cordatum, Spatangus purpureus and Brissopsis lyrifera, and also in small quantities in some specimens of Arbacia lixula. Liebman (1950) found similar cells in Arbacia punctulata.

The colour plate, Fig. 24, p.130 , shows some of these living cells from Echinocardium cordatum, together with red and colourless spherule amoebocytes.

These cells are the same size as the red spherule amoebocytes; the spherules are 1-2 micra in diameter, and are bright yellowish-green in colour. The possible nature of the pigment will be discussed later, p.131 ; it is not possible to release the pigment in the manner described for the red spherules, and there is no evidence that it is contained in a separable envelope.

The cells noted in Arbacia did not show active movement, but the cells from the irregular echinoids were more mobile. Unfortunately, it has not been possible to study these cells in detail as the irregular echinoids are difficult to obtain and keep, and there are few of these cells in Arbacia.

MOVEMENT OF THE SPHERULE AMOEBOCYTES

Previous observations

Liebman (I950) described movement of the spherule amoebocytes of Arbacia punctulata as being effected by emission of blunt lobopods into which the granules quickly followed. He also noted that the cells frequently have a trail of cytoplasm or granules in their wake, which led him to make a striking comparison with the appearance of 'rat-tailed maggots'! Other authors, for example, Boolootain and Giese (I958) have described these cells as having 'eruptive' or guttata pseudopodia. This is presumably due to the abrupt movement of the inclusions into the pseudopodium, see later, and it must be stressed that, if applied to the pseudopodium, this is really a mis-nomer,* as the actual formation of the pseudopodium precedes the 'eruption' and is a more gradual process.

Original observations

The amoebocytes I have observed move at a rate of about 0.I6 micra/second, but the speed varies greatly, especially if the cell encounters obstacles.

In fast, unimpeded locomotion the cells assume a rather standard, elongated form; tapering, with the broad end anterior, Fig. IO, p.48 . Locomotion may be seen to be effected by the formation of one or more pseudopods. Initially these are transparent and devoid of spherules. These inclusions, and sometimes even the free-moving nucleus, subsequently pass into the enlarging pseudopodial area, and a fresh pseudopodium is formed.

*a *and in amoebae the hyaline cap is invaded by spurts of endoplasm, Allen (1961)

Typically, there is a 'trail' of cytoplasm and small inclusions at the posterior end, which will be referred to as the 'tail'. The contents of this 'tail' region are usually small colourless inclusions, with an occasional small red spherule in the case of red spherule amoebocytes. This region, Fig. IO, p. 48 , often assumes a filiform appearance.

Active living cells of all 3 types of spherule amoebocyte are shown in Fig. 24, p. 83 . The 'transparent' pseudopodia may be seen in several of the cells, and one of the cells removed from the influence of the coagulating cells, is seen in 'fast' locomotion and shows the typical shape described above.

The pseudopodium

All following observations are based on freshly withdrawn coelomic cells; the use of the oil-immersion highpower objective necessitated the presence of a cover-slip, but the amount of coelomic fluid present was regulated to reduce pressure so that at least the form of the cell was not visibly distorted.

In 'fast' locomotion progression is essentially monopodial. The pseudopodium may be continually formed, to give the appearance of an advancing broad front, with spherules moving into the 'old' pseudopodium as the new one is formed, ahead of it; or it may be intermittently formed, with the spherules breaking through into the pseudopodium, followed by a brief interval before formation of the next pseudopodium. The sequence of events associated with this intermittent formation of the pseudopodium, which may occur at any point on the surface of the cell, except at the 'tail', see later, may be summarised as follows;

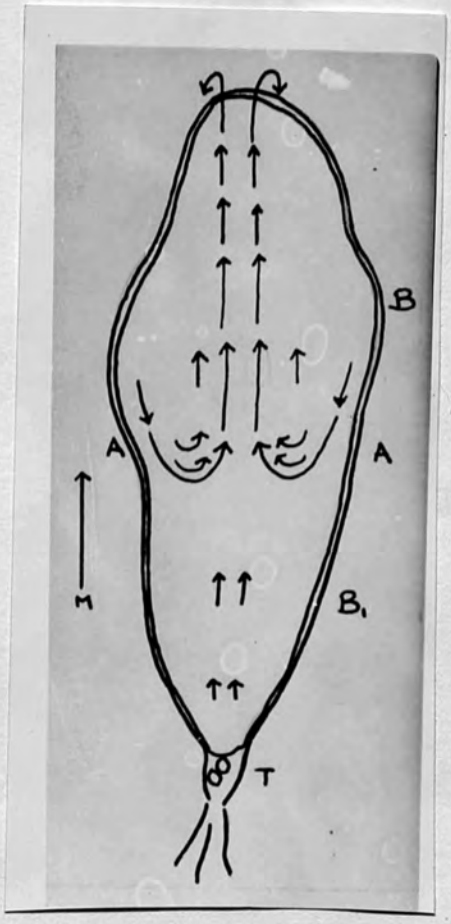
I. The colourless flap-like pseudopodium is formed, see Fig. I6, (this may be a hyaline cap, see Allen (1961)
2. One spherule passes into the pseudopodium, quickly followed by many others, and the pseudopodium is filled with these inclusions, with the exception of a narrow, clear zone at the anterior of the pseudopodium, Fig. I6, As the pseudopodium fills with spherules a constriction appears to pass back down the cell, Fig. I4, A-A, decreasing in speed and extent until it becomes obscured at the mid-region of the cell.
3. When the pseudopodium is filled with spherules a new one is formed, and another constriction wave appears to pass back.

The movements of the spherules behind the pseudopodium are of considerable interest, for they are tightly packed, and it would seem that their pattern of movement might be, to some degree, an index of whether this 'amoeboid' movement conforms with the type of movement observed in Amoeba and Chaos which has been analysed by several workers, including Mast, Pantin and Allen.

The movement of the inclusions behind the pseudopodium shows considerable variation. Fig. I4, however, is a fairly typical example of the paths of movement of the spherules as seen in an actively progressing cell. The area of greatest activity is in the mid-region of the cell, A-A. Spherules posterior to this region and at the sides, B-B, travel forward more slowly, while the 'tail' as Liebman (I950) noted, appears to lag. At the anterior region of the cell, inclusions often move to the 'undersurface' of the pseudopodial region, rather

FIGURE 14

FRESHLY WITHDRAWN RED SPHERULE AMOEBOCYTE OF DIADEMA ANTILLARUM: TO SHOW MOVEMENT OF SPHERULES IN 'CONTINUOUS' LOCOMOTION



A-A = Mid-region of cell, with considerable spherule activity. Note constriction.

B = Anterior lateral recruitment zone.

B = Posterior region, showing slow recruitment.

T = 'Tail'

M→ = Direction of movemnnt

→ = Direction of spherule movement

FIGURE 15

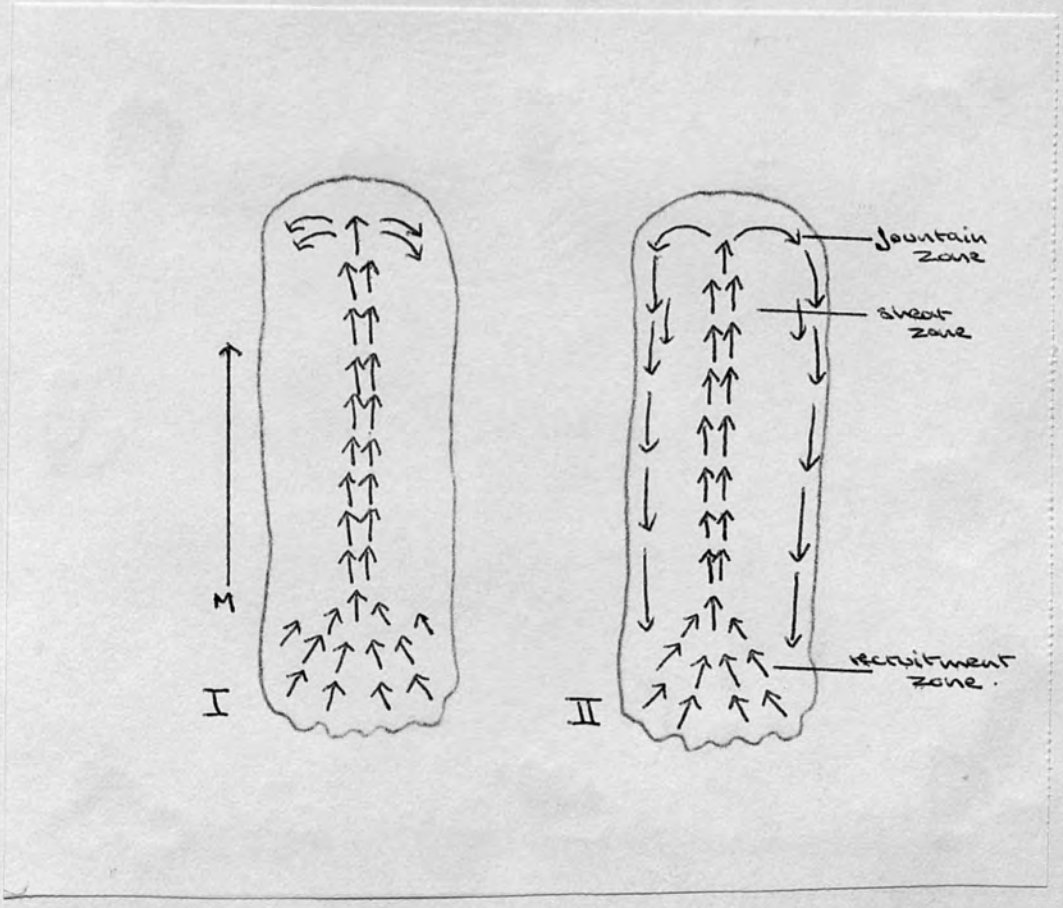


DIAGRAM TO SHOW STREAMING IN AMOEBAE, after Allen (1960)

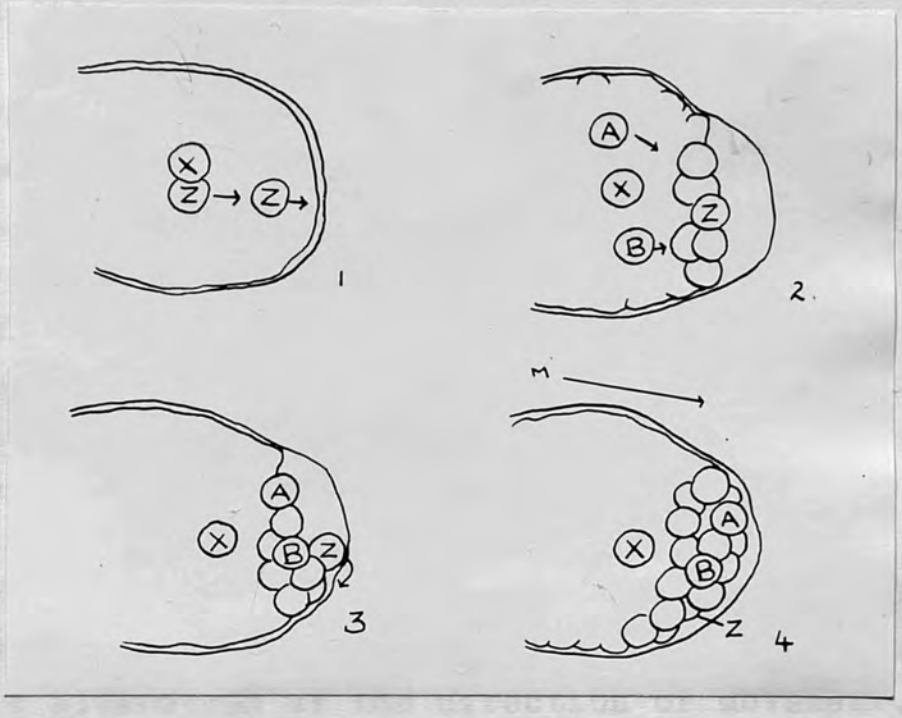
I Attached cell II Unattached cell

⟶ Direction of streaming

M ⟶ Movement of attached cell

FIGURE 16

PSAMMECHINUS MILIARIS: MOVEMENT OF SPHERULES AT PSEUDOPODIUM FORMATION



1. X and Z are two adjacent spherules. Z moves forward.
2. The pseudopodium is formed. X remains stationary, A and B move forward, Z is at the periphery of the pseudopodium.
3. Z moves into the pseudopodial area at breakthrough', and moves underneath succeeding spherules. X remains stationary (relatively).
4. A and B have moved forward into the pseudopodial area. Z is now underneath and to the side of the pseudopodial area. X still remains relatively stationary.

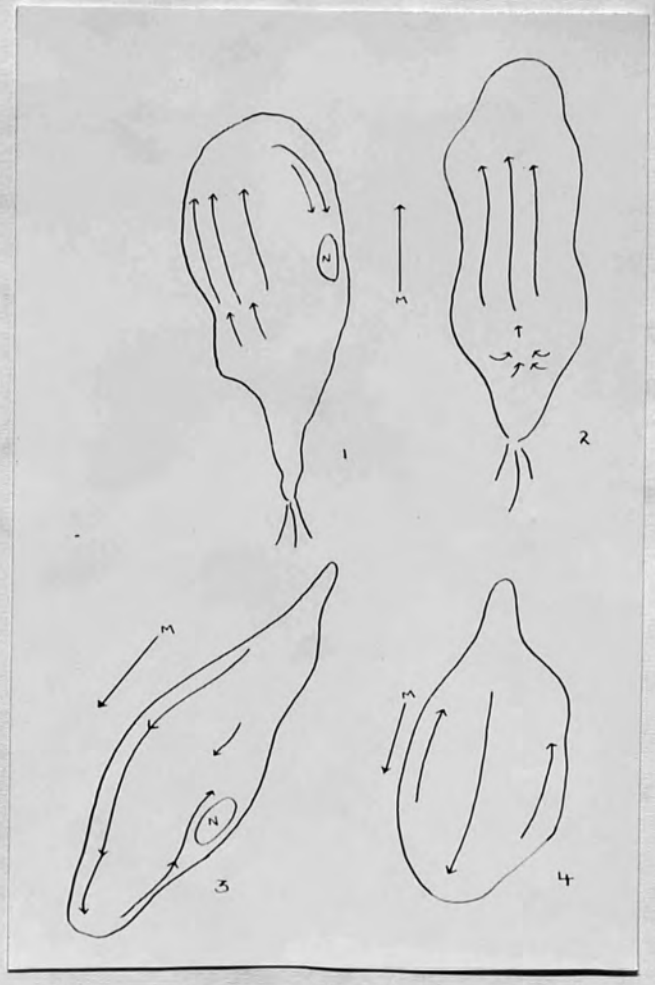
M ——→ direction of mmovement

than laterally, see Fig. I6, which shows the movement of some individual inclusions. However it must be stressed that great variation has been seen in the movement of the inclusions, in active and apparently normal cells; Fig. I7 shows the chief variations, expressed as gross movement of inclusions. It is based on observations of numbers (c. I00) of such cells.

'Fast' locomotion depends on successive formation of pseudopodia at the anterior end of the cell, and is essentially monopodial. However pseudopodia may be formed simultaneously in many places over the surface of the cell, although never at the 'tail'. Such 'polypodial' conditions are usually observed when the cells become obstructed, as in clot formation. Fig. II, p.50 , shows one such cell, which has 'rounded off' to some extent, such that the 'tail' region is obscured.

This facility for forming pseudopodia almost anywhere over the surface of the cell, ensures an effective means of rapid alteration of the direction of movement. The amoebocytes readily reverse the direction of travel by forming pseudopodia at the posterior end. Fig. I8 shows the sequence of events in a cell confronting an obstacle. Pseudopodia are formed laterally, I8, 2, after the anterior region confronts the obstacle. However the lateral area is also blocked, and new pseudopodia are formed at B, B , around the previous posterior region. The pseudopodium at B becomes the new dominant, and effectively reverses the cell. The 'tail' region itself, although close to the new anterior region, remains distinct, and the rest of the cell follows the new anterior region before the 'tail',

FIGURE 17



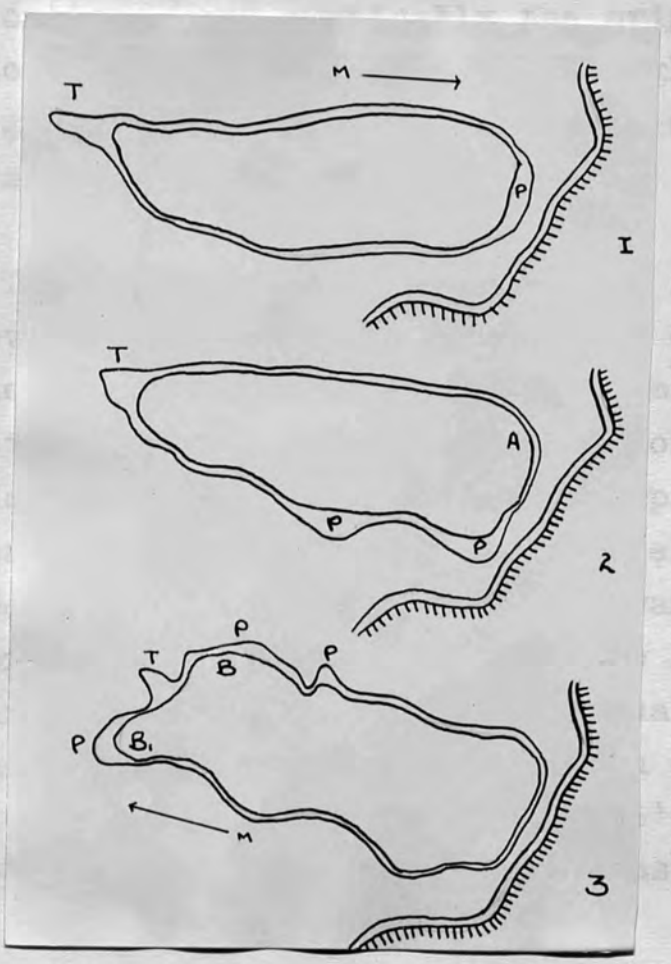
Variation in spherule movement observed in progressing, freshly withdrawn spherule amoebocytes of PSAMMECHINUS MILIARIS

M ——→ direction of cell movement

——→ direction of principal spherule movement

FIGURE 18

Reversal of a red spherule amoebocyte, from freshly withdrawn coelomic fluid of PSAMMECHINUS MILIARIS



T = 'tail'
P = pseudopodium
M ⟶ direction of cell movement

1. Amoebocyte, moving in direction shown, confronts obstacle.
2. There is turbulent movement at A: spherules apparently move at random. Pseudopodia form laterally.
3. Turbulent movement at B,B , are followed by formation of pseudopodia at the 'posterior' end, and effective reversal is accomplished when the pseudopodium at B becomes dominant

which then resumes a posterior position.

Another cell that was observed, from Arbacia lixula, in reversal, became almost split in two, with both portions streaming away from each other. The original 'tail' region formed a tenuous connection between the two portions, and eventually the original anterior portion reversed and streamed through this remaining 'bridge', so that the movement was, at last, effectively reversed.

To summarise, as far as can be ascertained, initiation of movement is manifest in the flow of protoplasm devoid of inclusions to form a pseudopodium*. Locomotion is effected when the inclusions pass forward into the pseudopodium*. There may be an accompanying contraction, of at least the anterior part of the cell, such that the greater part of the cell moves forwards. However, the posterior part of the cell tends to lag behind, and may continue thus for some time. Eventually however there may be what appears to be a contraction of the superficial zone as a result of which the 'tail' moves towards the cell-body, so that it resumes a less attenuated condition.

Theories of amoeboid movement

Amoeboid movement, particularly as manifest by Amoeba and allied genera, has attracted many workers. The most recent analysis, Allen (I96I), based on careful observation of the protoplasmic streaming, has been summarised in the 'fountain zone contraction hypothesis.'

* this may be equivalent to the 'hyaline cap', see Allen (I96I)

This is based on evidence that the endoplasm is pulled forwards by contractile tension developed in the anterior region, where the endoplasm becomes everted to form the ectoplasmic tube. There is a posterior recruitment zone, see Fig. 15, P.59

In attempting a comparison of amoebocyte movement with that of the amoebae previously studied it must first be emphasized that observations on amoebocyte streaming may indeed be partly aided by the large size of the spherules, here presumed to illustrate this motion, but the small size of the actual cell has hindered observation of fine detail. It has not been possible to differentiate ectoplasm and endoplasm in the spherule amoebocytes studied. The very thin clear area round the periphery of the cell, 'C' in Fig.14 and Fig.16, may be hyaline ectoplasm: it is impossible to be certain whether the clear 'pseudopodium', see Fig.16, initially formed, is ectoplasmic or equivalent to the hyaline cap of amoebae.

The patterns of streaming in amoebocytes, as detected by spherule movement, are varied, but even in the simplest monopodial amoebae streaming is complex and subject to wide variation in details, Allen (1961). The details of streaming in amoebocytes however may differ from the 'typical' streaming of amoebae, but are not necessarily incompatible with Allen's hypothesis. Such differences are the following:

1. There may be an anterior recruitment zone in an amoebocyte, see Fig.14, in which forward streaming emanates from the mid-region, with some slow posterior recruitment.

2. The apparent passage of a constriction, and apparently therefore an active contraction, periodically down the cell from the anterior pseudopodium to the mid-region of an actively progressing cell.

3. The lagging of the tail, and occasional contraction of this area towards the main body of the cell.

It is possible that these features are occasioned by less efficient locomotion perhaps caused by the conditions obtaining under a cover-slip. The lagging of the tail region would be directly caused by slow posterior recruitment.

THE DISTRIBUTION, INCIDENCE AND ORIGIN OF THE COELOMIC CELLS

THE DISTRIBUTION OF THE COELOMIC CELLS

The spherule amoebocytes are ubiquitous, being found throughout the echinoid tissues; in the haemal sinuses of the gut and gonads, the axial organ, test, spines and tube feet, besides the perivisceral and peripharyngeal coeloms, the watervascular and the haemal system.

It is difficult to ascertain whether the bladder amoebocytes and flagellated cells occur elsewhere than the coelomic and similar spaces, as they are less distinctive in appearance. The bladder amoebocytes may be capable of penetrating other tissues, although they are not very active in vitro.

The bladder amoebocytes are found in the perivisceral and peripharyngeal coeloms, and smaller proportions occur in the watervascular and haemal systems.

The flagellated cells occur in the perivisceral and peripharyngeal coeloms. They may also occur in the watervascular system, see below, but have not been found in the fluid examined from the haemal vessels.

It was only possible to estimate cell counts from the perivisceral and peripharyngeal coeloms; the methods used and details of results obtained will be discussed below, p. 69— . However, the following generalisations as to content of the principal spaces may be made;

I. The content of the perivisceral and peripharyngeal coeloms is probably identical; both contain all the cell-types described, p. 16 — 18 , in similar concentrations,

see p. 74

2. Fluid extracted from ampullae of the watervascular system contains red and colourless spherule amoebocytes, but as far as can be estimated, in smaller concentration than in the perivisceral and peripharyngeal coeloms, and bladder amoebocytes in much smaller concentrations. There were very few flagellated cells, and of those seen, it was not certain that they were not derived from the lining of the watervascular channels, see also p.87.

3. The internal marginal vessel of the haemal system of Echinus esculentus contained red and colourless spherule amoebocytes and a few bladder amoebocytes. Amoebocytes of both types were clumped around greenish granular material.

4. The siphon of E.esculentus contained a few spherule amoebocytes, which were however, 'rounded off', and non-motile, and were probably derived from the haemal vessels which pass around the siphon, see Fig. 31 , p.226, which would be punctured in withdrawing fluid from the siphon. There no other cells, but a quantity of fine granular material was present.

THE CONCENTRATION OF COELOMIC CELLS IN THE COELOMIC FLUID

Attempts at estimating the concentration of coelomic cells were principally confined to the perivisceral coelom as the larger volume involved reduced the probable error.

Methods

Accurate measurement of the numbers of cells in the perivisceral coelom is not possible, due to the coagulation

of the cells, which occurs rapidly on removal from the coelom. Measurement is also affected by the mobility of some of the cells and the tendency of some to float and others to remain on the substratum. These difficulties were noted by Endean (I958) who studied the coelomic cells of Holothuria leucospilota, 'coelomocytes present in the coelomic fluid showed a marked tendency to clump together when coelomic fluid was withdrawn from the body of the holothurian and thus vitiated attempts to make accurate counts of coelomocyte numbers by the use of the haemocytometer.' The use of fixation, see p.72 , or anti-coagulants to overcome coagulation effects, alters the form of some of the cells and obscures their identity.

Henri (I906) and Schinke (I950), used haemocytometers to estimate concentration of echinoid coelomic cells, (see below for details), but Schinke only used this device to obtain a uniformly thick film, resorting to cell counts per ocular field, as she found the scale unsuitable. She examined the cells quickly to avoid coagulation effects.

In the present study a Fuchs-Rosenthal haemocytometer was used, with a count of I0-20 squares per sample. Further details of the samples will be given as appropriate.

Previous estimates (for living material)

Henri (I906) used Thomas's haemocytometer to estimate concentration of cells per cubic millimetre of the perivisceral coelomic fluid of Paracentrotus lividus and Spatangus purpureus. He obtained the following values, p. 71 .

Estimate of cell concentration in the perivisceral coelomic fluid- Henri (I906)

Cell type	CELLS PER CU. MM. P.lividus			S.purpureus
Individual no.	I	2	3	
Bladder amoebocytes	7080	5600	4400	900
Flagellated cells	I20	250	240	5600
Red spherule amoebocytes	4o	600	340	I90
Colourless spherule amoebocytes	40	480	680	240
Green spherule amoebocytes				I20

The total maximum concentration for Paracentrotus lividus was therefore 8600 cells per cu. mm.

Yeager and Tauber (I935) published 'haemolymph cell counts' for 26 marine invertebrates, including Arbacia punctulata. The average count for the perivisceral fluid of 20 individuals, measured by a dilution haemocytometer, was II,300 cells per cu. mm.

Schinke (I950) found an average total of 3800 cells per cu. mm. of the perivisceral fluid of Psammechinus miliaris; comprising I9I0 bladder and filiform amoebocytes, 9I0 colourless spherule amoebocytes, 500 red spherule amoebocytes and 480 flagellated cells.

RESULTS OF PRESENT STUDY

In the present study the following results were obtained;

Firstly, the effect of fixation, see p.12 , was explored, to see if this was more satisfactory than estimates based on living cells, Table 5.

Table 5 Psammechinus miliaris, fresh animal, June 1962.
Width across ambitus 45 mm.
A= cells fixed with formalin vapour
B= living cells

CELL TYPE	CELLS PER CU. MM.			
	A		B	
Bladder & filiform a/cytes	42800	35%	91200	53%
Colourless spherule "	2800	2%	24000	14%
Red " "	11600	9%	27600	16%
Flagellated cells	67200	54%	29200	17%
TOTAL	124400		172000	

Although, therefore,'formalin vapour'avoided difficulties due to motility of the living cells, the large variation shown above, although it may represent a 'normal' variation under these artificial conditions, probably reflects identification confusion due to this same lack of motility. Thus in the living sample flagellated cells might be identified by their characteristic movement, but the

endoplasm of bladder amoebocytes may be mistaken for a flagellated cell in a group of fixed cells.

By way of comparison estimates of living cell numbers were made from a healthy animal that had been kept in the aquarium for I8 weeks, Table 6.

Table 6 Psammechinus miliaris, aquarium animal, received I8 weeks previous to test. June I962.
Width across ambitus 30 mm.
Living cells.

CELL TYPE	CELLS PER CU. MM.	
Bladder & filiform a/cytes	37200	35%
Colourless spherule "	8800	8%
Red " "	I9600	I8%
Flagellated cells	43200	39%
TOTAL	I08800	

Further estimates were made from the perivisceral and peripharyngeal coeloms of a ripe male, Table 7, p.74.

Table 7 Psammechinus miliaris, ripe male, July 1963.
Width across ambitus 28 mm.
Living cells.

PV perivisceral coelom PP peripharyngeal coelom

CELL TYPE	CELLS PER CU. MM.			
	PV		PP	
Bladder & filiform a/cytes	44800	68%	52000	54.5%
Colourless spherule "	6000	9%	14800	15%
Red " "	8000	13%	14000	14%
Flagellated cells	6800	10%	16000	16.5%
TOTAL	65600		96800	

Diadema antillarum; The % concentration of perivisceral coelomic cells was estimated to be;

Bladder & filiform amoebocytes 25%,
Colourless spherule amoebocytes 13%,
Red spherule amoebocytes 18% and
Flagellated cells 38%

From the tables and figures given it is seen that the percentage concentration of the various cell types in the

perivisceral coelomic fluid varies as follows;

Bladder & filiform amoebocytes	35-68%
Colourless spherule amoebocytes	2-14%
Red spherule amoebocytes	9-18%
Flagellated cells	17-54%

These figures may be contrasted with those of Liebman, (1950), who studied Arbacia punctulata, and found that of 1608 cells from the perivisceral coelomic fluid of 4 individuals, 50.4% were 'trephocytes' (= spherule amoebocytes).

DISCUSSION

The concentration of cells which I have measured in the perivisceral coelomic fluid of Psammechinus miliaris is much greater than some of the previously published estimations, see p.71.

However, the concentration of these cells per cu. mm. is not disproportionately large when compared with the volume occupied by these cells, see below, p.77.

It must be emphasized however that these findings have limited values. In the first place, as already noted, sampling is inclined to be erratic. Furthermore the technique is limited, and shortage of material has not allowed sufficient measurements to determine the extent of individual and seasonal variation.

It is interesting, nevertheless, to compare the concentrations obtained with figures published for

concentration of cells in body fluids of other invertebrates;

Yeager and Tauber (1935) measured cell concentrations of the 'haemolymph' of a number of marine invertebrates, with a haemocytometer technique. The following figures show the range of concentration obtained;

SPECIES	CELLS PER CU. MM.
Crago vulgaris	8300
Palaemonetes vulgaris	2700
Pagurus pollicaris	26000
Arbacia punctulata	11300
Asterias forbesii	6300
Venus mercenaria	9000
Ostrea virginica	22400
Amphitrite ornata	47400
Phascolosoma gouldii	78900
Limulus polyphemus- adult	30000
young	14600

Endean (1960) calculated that the blood of the ascidian Phallusia mammillata, contained 68,000 cells per cu. mm., These concentrations are, of course, very low as compared with the concentration of cells in human or vertebrate blood, the average human blood concentration being 4-5 million erythrocytes per cu. mm.

THE PACKED CELL VOLUME

It is extremely difficult to measure the total volume occupied by the coelomic cells of Psammechinus miliaris because of the small size of the individual, and the relatively small volume involved. Measurements were therefore principally confined to the larger species, Diadema antillarum.

The volume occupied by the freshly clotted cells was measured by the displacement of fluid in a cylinder measuring to 0.02 ml. Cells were not centrifuged to a 'standard' R.P.M. because it was felt that the small amount of material would be reduced disproportionately at transference to and from the centrifuge. The results obtained, Table 8, represent minimum values for the individuals as a certain loss was unavoidable.

TABLE 8

SPECIES	INDIVIDUAL NOM	VOLUMES in ml.		
		FLUID	CELL	CELL as % FLUID
P.miliaris	I	7.45	0.04	0.5%
	2	2.00	0.02	I.0%
D.antillarum	I	9.37	0.I2	I.2%
	2	I6.57	0.25	I.5%
	3	8.33	0.05	0.6%
	4	7.35	0.I0	I.3%
	5	I6.35	0.30	I.8%
	6	24.I5	0.65	2.6%
	7	23.55	0.45	I.5%

If the average volume of one of the coelomic cells is assumed to be 125 cu.micra, then the following calculations may be made:

P.miliaris

Calculated concentration per cu.mm. = 42,000-80,000 cells.

D.antillarum

Calculated concentration per cu.mm. = 48,000-2,500,000 cells, with an average concentration of 140,000 cells per cu.mm.

In summary, the volume of coelomic cells in the perivisceral coelomic fluid of P.miliaris and D.antillarum are an average of 0.6% and 1.78% of the fluid volume respectively.

THE WEIGHT OF CELLS OF THE PERIVISCERAL COELOM

Table 9, p.79, summarises the weight relationships of the coelomic cells of mature animals to the other major tissues. The coelomic cells varied from 1-4% of the dry weight of the gut and gonads.

TABLE 9

THE WEIGHT OF THE PERIVISCERAL COELOMIC CELLS COMPARED WITH THE WEIGHT OF OTHER TISSUES OF PSAMMECHINUS MILIARIS

All weights are in grammes

INDIVIDUAL NO.	1	2	3	4	5
Diameter of animal	40 mm	35 mm	30 mm	25 mm	23 mm
Date	7.62	8.62	8.62	8.62	8.62
Sex	Male	Male	Male	Male	Female
Total wet weight	25.2	18.73	7.01	9.26	5.89
Wet weight coelomic fluid	5.89	3.48	1.135	0.49	0.264
Wet weight coelomic cells	0.67	0.065	0.075	0.036	0.02
Dry weight coelomic cells	-	0.005	0.005	0.003	0.001
Wet weight gut	-	0.634	0.426	0.388	0.235
Dry weight gut	-	0.153	0.096	0.087	0.050
Wet weight gonads	-	1.687	0.838	0.788	0.255
Dry weight gonads	-	0.283	0.137	0.190	0.052
Coelomic cells % dry weight of gut & gonads		1	4	< 1	1

THE ORIGIN OF THE COELOMIC CELLS

Historical

Geddes (I880) who first described the coelomic cells of echinoids in detail, noted a resemblance between the flagellated cells and the smallest of the amoebocytes. He also described the occurrence of intermediate forms. He believed that the flagellocytes were derived from the coelomic epithelium, but gives no opinion as to the derivation of the colourless spherule amoebocytes and had the rather unusual notion that the red spherule amoebocytes are derived from non-nucleated masses of yellowish-green material , see p. 225 (and p. 69) found in the haemal system.

Prouho (I887) presented evidence that the flagellated cells of Dorocidaris papillata (now = Cidaris cidaris) are produced in the sub-madreporic vesicle.

Cattaneo (I89I) introduced a more radical approach in a paper primarily devoted to crustacea*, suggesting that the flagellated cells might be protozoan parasites. Cuénot, (I900, I9I2), supported this idea and enlarged it. The question of the origin of these cells henceforth became involved with the more radical question of their nature, to which reference will shortly be made.

Kindred (I924) found that it had not been proven that the red spherule amoebocytes are derived from other cells by the ingestion of pigment from food, and believed that they might be directly derived from the pigmented cells of the larval echinoid.

Liebman (I950) believed that the flagellated cells

* not obtainable

are detached cells of the coelomic epithelium, and stated that he never observed division of coelomic cells in his hanging drop preparations. He thought that the 'fibroblasts' (= filiform amoebocytes) and 'macrophages' (= bladder amoebocytes) were derived from the flagellated cells and that the green spherule amoebocytes were also produced from the flagellated cells. The colourless spherule amoebocytes and the red spherule amœbocytes were thought to be derived from the green spherule amoebocytes because the colourless spherule amoebocytes sometimes have a greenish hue, see comment p. 47 , and the red spherule amoebocytes 'sometimes have green inclusions.'

Schinke (1950) followed Cattaneo's and Cuénot's suggestion, see below, that the flagellated cells are parasitic; 'Cuénot und Cattaneo vermuten, das die Giesselzellen, sondern Parasiten sind.' In investigating the origin of the other coelomic cells she reported that Psammechinus miliaris could completely and repeatedly regenerate coelomocytes in the absence of gut, axial organ, gonads or Aristotle's lantern. She therefore concluded that coelomic cells were formed either in the connective tissue matrix of the test; 'Matrixgewebe', in the endothelium of the watervascular system or in the coelomic peritoneum. She further stated that histological investigation of the latter 2 types of tissue did not reveal any kind of cell which she could regard as a coelomocyte precursor, but the 'Matrixkerne' of the test were regarded as a more promising source. However no mitotic figures were evident in any of these tissues; 'In keinem Fall waren Mitosen oder Amitosen zu beobachten.'

To substantiate her ideas she made calculations of the number of coelomocytes regenerated after each withdrawal and compared them with the gradual decrease of nuclei in the test, in sections of known dimensions. The correlation was found to be good, and Schinke therefore concluded that this tissue was responsible for much of the regeneration observed. She believed that amoebocytes with red and colourless inclusions are derived by transformation of the cells in the test, and that the leucocytes (= bladder amoebocytes), are in turn derived from the colourless spherule amoebocytes.

THE NATURE OF THE FLAGELLATED CELLS

As already noted, Cattaneo's (I89I) suggestion that these cells are parasitic was favoured by Cuénot (I900, I9I2). He assigned them to the genus Oikomonas (Saville Kent), with the specific name echinorum (I900), but he was a little doubtful about the suitability of this classification because the genus is characterised by an ability to become temporarily attached to the substratum, which he never observed in the coelomocytes. His first, (I900) description was very brief, and was later (I9I2) amplified. I append his diagnostic description of Oikomonas echinorum Cuénot, Appendix I. These cells clearly correspond with those I have already described, p. 31.

Cuénot (I9I2) already expressed doubts as to the validity of considering the cells to be parasites, and reaffirmed this as late as I948, saying; 'On ne sait trop si ce sont des elements normaux du liquide coelomique

ou bien des Flagelles parasites du genre Oicomonas.' Despite this Hyman misrepresents the situation, saying, (I955), 'Cuénot (I9I2) mentions a little flagellate, Oikomonas echinorum, (probably incorrectly named) common in the coelomic fluid of urchins on the French coast, apt to be confused with the vibratile cells of this fluid.'

Schinke (I950) however, accepts the original thesis without comment, see quotation p. 81 , whereas Boolootian and Giese (I958) are non-commital, and state that the flagellated cells might be protozoan parasites, and that it is an interesting problem.

It is, indeed, an interesting problem, and a very difficult one, for the solution depends ultimately on a demonstration of the origin of the flagellated cells, an achievement which has not yet even been adequately attempted; for most authors, see p. 31 , have assumed, as did Geddes (I880) that the flagellated cells are truly echinoid, possibly derived from the coelomic peritoneum, and have not enquired further.

Although there is little direct information on this subject there is circumstantial evidence of some importance, which may be divided into two categories, that which is related to the protozoan and that which is related to the echinoid affinities of the flagellated cells.

I. The flagellated cells as protozoa

First, as regards their supposedly parasitic nature, Cuénot (I9I2, I948) admitted that their occurrence in almost every echinoid investigated 'n'est pas très favorable à l'hypothèse parasitaire.' It is also noteworthy that they have never been observed to divide.

Second, concerning their supposedly protozoan character, Prof.H.Sandon, (personal communication) thinks that the flagellum length varies rather too much and that the nucleus is too large in the flagellated cells of Diadema antillarum, to be Oikomonas or any other flagellated protozoan.

Further, Tartar (I950) found that dilute solutions of nickel sulphate would stop the activity of the cilia of all species of Paramecium studied, also Spirostomum, Condylostoma, Stentor and 'many others', and that the flagella of euglenae were likewise immobilised. In fact, as Seaman (I955) noted, this substance seems to specifically retard ciliary and flagellar structures in protozoa, but has no effect on similar metazoan structures, such as the cilia of rotifers and oysters. Experiments I performed in I960 showed that normal solutions of nickel sulphate did not affect the ciliary beat of Mytilus gill but stopped Epistylis. In I96I it was found that 0.0I% nickel sulphate in tap water stopped Euglena species, but had no affect on Cirratella (?), a rotifer. 0.03% nickel sulphate stopped a dinoflagellate, but had no affect on the flagellated cells of Diadema antillarum, or the flagella of the coelomic peritoneum.

Finally, some remarks on the classification of these cells as oikomonads may be added:

Oikomonads, as characterised by Saville Kent (I880), and redefined by Hollande (I948), see Appendix I, possess an anterior flagellum, usually longer than the body, and may become temporarily attached to the substratum. Echinoid flagellated cells, on the other hand, have a posteriorly attached flagellum, which is usually more than twice the

length of the body. These cells, as Cuénot (I9I2) noted, have never been observed to attach to the substratum in the manner described for oikomonads.

Again, oikomonads have been described as being plastic and unstable in form, and some species have anterior asymmetrical projections. Although there is variation, see p.32-3, in the size and form of the echinoid flagellated cells, the form of an individual cell could not be described as plastic. The form is typically ovate or globular, and only becomes asymetrical under lethal conditions.

Moreover, modern taxonomists, e.g. Manton and Parke, (I960), have used the fine structure of the flagellum as a guide to classification of the flagellates; the presence of lateral and terminal 'hairs' being used as a taxonomic feature. Chrysomonads, to which group the Oikomonadina are assigned, have pantonematic flagella,or, where the organism if biflagellate, one flagellum is pantonematic and one is acronematic. There is no evidence of any mastigonemes on the flagellum of the echinoid cells, (as indeed would be expected if they were truly metazoan.)

As far as can be determined therefore, Cuénot's suspicion (I9I2),that his classification was incorrect appears to be fully substantiated. The echinoid flagellated cells do not correspond with the descriptions of oikomonads.

2. The possible relation with the peritoneum

As has already been mentioned Geddes (I880), and subsequent authors have considered that the flagellated cells may be derived from the ciliated coelomic

peritoneum.

I have attempted to compare these cells with the coelomic cells in order to elucidate a possible relationship. However, the coelomic peritoneum is not easily studied in fixed or living preparations because the cells are very small. It was possible however to measure the overall diameter of the cells (which are globular) in specimens of Echinus esculentus, from which samples of the epithelium are more readily obtained than from the small Psammechinus miliaris.

The average diameter of 50 living flagellated cells from the perivisceral coelom was 6.36 micra, with an variation of 4.4-8.8 micra, whereas the average diameter of 50 living ciliated peritoneal cells was 4.84 micra, with a range of variation of 3.3 to 7.7 micra. An attempt was made to obtain similar measurements for P.miliaris. It was extremely difficult to detach peritoneum in a suitable condition, and, eventually, detached cells from the inner surface of ampullae were examined. These were estimated to have a diameter of 3.3-4.4 micra, whereas the coelomic flagellated cells had a diameter of 3-9 micra, with an average of 5.85 micra.

The nucleus of the peritoneal cells appears to be relatively large. Indeed, in fixed preparations, very little cytoplasm is detected. The 'cilia' are quite long, probably at least 40 micra, but accurate measurements have not been possible.

From the rather limited data obtained therefore it seems that the coelomic peritoneal cells are smaller than the flagellated coelomic cells in average measurements, but overlap in the case of individual cells. It is, therefore, possible that the latter derive from the

former. However, this is only one aspect of the situation. It was not possible to determine the detailed morphology of the peritoneal cells, as they are small and difficult to visualise.

Perhaps the most significant observation as to the possible affinities of the flagellated and peritoneal cells is the fact that where the peritoneum was ruptured and peritoneal cells became detached, it was impossible to determine whether individual cells were 'peritoneal' or bona fide 'coelomic flagellated cells.'

THE ORIGIN AND RELATIONSHIPS OF THE COELOMIC CELLS

We may now consider the origin of the coelomic cells in general. The question of the origin of the cells in body fluids of animals is perennially difficult. According to Holmes (I96I) the presently used criteria for determining 'lymphoid' tissues are wholly inadequate. So far as I have been able to determine few of the many supposed sites of origin of invertebrate coelomic and blood cells are established by reliable evidence. Thus, as far as echinoderms are concerned, Prosser and Judson, (I952), mention sites of haematopoiesis in the haemal system of Stichopus californicus, but their reasons for this statement are not presented.

A very important contribution to studies of the origin of echinoid coelomocytes was that of Schinke, (I950), already reviewed, p. 81 . Her findings are of great relevance but include some puzzling features. Thus, her estimate of cell numbers, which she calculated per cu. mm. of coelomic fluid, was distinctly low for normal animals, especially when compared with the figures given

by Henri (I906), for Paracentrotus lividus, which has, in my experience, far fewer coelomic cells than Psammechinus miliaris. Schinke's estimates of concentration for the latter species, based on an average from at least 6 individuals, was 44% of the concentration Henri calculated, from 2 individuals, and 5.5% of the lowest concentration I have calculated for P.miliaris, see p.71-5.

It is, moreover, difficult to understand how Schinke's experimental animals survived the injection of sea water which she used to replace the coelomic fluid. Sea water has a higher pH than the coelomic fluid, and causes degenerative changes in the body tissues, as I have observed when examining living preparations. In view of this I attempted to repeat Schinke's experiments, using Psammechinus miliaris and Echinus esculentus. Of I7 P.miliaris from which coelomic fluid was removed, via the peristome, (this was considered to be less damaging), and replaced with sea water, none survived more than 4 days. The average life of I0 E.esculentus in which the coelomic fluid was replaced by sea water, was 3.2 days. In the experiments described by Schinke however such animals survived for much longer periods. She records maintaining some operated animals for I8 days.

Schinke did not eliminate the likely replacement of the coelomic amoebocytes by migration from remaining tissues, most of which must have contained considerable numbers of these cells. Neither does she comment on the replacement, which her figures show, of the flagellated cells. If these cells are parasitic, as she assumed, then their replacement after removal of the coelomic cells either implies re-infection in a very short time, with a phenomenal multiplication - and no division of these

or any of the coelomic cells has ever been observed, - or multiplication by a residuum of cells, from a source different from that which she considered responsible for replacing the amoebocytes. If the latter explanation were true then it demonstrates a possible source of coelomic cells which her experiments failed to eliminate.

It is interesting to recall that Kindred (I924) found that leucocytes of Arbacia punctulata seemed to be involved in skeletal regeneration after injury. He says, 'it is well known from the observations of Théel and others that in the test of Echinoidea the leucocytes form syncytia within which is secreted (intracellularly) the spicules which fuse and form the stereom.' It seems therefore that some at least of the dermal cells are derived from leucocytes, and might readily return to the coelom, as Schinke believes.

Schinke's conclusions that the coelomic amoebocytes are derived by transformation of cells from the dermal region may indeed be valid. I have been unable to repeat her experiments however, and it is clear that tissues such as the peritoneum and the haemal tissue, which she did not seriously consider as possible sources of amoebocytes might merit more attention.

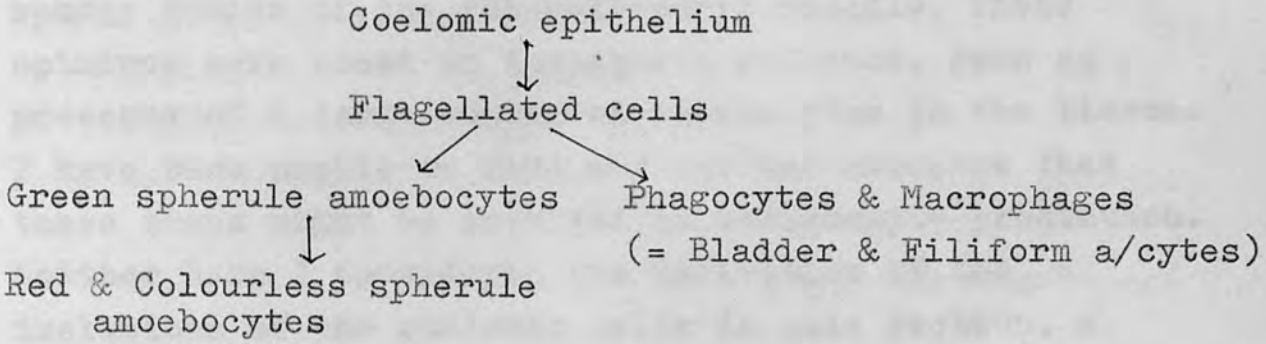
There is no need to assume that the coelomic cells are closely inter-related, and some similarities may merely reflect similarity of environment and 'convergence'. However, there are similarities which cannot be fortuituous and may truly indicate a close relationship. It is perhaps easiest to relate the red and colourless spherule amoebocytes, and it has long been considered that the red cells might be derived by pigmentation of

the colourless cells. This idea might well be confused with the reports that echinochrome exists in a reduced state in some echinoids, so that 'colourless' spherule amoebocytes may become red on exposure to air, see Jacobson and Millott (1953). It seems unlikely that the red spherule amoebocytes are directly derived by pigmentation of the colourless spherules, for the inclusions seem to be different both in chemical constitution, p. 164-165, and in structure, p. 51 and p. 148. The colourless inclusions are 'solid', whereas the red spherules appear to be capsules enclosing a viscous material incorporating the pigment. For these reasons it is unlikely that the red spherules are directly derived from the colourless ones. However these amoebocytes are so similar in form and locomotion that they may be derived from a common source.

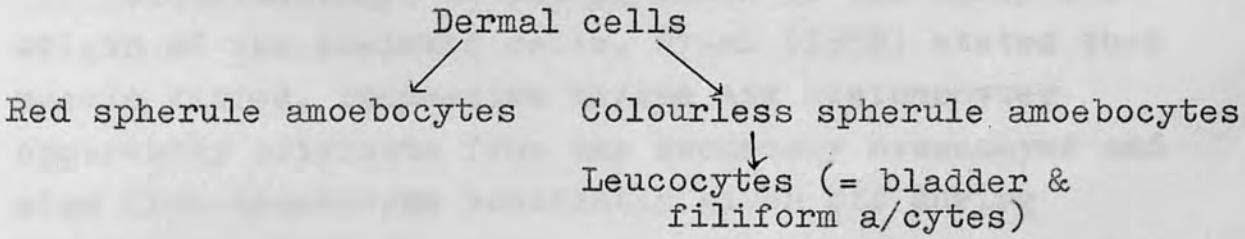
I have observed small colourless inclusions in the 'tail' region of red spherule amoebocytes, see p. 56, but they have resembled the small inclusions of some flagellated cells rather than the large inclusions of the colourless spherule amoebocytes. Indeed, I have observed two pink inclusions in a flagellated cell, and some flagellated cells contain larger, angular colourless inclusions, apparently identical with those of the colourless spherule amoebocytes. Moreover, see p. 148, some flagellated cells have a similar chemical 'inclusion' constitution to that of the colourless spherules. Flagellated cells which have lost their flagellum might readily be mistaken for small colourless spherule amoebocytes, or as Liebman (1950) thought, small bladder amoebocytes.

Liebman (1950) favoured the following scheme of

inter-relationship in Arbacia punctulata:



Schinke (1950) however concluded that, in P.miliaris, the bladder and filiform amoebocytes were derived from the spherule amoebocytes; the flagellated cells she believed to be parasitic:



It is not yet possible to decide, finally, which, if either, of these two opinions is correct, but I believe that Liebman's may prove to be. As already stated, p. 83 et seq. the evidence that the flagellated cells are parasitic appears somewhat flimsy, and indeed is outweighed by evidence of their echinoid origin for derivation of these cells from the coelomic peritoneum appears possible. Again, derivation of the coelomic cells from the coelomic peritoneum is easier to envisage than origin from the more enclosed and remote dermal region, not the least because proliferation is a widespread attribute of peritoneal cells.

I have not dealt here with the historical opinions that coelomic cells are derived from the axial organ, spongy bodies or the sub-madreporic vesicle. These opinions were based on inadequate evidence, such as presence of a large number of amoebocytes in the tissue. I have been unable to find any further evidence that these areas might be involved in coelomocyte production. Neither have I considered the derivation of the inclusions of the coelomic cells in this section, a problem which is undoubtedly relevant to their possible origin, but which is better considered later, p. 118.

Embryonic origin of the coelomic cells

Unfortunately, little is known of the embryonic origin of the coelomic cells. Hyman (1955) states that muscle fibres, connective tissue and coelomocytes apparently originate from the secondary mesenchyme and also from mesenchyme constantly given off during development from the walls of coelomic spaces. She does not disclose the source of information however, which is regrettable in view of the interest of a possible origin of these cells from the primitive coelomic 'peritoneal' region.

In this context larvae of Psammechinus miliaris were reared, and although it was not possible to maintain them beyond the pluteus stage, some information was obtained.

Firstly, it was found that with this species, which has non-pigmented eggs, pigmented cells first appear in the primary mesenchyme of the gastrula, which is similar

to the findings of Young (1957), on Lytechinus variegatus. At the gastrula stage the pigmented cells are only faintly pink; the pigment appears to be dispersed throughout the cell and is not enclosed in inclusions, Fig. 22, p. 124 At the pluteus stage pigmented cells are much more numerous, and the pigment is concentrated in inclusions, Fig. 23, p. 125 . Young (1957) states that the 'echinophores' are amoeboid, but I was unable to detect movement comparable to that exhibited by amoebocytes. This, and the fact that the pigmented inclusions are small and irregular in shape in these cells, distinguished them from the adult red spherule amoebocytes. It is, however, possible that these cells are direct fore-runners of the spherule amoebocytes. I was unable to identify any other cells comparable to amoebocytes or coelomocytes of the adult, in the pluteus.

PHAGOCYTOSIS AND COAGULATION

PHAGOCYTOSIS AND COAGULATION

PHAGOCYTOSIS: Historical

Durham (I888) noted that 'insoluble granules' introduced into the body cavity of the asteroid, Asterias rubens, were ingested by leucocytes which migrated to the exterior through the dermal branchiae. He did not repeat the experiments with echinoids, but suggested, (I892), that they would react similarly. Cernovodeanu and Henri (I906) studied Spatangus purpureus and Strongylocentrotus (= Paracentrotus) lividus. They found that bacteria injected into the perivisceral cavity were phagocytosed by the 'amoebocytes avec longs prolongements'.

Awerinzew (I9II) found that coelomic cells with ingested carbon or carmine would leave the body via the gills in Strongylocentrotus drobachiensis. Kindred (I92I) investigated cells of Arbacia punctulata. He injected sea water suspensions of carmine and indian ink into the perivisceral fluid and examined samples of the perivisceral fluid at intervals. He found that the 'leucocytes' (i.e. bladder & filiform amoebocytes) were phagocytic, taking up carmine within 30 minutes of its introduction into the coelom. None of the other cells were found to be phagocytic. He noted that 2 weeks after the injection some of the leucocytes in the perivisceral cavity still contained carmine. Bookhaut and Greenburg, (I940) studied Mellita quinquiesperforata, and found that the 'leucocytes' (= bladder & filiform amoebocytes), and 'amoebocytes with scattered brown particles' phagocytosed

carmine particles.

Liebman (I950) described 4 types of phagocyte from the perivisceral fluid of Arbacia punctulata; flagellated, amoeboid, fibroblast and petaloid phagocytes. He presents no convincing evidence of the phagocytic capacity of the flagellated cells and says, 'both in vitro, as well as in fixed preparations, the cytoplasm occasionally shows trephocytic granules, suggesting a certain phagocytic capacity'. He records that the petaloid cells (= bladder amoebocytes) show the highest phagocytic ability, whereas in the fibroblasts (= 'extended' filiform amoebocytes, see p. 108), the 'phagocytic capacity appears to be considerably reduced'. He described the process of phagocytosis as follows; 'the amoeboid forms phagocytose in the familiar way of engulfment with pseudopods'. He attributes the high phagocytic capacity of the petaloid cells to their ability to act unimpeded in 3 dimensions, and reported that whole living trephocytes (= spherule amoebocytes) were repeatedly observed to be attacked by single, or occasionally several, phagocytes, and thought that this represenbed a nutritive function.

Stott (I952) recorded that trypan blue injected into the coelom of Echinus esculentus, was taken up by amoebocytes and that after I4 days it was found in the haemal and watervascular systems.

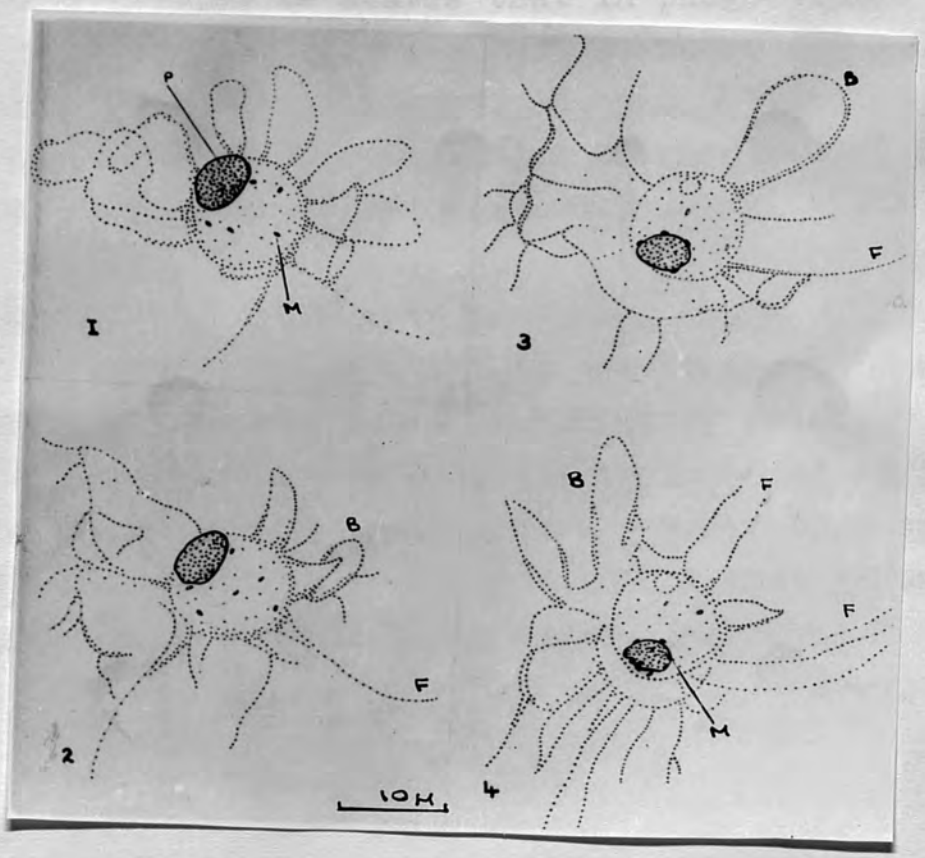
It is, therefore, generally agreed that the cells now called bladder amoebocytes, are actively phagocytic. Reports that other cells show phagocytosis are uncorroborated. The phagocytic capacity has been associated with both excretory and nutritive functions.

Thus Liebman (1950), as already noted, connected phagocytosis with nutrition, and Stott (1955) believed the phagocytes invade the gut lumen and become involved in intracellular digestion, see discussion later. Hyman (1955) associated the activity of phagocytic amoebocytes with reports of accumulation of inert substances in the axial organ, and suggested that this organ acts as an 'excretory' organ, material being accumulated there, by phagocytic activity, before being eliminated via the stone-canal and madreporite.

Observations on phagocytosis

The bladder amoebocytes, and also the filiform amoebocytes are the only coelomic cells which I have observed to actively take up particles, such as carmine and iron saccharate. It is not easy to observe the process of phagocytosis, as the cells are transparent and have very fine processes. Observations made with the oil-immersion objective show that particles are apparently taken up between the ectoplasmic processes, without penetration of the ectoplasm, and are passed rapidly to the perinuclear and endoplasmic region, which they penetrate. There is no evidence of vacuole formation. The ingested particle has been observed to become surrounded by small refractile inclusions which may be mitochondria, Fig. 19. Several particles may be taken up by one cell, and as already mentioned filiform cells do show phagocytosis. However, as explained later, the filiform condition is a pre-coagulation change, and filiform cells do not retain the phagocytic capacity for very long. Fig.19 illustrates a cell which is largely

FIGURE 19



PSAMMECHINUS MILIARIS: Living bladder amoebocyte undergoing slow coagulation change Note enclosure of phagocytosed particle. Cell observed in freshly withdrawn coelomic fluid, under cover-slip, with oil-immersion lens. The nucleus was not visible

1. 0 mins. 2. 10 mins. 3. 35 mins. 4. 50 mins.

B = bladder process F = filiform process

P = phagocytosed particle M = mitochondrion (?)

filiform and still phagocytic.

The phagocytosis shown by these cells appears to be very similar to that described for vertebrate leucocytes by Harris (I96I). He states that in phagocytosis by these cells the particle enters a cell directly and not within a vacuole of extracellular fluid, and apparently enters the cell membrane by penetration as no perceptible invagination of this membrane occurs.

Chemotaxis

The bladder amoebocytes do not appear to show progressive movement so it is extremely unlikely that they show a chemotactic response to injected or 'foreign' particles, unless the particles are very close to the individual cells. It seems more likely that contact with such particles elicits a phagocytic response.

COAGULATION

Historical

Geddes (I880) first described coagulation, of the coelomic cells of Toxopneustes (=Paracentrotus) lividus and Echinus sphaera; 'on ne voit pas de fibrine, mais les corpuscles blancs que nous venons de décrire s'attachent l'un à l'autre, et forment des tas irréguliers ou des masses ne forme de mures,' The 'corpuscles blancs' he described as having long pseudopods, filiform and ramifying, in some cases united to form rings, p.23.

Schäfer (I883) noted inhibition of coagulation by magnesium sulphate, with coelomic fluid of Echinus esculentus, and stated that coagulation was not solely due to plasmodium formation. Théel (I896) studied cells from Paracentrotus lividus and Echinus esculentus. He concluded that the 'white corpuscles' always tend to fuse, but are prevented from doing so in vivo by ciliary currents.

Michel (I888) found that Geddes was incorrect in attributing coagulation to plasmodium formation, for he found that there was no fusion of cytoplasm. Conversely, Cuenot (I89I) states that plasmodium formation occurs, and Dekhuysen (I90I) regarded the filiform pseudopodia, involved in coagulation, as lifeless extrusions. Henri, (I906) made some elaborate comparisons between the coagulating cells and the behaviour of latex in certain conditions!

Kindred (I92I) investigated clotting of the perivisceral fluid of Arbacia punctulata. He found that

clumping of the leucocytes (=bladder amoebocytes) gave rise to a clot, but that in hanging drops typical clot formation did not occur except where the leucocytes came into contact with the glass. He described coagulation changes as follows; 'long filamentous processes are produced by the shrinkage of the membraneous processes' (bladder pseudopodia) 'these filamentous processes adhere to those of neighbouring leucocytes, forming a delicate mesh to which the colourless and pigmented amibocytes adhere.' He was unable to conclude whether true plasmodia were formed.

Donnellon (I938) also studied coagulation in Arbacia punctulata. He found that clotting was dependent on the coelomic cells, and could not occur after these had been filtered off. He determined clotting times under various conditions, the endpoint being the time when the corpuscles 'were precipitated in typical clot formation, made more distinct by the accompanying pigment liberation.' His normal controls usually took 500 seconds to reach this endpoint. He found that extracts of Arbacia, molluscs etc., were effective in hastening clot formation, as were potassium, calcium, strontium and barium ions. Magnesium chloride, I0% peptone, citration and oxalation were found to prevent clotting. Donnellon believed that normal clotting was induced by an injury substance, and that this effect is not due to potassium, but can be duplicated by it. He considered that potassium and calcium might function indirectly to produce the injury substance.

Bookhaut and Greenburg (I940) examined cells of

Mellita quinquiesperforata, and found that the 'leucocytes with lobed pseudopodia' (= bladder amoebocytes) were the only cells to take part in the formation of a clot.

Davidson (1953) found that with Echinarachnius parva, clotting occurs after tissue injury, and the clot is formed exclusively of cellular elements. He also found that calcium removal, 'before white cell breakdown', inhibits clot formation. He thought that the tissue factor was released in the absence of calcium, but was then ineffective.

Boolootian and Giese (1959) investigated the clotting of the coelomic fluid of a number of echinoderms, including the sea urchins, Strongylocentrotus purpuratus, S.franciscanus, S.fragilis, and the sand-dollar Dendraster excentricus. They stated that in all cases the cells which initiate the clot are the filiform amoebocytes, but also state, although they admit temporary agglutination of these cells in sea urchin coelomic fluid, that clotting in sea urchins depends on 'hyaline hemocytes' which rupture to form fibres. Clotting of the coelomic fluid of D.excentricus was found to be dependent on the presence of calcium ions and to be a cellular agglutination in which each cell maintained its identity. Calcium removal did not inhibit coagulation of Strongylocentrotus coelomic fluid.

They suggested that the clot in Dendraster is formed by development of a sticky layer on the surface of the amoebocytes, perhaps as the result of the liberation of a tissue factor. Coagulation of the coelomic fluid of Strongylocentrotus species was inhibited or delayed by substances which form mercaptides with sulphydryl groups

or maintain a reduced state. They therefore suggested that the mechanism of agglutination in these animals is based on the formation of disulphise bonds between cells.

THE ROLE OF THE BLADDER AND FILIFORM AMOEBOCYTES IN COAGULATION

It is clear that there has been considerable confusion as to the role of these cells in coagulation, although Hyman (I955) states that 'all observers agree that the coelomic fluid clots shortly after shedding and that the clot consists of the phagocytic type of coelomocytes whose pseudopods more or less fuse to form a mesh in which the other amoebocytes become entangled.'

The confusion has principally arisen because some authors have described the filiform amoebocytes as distinct and separate types of cell. Geddes (I880) clearly recognised a connection between these cells and the amoebocytes with 'ring-like' pseudopodia, now known as bladder amoebocytes, but some subsequent authors have not accepted this relationship. Boolootian and Giese, (I958) stated that in asteroids, the two cell-types, bladder and filiform amoebocytes, undoubtedly represented phases of the same cell, the bladder amoebocytes being only found in fresh coelomic fluid. However, in their (I959) paper on coagulation, although they state that the filiform amoebocytes, when present, are responsible for coagulation, they do not enlarge on the relationship, and also describe 'hyaline haemocytes' in the regular echinoids studied, which are said to be responsible for

fibre formation whereas the filiform amoebocytes in these species only give temporary agglutination.

In the echinoids I have studied the filiform amoebocytes are a pre-coagulation phase, derived from the bladder amoebocytes under certain conditions.

In the regular echinoids studied, clotting of the coelomic fluid in vitro may be described as occurring in 3 stages;

First, bladder amoebocytes change form; they become 'filiform' and elongated, and tend to become entangled and trap other cells. It is not certain whether they truly become 'sticky' and whether there is active agglutination.

Second, the preceding activity becomes evident as macroscopically visible strings or clumps, often within 3 minues of withdrawal.

Third, at a very variable time after the initiation of the clotting mechanism the clot or clots tend to round off, and contract into a more compact mass. At this stage the clot tends to become darker in colour, turning brown, and, eventually, with bacterial action, green.

Coagulation can therefore be detected and studied at two levels; the microscopic and the macroscopic. Although the involvement of the 'phagocytes' or 'leucocytes' has long been recognised, workers on the coagulation mechanism have usually studied the macroscopic rather than the microscopic effects. This may have led to error where cells have been 'killed' rather than inhibited by reagents or

where, as is often the case, there is considerable variation in the speed and extent of the appearance of the macroscopic clotting.

THE MICROSCOPIC CHANGE OF FORM

The bladder amoebocytes, which have already been described, p. 23 , show fairly continuous but slow change of form on a glass slide.

When coagulation occurs on removal of the coelomic fluid from the urchin, the bladder amoebocytes become 'filiform' and strongly resemble 'untidy' heliozoa. If these filiform cells are examined carefully it becomes evident that at least some of these filiform processes represent the visible axial radii of the elongated and very much thinner bladder pseudopodia, the margins of which are very difficult to see. Further observation reveals a process of attenuation which gives rise to this effect. Fig. 19 shows the gradual transformation of a cell from the bladder-filiform stage, still actively phagocytic, to a more filiform condition. In this cell it was possible to detect that the 'filiform' appendages were often, as already described, visible parts of the flattened 'bladders'. However this may not always be the case, and some of these processes are truly filiform, for they may be independently agitated by vibrations in the surrounding fluid, which would not be possible if Goodrich (1919) were wholly correct. He stated that filiform processes, described by various authors from invertebrate 'leucocytes' do not exist; 'they - are merely representations of optical sections of an extensive membrane, folfed with rounded surfaces. Very soon, however, after the coelomic

fluid has been removed from the animal the membrane begins to stretch out wing-like films in various directions, and these soon acquire a jagged edge with sharp points.'

The change of form of the 'bladder' amoebocyte, illustrated in Fig.I9 was accomplished in about 60 minutes, but in 'normal' coagulation such a change is effected in a very much shorter time; about I minute, and consequently the details of the change are very difficult to record.

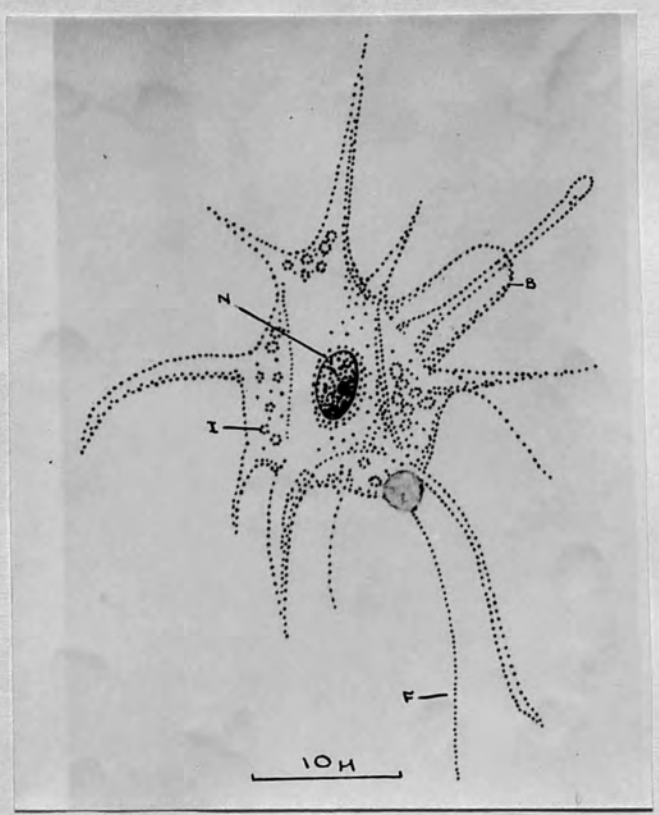
A typical filiform cell produced by the 'normal' coagulation change is shown in Fig.20. The nucleus is still visible but the endoplasm and ectoplasm are not so clearly differentiated as with a bladder amoebocyte, see Fig. I, p.24 , Fig. 3, p.30.

In 'normal' coagulation the filiform cells become further elongated and attenuated, often orientated in a two way fashion, to form threads of cells, and the nucleus becomes difficult to detect, Fig. 2I. This stage is probably equivalent to the cells Liebman (I950) describes as 'fibroblastic phagocytes', for the process simulates fibre formation.

The visible manifestations of coagulation

As already explained, p.104 , the microscopic changes and activity become macroscopically evident when minute clumps and 'strings' of the elongated and entangled cells are formed. It is not possible to state whether plasmodia are formed, but the effect is very similar. The attenuated membraneous processes of the bladder amoebocytes certainly tend to become linked;

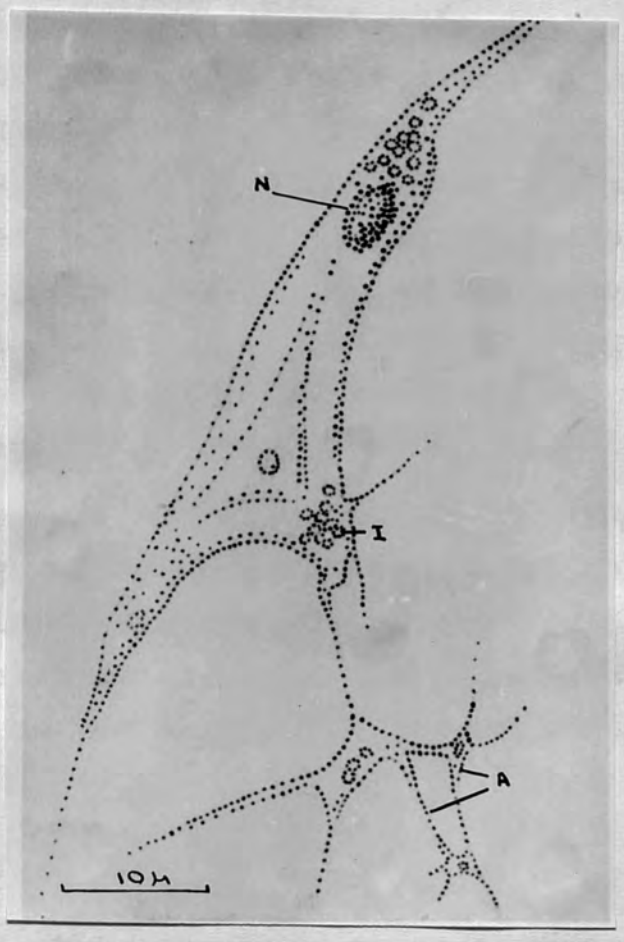
FIGURE 20



DIADEMA ANTILLARUM Living filiform amoebocyte, observed in freshly withdrawn coelomic fluid, under cover-slip, with oil-immsersion lens

N = nucleus I = inclusions F = filiform process
B = bladder process

FIGURE 21



PSAMMECHINUS MILIARIS 'Fibroblast' : filiform amoebocyte transformed by final stage of the coagulation change

N = ill-defined nucleus I = inclusions
A = attenuated cell-processes

whether there is an active attraction or fusion is, as yet, impossible to say. Many other coelomic cells are enclosed and trapped by the processes, but, judging from the facility with which spherule amoebocytes may be observed to glide over the surface of a clot, such 'clots are not 'sticky'. Some clots are found to include most of the coelomic cells of the fluid, excluding only some flagellated cells. This is possibly explained by the provision of a sub-stratum for the spherule amoebocytes; these cells probably move onto the coagulating cells because a sub-stratum is provided, and many of the spherule amoebocytes would then be entangled.

Stage 3 of coagulation, see p. 104, occurs when the strings and clumps of coagulated cells tend to contract into more compact masses. This 'contraction' varies considerably in extent and effect, and is principally dependent on the initial degree of contact between strings of cells.

THE MECHANISM OF COAGULATION

INHIBITION OF CLOTTING

I. The importance of calcium

It has long been known that the removal of calcium ions by oxalate or citrate is an effective anti-coagulant for mammalian blood, and students of the coagulation of invertebrate fluids therefore explored the importance of calcium to these clotting mechanisms.

Donnellon (1938) found that coagulation of the

coelomic fluid of Arbacia punctulata was calcium dependent, and Davidson (1953) found the same with Echinarachnius parva. However although Boolootian and Giese (1959) found that clotting was calcium dependent in Dendraster excentricus, they found that calcium removal did not affect coagulation of the coelomic fluid of Strongylocentrotus species.

It should be noted that although similar concentrations of sodium citrate were used by these authors, e.g., 0.3M by Donnellon and 0.14M by Boolootian and Giese, the former used a ratio of twice the volume of coelomic fluid, whereas the latter used a ratio of 1 part anti-coagulant to 9 parts coelomic fluid.

In the present study it was found possible to inhibit clotting of the coelomic fluid of Diadema antillarum, Paracentrotus lividus and Arbacia lixula by the addition of sodium citrate to make an approximately 0.35% solution, but the fluid of Echinus esculentus, Psammechinus miliaris and Spatangus purpureus was not affected.

The procedure adopted by Boolootian and Giese (1959) when using Sequestrene, potassium oxalate and sodium citrate as anti-coagulants, was repeated with P.miliaris, and again, little inhibition was detected.

I thought that the effectiveness of the chemical might be a factor of the speed of clotting. However, with P.miliaris, introduction of citrate into the coelomic fluid by injection through the peritome or withdrawal of coelomic fluid into a citrated syringe did not delay clotting, and it is concluded that calcium is not an important factor in the coagulation of the coelomic fluid of this species.

2. Inhibition by other chemicals

Boolootian and Giese (1959) recorded inhibition of clotting of the coelomic fluid of Strongylocentrotus species by 0.01 M cupric chloride, 0.01 M mercuric chloride, 0.01 M parachloromercuribenzoate, 0.01 - 0.1 M cadmium sulphate and 0.05 M sodium thioglycollate. They found that coagulation was delayed by 0.19 M sodium bisulphite, 0.29 M sodium hydrosulphite, 0.07 - 0.08 M cysteine hydrochloride and 0.06 M glutathione. These authors do not however, report on the microscopic effect of these reagents, some of which are known 'fixatives' and it was therefore decided to investigate the affect of some of these reagents on the coelomic cells of P.miliaris:

a. 0.01 M mercuric chloride

When used in a 1:9 ratio, as by Boolootian and Giese, this substance certainly inhibited coagulation of the coelomic fluid, but it also killed the coelomic cells, as shown by complete cessation of movement by the amoebocytes and the flagellated cells. The cells became less translucent, and nuclei and inclusions became more apparent.

b. cadmium sulphate

Boolootian and Giese used 0.01 - 0.1 M solutions. I found that addition of 1 part 0.01 M cadmium sulphate to 9 parts of coelomic fluid did not affect clotting or the activity of theccells, but a slightly higher concentration of the cadmium killed the cells.

c. sodium hydrosulphite

0.06 M sodium hydrosulphite immobilised and decolourised the coelomic cells.

It was concluded that some of the reagents, that is, mercuric chloride, cadmium sulphate and sodium hydrosulphite, reported to 'inhibit' clotting, did so because they fixed or killed the coelomic cells before clotting occurred.

THE INITIATION OF THE COAGULATION CHANGE

Removal of coelomic fluid from the perivisceral cavity does not inevitably result in clotting, nor are all the bladder amoebocytes necessarily involved when clotting does occur. The amount of clotting varies both with the species and the individual, and may also be dependent on the amount of contamination, both by sea water and by injured tissues. Clotting of the coelomic fluid of Paracentrotus lividus is usually slow and incomplete as compared with coagulation of the coelomic fluids of Arbacia lixula, Diadema antillarum and Psammechinus miliaris.

Uncoagulated and apparently normal cells have been examined, when available, to try and determine what factors initiate the coagulation change.

Diadema antillarum non coagulated cells

I. The effect of tissue extract, preparation after Donnellon (1938), - no change in cells detectable.

2. Addition of I% potassium chloride, - the bladder amoebocytes very quickly became filiform and apparently

normal coagulation ensued.
3. Addition of I% potassium chloride dissolved in sea water, - bladder amoebocytes remained uncoagulated.

Paracentrotus lividus non coagulated cells
I. Addition of I drop 0.5% potassium chloride, dissolved in glass-distilled water, - filiform cells were produced in IO minutes, flagellated cells remained fully mobile. (N.B. distilled water, as already mentioned causes cytolysis and immobilises the cells)
2. Addition of 0.5% calcium chloride, - cells became slightly more filiform, not nearly such a marked change however, as was obtained with the KCl.
3. Addition of 0.8% sodium chloride, - cells were not changed to the filiform state, the maximum effect was a slight extension of the cells.
4. The cells were gently agitated with coelomic fluid for about I minute, - there was no detectable affect.
5. The cells were similarly agitated with sea water, - coagulation occurred.

These observations were unfortunately limited by the scarcity of the uncoagulated cell condition. However it seems that cells in this condition can be induced to show the typical phase change, and apparently normal coagulation, by the addition of certain ions or sea water.

The possible significance of these observations is discussed below.

DISCUSSION

The bladder amoebocytes effect clotting in all the species I have studied, by elongating to form filiform

cells, which further elongate and entangle other cells to form a clot. Hyaline haemocytes, found by Boolootian and Giese (1959) in Strongylocentrotus species, which rupture to form fibres, have not been detected. Inhibition of clotting which these authors report may represent fixation affects rather than physiological blocks, and although clotting may depend on the SS linkage which these authors envisage, (and reversal of clotting inhibition which they observed appears to support this idea), some of the reagents they used; namely mercuric chloride, cadmium sulphate and sodium hydrosulphite, were lethal to cells of Psammechinus miliaris.

Donnellon (1938) found that potassium had a marked accelerating affect on coagulation, and thought that both this and calcium might have an indirect effect in producing the injury factor which he assumed was necessary to give coagulation.

An injury factor may be necessary for coagulation, but it is not the only factor because non-coagulated cells which cannot be induced to become filiform by addition of tissue extracts or by agitation will become filiform after addition of potassium chloride, sea water, and, to a lesser extent, calcium chloride. Sodium chloride has no effect. Severe agitation, as produced by high speed centrifugation does simulate normal coagulation - before compaction of the coelomic cells occurs - such that bladder amoebocytes become filiform. However if a whole urchin is centrifuged at the same speed the coelomic cells do not show coagulation changes, which indicates that another factor, besides mechanical agitation was responsible for the coagulation of the coelomic cells in vitro.

The removal of calcium does not affect coagulation of some species, and potassium has not been shown to induce coagulation in all the species studied. However, the effect it does have; a selective induction of filiform pseudopodia, with no accompanying damage to other cells, may be associated with fundamental properties of membranes relative to the anion:cation balance.

Interesting parallel observations have been made, on protozoa. Mackinnon and Hawes (I96\ ) quote Verworn; 'In a famous and often quoted experiment Verworn (I899) showed that the form of the pseudopodium is affected by the environment; thus a limax amoeba, on the addition of KOH to the medium, developed, in place of its normally single lobopodium, a number of slender, radiating pseudopodia.' Willmer (I960) studied Naegleria gruberi, which changes from the amoeboid to the flagellate form on dilution of the medium. He has shown that the cation content of the environment determines this change; 'if the medium is such that cations tend to be lost from the cells of Naegleria, then the flagellated form is assumed, and alternatively, the amoeboid from becomes the appropriate form when cations tend to be gained rather than lost by the cells.'

If, as seems likely, change in the cation environment selectively affects the bladder amoebocytes, in a manner similar to that described for Naegleria, it is pertinent to query the nature, derivation, threshold concentration and membrane affect of such ions. At the present moment it is impossible to do more than speculate.

Principal changes in the cation environment would be from two sources; injured tissues and sea water. As far

as can be ascertained the latter has more effect on coagulation. However, published data, see below, TableIO, shows very little difference in cation content of the coelomic fluid as compared with sea water. The table is taken from data compiled by Boolootian (I96I), and some of the figures may be inaccurate. The most striking difference between sea water and coelomic fluid appears to be the higher pH of the former, and although this may have some influence on coagulation it is not an essential factor in the initiation of the coagulation changes as isosmotic solutions of certain salts have different effects.

TABLE IO

The cation content of Plymouth sea water and the coelomic fluid of 3 species of echinoid Boolootian (I96I).

	Calcium	Potassium	Sodium (mM/L)
Plymouth sea water	I0.2	9.9	465
Echinus esculentus	I2.8	I4.I	530
	I0.0	9.6	444
Paracentrotus lividus	I3.0	I2.7	
Strongylocentrotus drobachiensis	9.56	9.7I	420
	8.82	9.59	46I

THE FUNCTION OF THE SPHERULE AMOEBOCYTES

THE FUNCTION OF THE SPHERULE AMOEBOCYTES

The possible function of the spherule amoebocytes has aroused considerable interest, but apart from ascertaining a role in pigmentation, little has been established.

HISTORICAL

MacMunn (I885), who first described the characteristics of the red pigment in the coelomic cells of echinoids, believed it to have a respiratory function, but this has never been proved.

Cuénot (I89I) thought that the spherules of the amoebocytes are derived by the absorption of nutrient material from the food, but later (I897), he thought that the inclusions represent end-products of metabolism, which are carries to the exterior by the cells. Kindred (I926) inferred that the amoebocytes with spherules are derived from leucocytes (= bladder amoebocytes) which had ingested food, and believed that the colourless spherule amoebocytes have a nutritive function, releasing material to the tissues. Liebman (I950) termed the spherule amoebocytes 'trephocytes' in the belief that the inclusions are nutritive, and thought that this interpretation was substantiated by some observations he made on the relation of these cells to the devloping eggs, in Arbacia punctulata, and their relation to the phagocytes.

Jacobson and Millott (I953) have shown that the coelomocytes may form the black melanic pigment found in the skin of Diadema antillarum.

Stott (1955) found no evidence to support the idea, suggested by Oomen (1926), for holothurians, that the colourless spherules contain the precursors of digestive enzymes. Stott thought that the spherules were directly derived from nutritive materials, 'soaked up' in the gut lumen, by these cells, in Echinus esculentus.

DISCUSSION

Knowledge of the pigmentation of the coelomocytes and the composition of the inclusions is obviously of great importance in elucidating the role of these cells. Much work has been done on the pigments of echinoids, and the relevant material is discussed below, p. 120. Similarly there has been considerable interest in the nature of the inclusions, but some of the studies have lacked precise analytical method. It was therefore considered that a further investigation should be undertaken, utilising modern histochemical techniques, supplemented by chromatography. A review of the previous work, and the results and conclusions of the present study, are given below, p. 132.

PIGMENTS OF THE SPHERULE AMOEBOCYTES

THE RED PIGMENT OF REGULAR ECHINOIDS

MacMunn (I885) first described the characteristics of the red pigment found in echinoid coelomic cells. He extracted pigment from the coelomic fluid of Echinus esculentus and Psammechinus miliaris. He believed it to have a respiratory function.

Red pigments with similar characteristics are widely distributed in echinoids; they have been shown to have the characteristics of hydroxy-naphthoquinones in every species of regular echinoid that has been investigated. Several different bu similar naphthoquinones may be simultaneously extracted from one individual; from the gut, test, coelomic cells etc., but pigments mostly studied are those extracted from the test, spines and eggs. The pigments are usually described and characterised by reference to their site of occurrence, solubilities, absorption maxima in different solvents, their behaviour on chromatograms, etc. Recent reviews of the data obtained are given by Fox, (I953), Thomson (I957), and Fox and Vevers (I960).

Millott (I957) studied the red pigments of Diadema antillarum, and found that the principal red pigment extracted from the tissues of this animal, although unstable, had the characteristics of Echinochrome A. Absorption maxima of the crude pigment extracted from the coelomic fluid of adult urchins, in acidified ether, were 258-9, 340 and 466, and from the test; 257-62, 340 and 467-70, in millimicra.

Yoshida (I959) found Spinochrome E in the coelomic fluid of Psammechinus miliaris; the absorption maxima of crude acidified ether extracts from the test were 269, 3I5-I8 and 480 millimicra.

Complete characterisation of the pigments is beyond the scope of the present study, but absorption data for crude acidified ether extracts from D.antillarum were obtained, for comparison with the data given by Millott, (I957), see above. Maxima obtained on a Unicam spectrophotometer, from the test and coelomic fluid extracts respectively, were; 254, 339 and 460-70, and 253.5, 330 and 460, in ethereal solution.

Living cells of 3 species were examined under a microspectrophotometer to give maxima in the visible range 400-600 millimicra. Maxima obtained were;

Psammechinus miliaris		550		500	470	
			5I0	500	480-5	455
Diadema antillarum		550	5I0-35		470-5	
Arbacia lixula	570-80		520	505	470	

These maxima are, understandably, somewhat different from those obtained from acidified ethereal extracts, but compare favourably with data compiled by Fox (I953), for maxima of extracts from the eggs and similar tissues of Arbacia and allied genera, in more neutral media. Under these conditions purified extracts of Echinochrome A has maxima at 535-26, 499-90 and 464-56, whereas Spinochrome' has maxima at 595-8I, 548-40 and 507.

The absorption maxima, and hence the visible colour vary with the pH of the medium, see Fox (1953). In neutral or alkaline solutions , or when combinedaas insoluble salts, certain echinochromes are purplish, while in acidic solutions they become red. Fox suggests that, on this basis, the colour of these pigments in vivo may indicate either the approximate pH of the tissues in which they are observed, or their state of combination; 'e.g., as calcium salts in the purple skeleton of Strongylocentrotus drobachiensis - and perhaps as largely free, buffered colloidal material in certain red aggregates found in soft tissues of the same species.'

Maxima obtained from the microspectrophotometric examination of living red cells show a slight shift towards the red end of the spectrum, as compared with extracts in acidified ether, see above. This and the slightly orange-red colour of the echinochrome in the coelomocyte spherules suggests that it may be in a medium of slightly acid pH, and that it is not in the form of the calcium salt. The constitution of the 'medium' of the echinochrome will be further discussed later, p. 164.

The origin of echinochrome and naphthoquinones in the coelomic fluid

The origin of the naphthoquinone pigments in echinoids has been a subject for much speculation, as most known quinones are derived from plants, and rarely occur in any quantity in animal tissues. Fox (1953) therefore believes that echinoid echinochromes etc. are derived from algal sources, and that the echinoid stores this material en route to its excretion, in the skin, test and other structures, in a manner comparable to the treatment of

excess carotenoids by some other animals, e.g. polychaetes.

The idea that naphthoquinones in echinoids are waste material en route to excretion is not, however, supported by two facts; firstly, echinochrome is present in the eggs of some species - although this might be analaguous to the 'excretion' of haemoglobin via the eggs of some cladocera. Secondly, Young (I957) found that in the development of Lytechinus variegatus, which has no pigment in the eggs, echinochrome first appears at the gastrula stage. He concludes that since 'the pigment is elaborated long before the organism begins to take in food material the pigment must be synthesised from pre-existing materials.' The development of pigmentation in Psammechinus miliaris, described below, appears to be very similar.

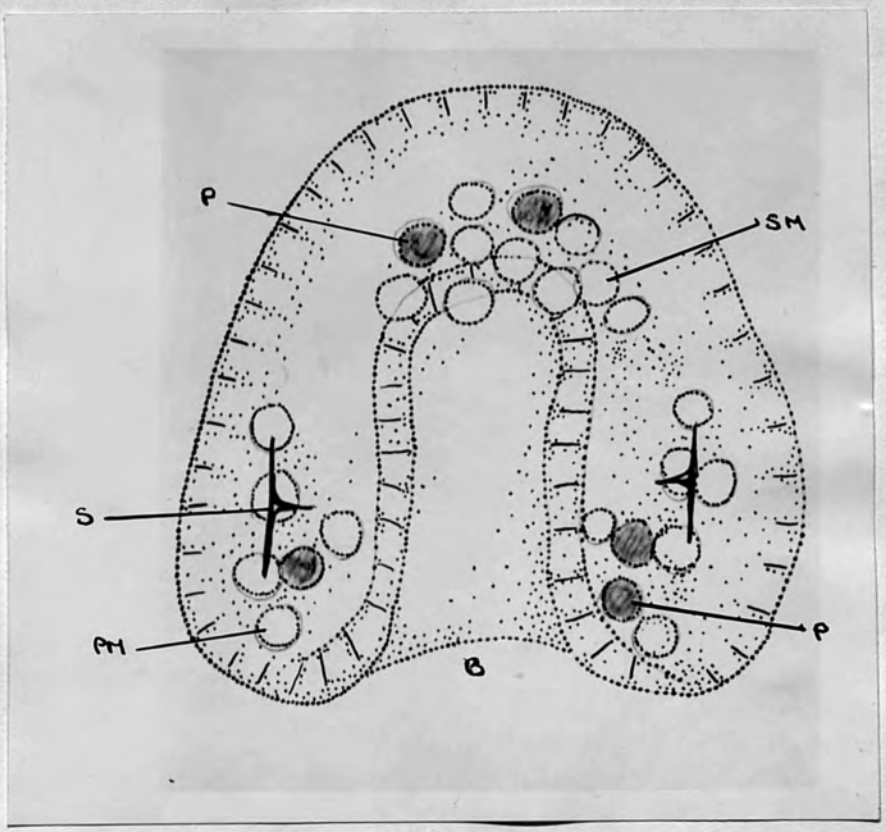
Development of pigmentation in Psammechinus miliaris

The ovum of P.miliaris is non-pigmented, unlike that of Arbacia species, which is coloured by echinochrome-containing inclusions scattered in the cytoplasm.

Development was studied from the time of fertilisation, the gametes having been obtained by dissection. Pigmentation, as already noted, p. 93 , was first observed at the gastrula stage; in one or two cells of the primary mesenchyme, round the blastopore, and in one or two of the secondary mesenchyme cells in the presumptive mouth region. These cells were coloured a faint and uniform pink, see Fig.22, there was no sign of granulation, and although the colour was faint it was observable under the high power dissecting lens.

One batch of embryos was kept without food, while the remainder were fed with an algal culture.

FIGURE 22



PSAMMECHINUS MILIARIS: GASTRULA

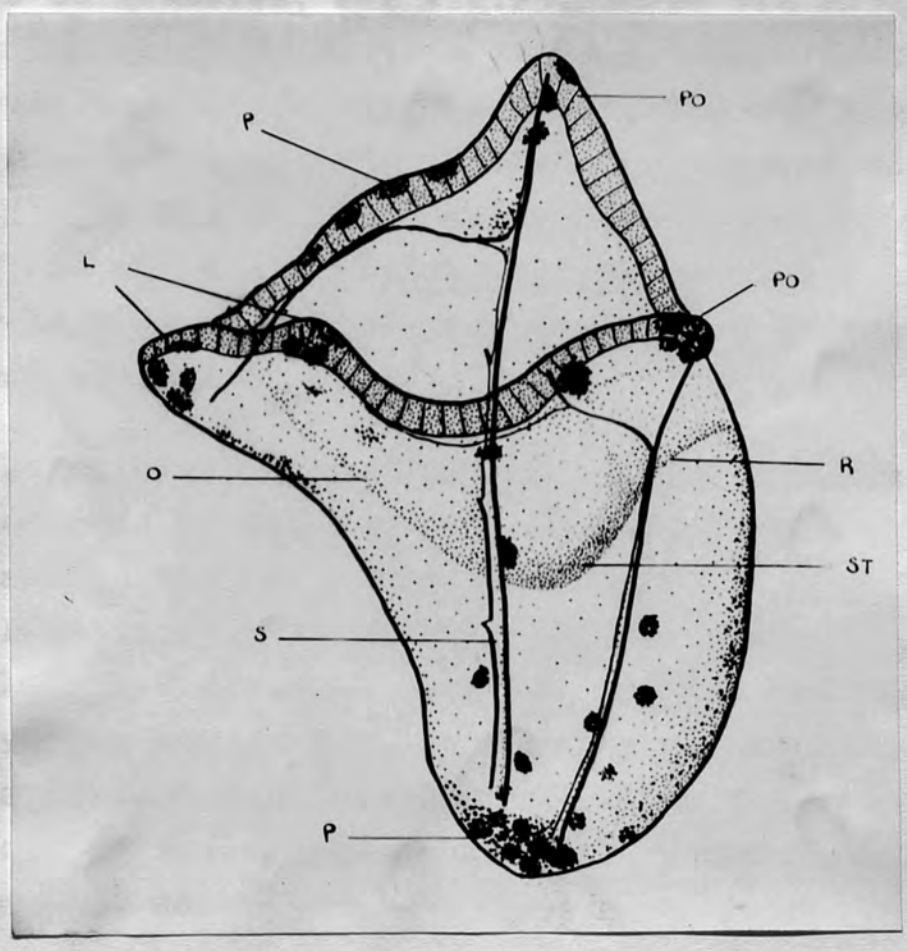
drawn from living specimen, X 150

B = Blastopore P = Pigmented cell S = Spicule

PM= Primary mesenchyme SM= Secondary mesenchyme

FIGURE 23

PSAMMECHINUS MILIARIS: 7 DAY PLUTEUS, REDRAWN FROM LIVING SPECIMEN WHICH WAS SLIGHTLY DISTORTED DUE TO COMPRESSION ×100



L = Lateral arms O = Oesophagus P = Pigment
PO = Posterolateral arms R = Rectum S = Spicule
ST = Stomach

Pigmentation increased in all the plutei to the same extent, whether they were fed or not. It was concentrated in the ciliary band regions, particularly at the tips of the arms and round, and at the base of, the spicules. There was still no difference between the pigmentation of the starved and fed plutei after 7 days.

The pigment in the pluteus is in the form of small masses of granules, see Fig.23. These are smaller and less regular in shape than the red spherules of the red spherule amoebocytes of the adult, and the colour is rather more orange. The pigment does appear to be contained in definite cells, which may well be amoeboid as Young, (I957) found in Lytechinus variegatus, but I have not observed tham in motion, see discussion on the origin of amoebocytes, p. 93 .

It is clear from this and earlier studies that echinochrome is not directly derived from algal pigments, at least in the early plutei.

Finally, it may be observed, as Thomson (I962) remarks, that although some sea urchins are mainly herbivorous and others are chiefly carnivorous, both groups produce naphthoquinones, which suggests 'that the echinoid quinones are endogenous', although this is not necessarily so.

Possible functions of naphthoquinones in echinoids

I. Respiration MacMunn (I885) who first described echinochrome, believed it to be a respiratory pigment similar to haemoglobin. Cannan(I927) found no evidence to that it could from a dissociable compound with oxygen, and suggested that it might function as an intermediate in

cellular oxidative processes, because the oxidation-reduction potential is very low. Cook (I928), however, found that small amounts of oxygen were given off with potassium ferricyanide. This 'small amount' is no greater than that dissolved in sea water, but Cook pointed out that it is not possible to remove oxygen from solution by ferricyanide. Objections to this work have been based on the fact that he was using cellular material which probably contained other active systems. Fox and Vevers, (I960) summarise the literature on respiration as follows; 'there is no evidence for this' (MacMunn's) 'suggestion and later work has never been able to show absorption of oxygen by this pigment.'

Cannan's suggestion, (I927), see above, that echinochrome might function as an intermediate in cellular oxidative processes, rather than as an oxygen carrier like haemoglobin, has been partly amplified by Baldwin (I948). He suggests that echinochrome, which is readily oxidised and reduced, might act as an oxidative system superceding the cytochromes. It is becoming increasingly evident that many cells and organisms contain oxidative systems that are resistant to cyanide and carbon monoxide. However, cytochromes are well known constituents of sea urchin eggs, and there is no doubt that fertilised sea urchin eggs utilise the cyanide-sensitive cytochrome system, although it is possible that the system is not so important prior to fertilisation. I have found that a sea urchin can live for at least 2 days, however, in IO. -4 cyanide solution, sufficient concentration to kill animals such as fish in a short time. The possible significance of echinochrome in cellular oxidation remains an exciting if remote

possibility.

2. Echinochrome as a sperm attractant Hartmann, Schartau, Kuhn and Wallenfels (1939) stated that dilute solutions of pure echinochrome would effect activation and chemotaxis of Arbacia sperm. Tyler (1939), however, who worked with Strongylocentrotus purpuratus, found no such effect. It should be noted though that this latter species is reported to have gonads devoid of echinochrome, whereas the eggs of Arbacia yield quantities of this pigment. Later work, however, does tend to disprove that echinochrome plays an active role in activation and attraction of the sperm. A full review is given by Brachet (1950).

3. Echinochrome and light sensitivity Finally it must be noted that Millott and Yoshida (1957) found that the maximum sensitivity of Diadema antillarum to light correlates with the absorption maxima of Echinochrome A after extraction. He* has not established that this pigment is of importance in light perception, and there are many difficulties in establishing such significance but it may yet prove to be so.

* Millott

THE RED PIGMENTS OF IRREGULAR ECHINOIDS

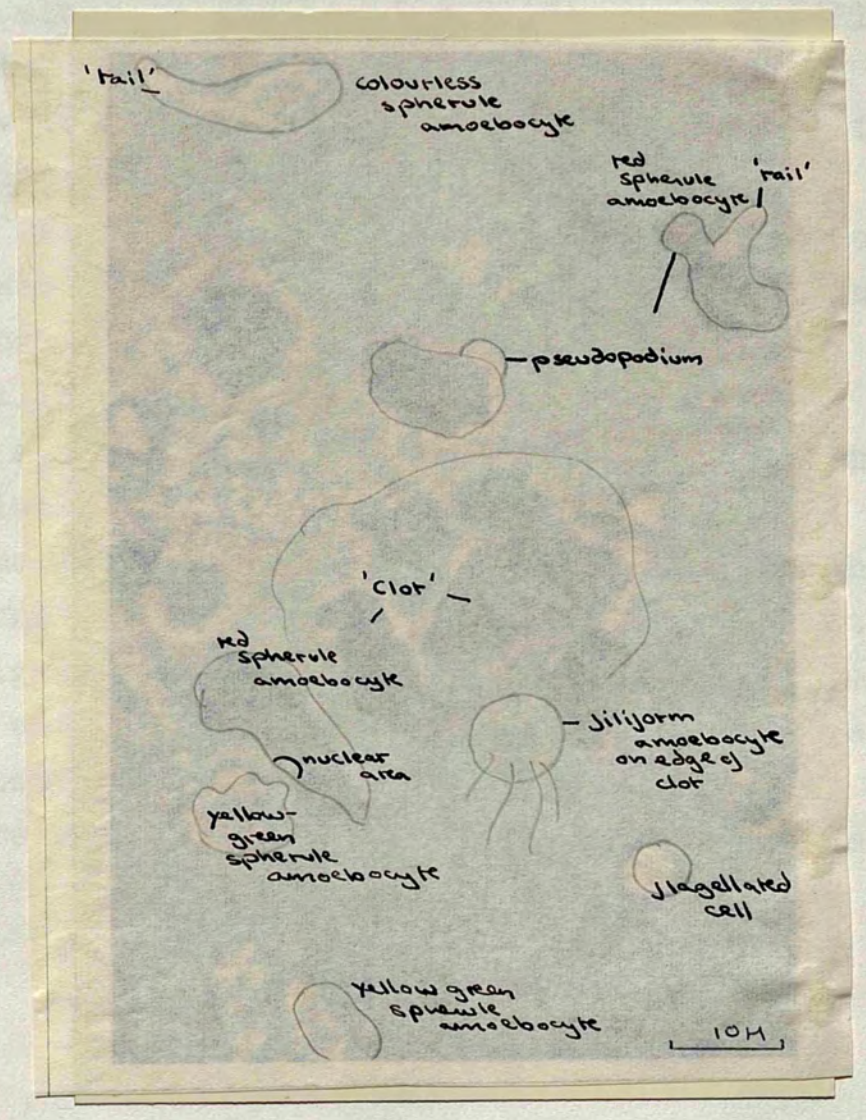
I have not been able to investigate the coelomic cells of irregular echinoids in detail, as they are difficult subjects for aquaria. Red pigments of the coelomic cells of urchins I have examined, viz:

Brissopsis lyrifera, Spatangus purpureus and Echinocardium cordatum are of two kinds. A 'true' red pigment is found in some of the spherule amoebocytes' inclusions, which is very similar to that found in the coelomic amoebocytes of regular echinoids. A purplish red pigment also occurs, in some of the 'bladder' amoebocytes of these species. It is enclosed in a few discrete inclusions similar to those of the spherule amoebocytes. It sometimes appears almost black. Unfortunately I have been unable to investigate it.

The red pigment of the spherule amoebocytes is leached by some solvents e.g. distilled water, ethanol, but is not completely decolourised by sodium hydrosulphite. It is interesting to recall that Goodwin and Srisukh (I95I) recorded the carotenoids echinone and lutein from the coelomic fluid (presumably including the cells) of Echinocardium cordatum, and it may well be that at least some of the red pigments of these heart urchin coelomic cells are carotenoids rather than naphthoquinones. I have not been able to obtain sufficient material to investigate this possibility using absorption data etc.

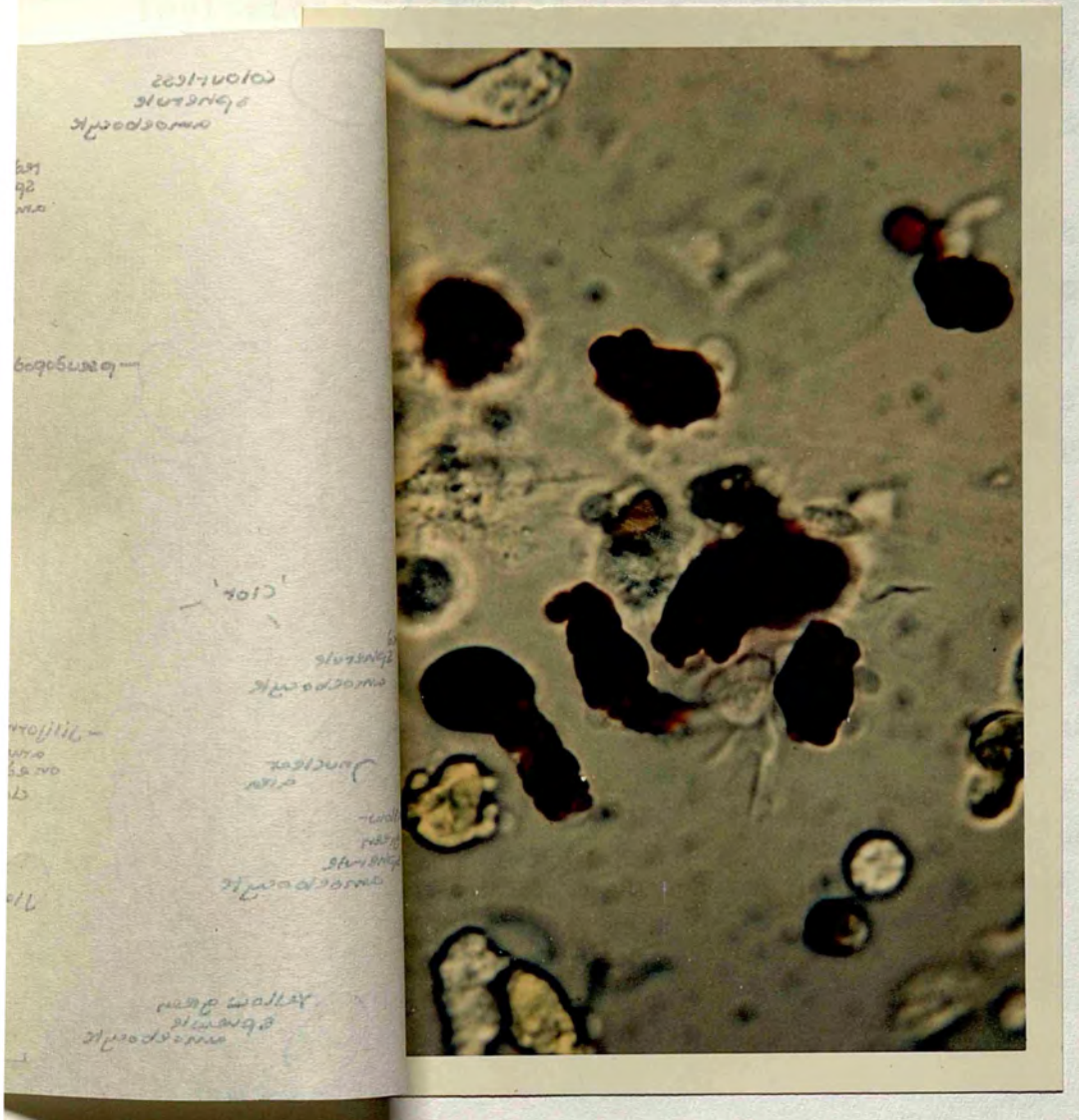
Red spherule amoebocytes of Echinocardium cordatum are illustrated in Fig. 24, whilst actively moving. The inclusions containing pigment are clearly visible, but the colour is rendered incorrectly; the print having a blue overtone.

FIGURE 24



ECHINOCARDIUM CORDATUM: LIVING CELLS, in a sample of partly clotted coelomic fluid, examined on a slide under a cover-slip.

FIGURE 24



ECHINOCARDIUM CORDATUM: LIVING CELLS, in a sample of partly clotted coelomic fluid, examined on a slide under a cover-slip.

THE GREEN PIGMENTS OF IRREGULAR ECHINOIDS

The irregular echinoids examined also contained a proportion of spherule amoebocytes with yellowish-green inclusions. A few similar cells were found in some specimens of Arbacia lixula. Green spherule amoebocytes are shown in Fig. 24, from the coelomic fluid of Echinocardium cordatum.

The green pigment is insoluble in acetone and ethanol, and was not removed or replaced by any of the fixation or staining procedures used, which included the use of distilled water, alcian blue and eosin. The inclusions were slightly blackened by Sudan black B. This rather insoluble pigment might possibly be melanic or chromolipoid. Lillie's (1956) Nile Blue method with acetone extraction for differentiating the two groups of pigment, indicated that the pigment was melanic rather than chromolipoid. However, the material was much too limited to conduct more satisfactory tests, and the nature of the pigment could not therefore be determined.

THE COMPOSITION OF THE INCLUSIONS OF THE SPHERULE AMOEBOCYTES: (CONSTITUENTS OTHER THAN PIGMENTS)

The composition of the inclusions of the amoebocytes has been studied by a number of authors, notably Kollmann (I908), Ohuye (I937) and Liebman (I950), but in general their conclusions were necessarily tentative, and Hyman's (I955) comment that the nature of the inclusions in the coelomocytes has not been definitely ascertained, is fully justified.

The following summarises the published work on the composition of the inclusions;

Cuénot (I89I[b]) considered the colourless spherules to be albuminoid, 'par les réactifs caracteristiques (iode, fuchsine, acides etc.)' However as Lison (I960) points out, the yellow colouration given with iodine or colouration by picric acid or eosin, has no value.

St.Hilaire (I897), in a paper I have been unable to consult, is reported by Liebman (I946) as stating that the colourless spherules are composed of lecithin and protein, and the red ones pigment, lecithin, protein and an osmiophilic substance.

Kollmann (I908) believed the colourless spherules to be basophilic and the red ones acidophilic. He stated that the former contain an albuminoid substance insoluble in alcohol, and an alcohol soluble lecithin, and that the red spherules are of a more complex nature, 'elles se composerait d'un pigment, d'un substance grasse, d'une albuminoide colorable, enfin d'un lécithine.'

Kindred (I926) found that the colourless spherules

are basophilic to amphophilic, whereas the red spherules are acidophilic, staining a 'brilliant red' in Ehrlich's triacid stain after Helly fixation. This interpretation needs re-examination because Zenker-formol or Helly tends to preserve echinochrome in situ, and this retained pigment, rather than a 'stain' might be responsible for the 'brilliant red'. Kindred also noted the effect of osmium on the spherules; when fixed in fumes of 2% osmic acid, 'the spherules of the amoebocytes are preserved and are sharply outlined in black.' He interpreted this appearance as meaning that the spherules are composed of an envelope of lecithin containing protein.

Ohuye (I937) investigated coelomic cells of Temnopleurus hardwickii, and recorded the following observations on the 'coarsely granular' (= colourless spherule) amoebocytes; he found that these cells failed to stain with Sudan 3, with Scharlach R and with Best's carmine. However, with Millon's reagent the spherules became dirty yellow or grayish in colour, and with 2% osmic acid he noted that these amoebocytes always assumed a dark appearance. Close examination revealed that this effect was due to the appearance of very fine particles in the cytoplasm between the spherules.

Bookhaut and Greenburg (I940) stated that the red spherules of Mellita quinquiesperforata contain fatty material on the basis of tests with osmic acid and Sudan 3.

Liebman (I950) examined the coelomic cells of Arbacia punctulata. He found that the green, red and occasionally the colourless spherules were stained yellow after Mallory's staining, and therefore suggested that they

contain 'phospholipin'. He rarely found any indication of the presence of glycogen with Langhan's iodine and Best's carmine, checked with a ptyalin control. He believed the basophilia of the colourless inclusions might indicate the presence of R.N.A., whereas he obtained a positive reaction to mucicarmine, and therefore assumed that a mucin was present.

In none of these papers is it clear that the author has successfully differentiated between the red and colourless spherule amoebocytes after fixation and staining. It is considered essential, in the present study, to be able to distinguish each type of cell. This is not easy because the echinochrome is usually leached out or obscured during processing, and the small difference in size of the inclusions is not necessarily maintained, due to differential swelling or shrinkage. Conversely, some fixatives, for example, those containing formalin, tend to preserve the red pigment in the cells. This may cause confusion under certain circumstances, as when using Sudan 3, because the colour imparted by this dye is so similar to that of the naturally occurring pigment. There is no evidence that any of the previous authors realised this.

However, the fact that formol fixation tends to preserve pigment in the red spherule amoebocytes can be utilised to enable accurate localisation of the two types of amoebocyte after fixation. The following method was evolved;

Method of identifying types of amoebocyte in fixed preparations

The cells are treated as follows;

I. The coelomic fluid and cells are pipetted gently onto a slide, which is then placed in formalin vapour for IO-I5 mins., see p.\a.
2. The fluid is then dried on the warm part of the 'hot-plate', for 7-IO mins, (about 35° C).
3. The slide with the dried film on it is washed with IOO% ethanol for 5 mins., with 3 changes, the alcohol being poured directly onto the slide.
4. The slide is rinsed very quickly in distilled water.

The proportions of ethanol and distilled water are altered to remove salt crystals without disturbing the pigment. Ethanol removes some, whereas distilled water removes it much more efficiently, but also removes the pigment, if too much is used.

5. Dry rapidly and completely.

This procedure preserves the pigment in the red spherules. The position of the red spherule amoebocytes may then be noted, and a quick 'map' is made of these and the colourless spherule amoebocytes in a suitable area. Reactions with various stains and techniques can thus be traced to the red or colourless spherule amoebocytes.

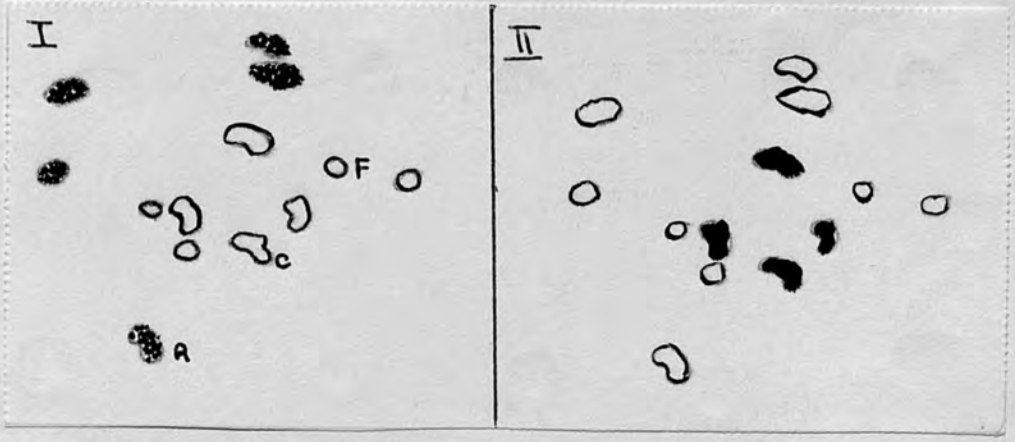
Fig.25 A shows how 'maps' of cells corresponded

FIGURE 25 A,B

A. DIADEMA ANTILLARUM

I.'Map' of fixed coelomic cells, see procedure p.135. F = Flagellated cells, C = Colourless spherule amoebocytes, R = Red spherule amoebocytes.

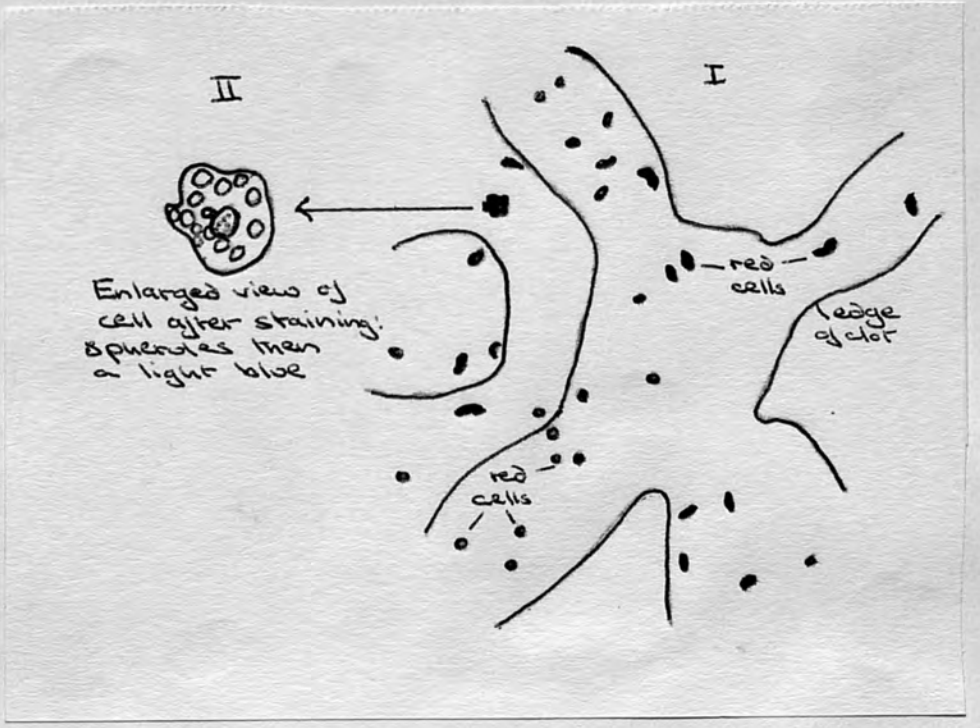
II. The same cells after staining with mercuric chloride bromphenol blue, see p.142. N.B. Colourless spherule amoebocytes are chromophilic.



B. DIADEMA ANTILLARUM

I. 'Map' to show red spherule amoebocytes after fixation, see p.135.

II. Enlargement to show appearance after Mallory's stain.



with staining after the use of mercuric chloride bromphenol blue, Mazia, Brewer and Alfert (1953), see also p. 142 , and Fig. 25B shows a 'Mallory trichrome map'. Unfortunately it has not been possible to obtain detailed photographs of the cells at the 'map' stage since they cannot be covered with a cover-slip.

GENERAL STAINING REACTIONS

The species used for the preliminary investigations were Diadema antillarum, Arbacia lixula and Paracentrotus lividus.

The following reactions are of interest;

I. Haematoxylin (Ehrlich's, Delafield's and Haidenhain's) and Eosin (aqueous and alcoholic)

It was found that the inclusions of the colourless spherule amoebocytes were eosinophilic, whereas those of the red spherule amoebocytes tended to stain with haematoxylin, although not very densely.

2. Mallory's trichrome stain

The inclusions of the colourless spherule amoebocytes showed an affinity for the fuchsin, and stained red, whereas those of the red spherule amoebocytes stained with aniline blue, see Fig. 25B.

3. Leishman's and Wright's blood stains

It was not possible to obtain consistent results with either of these blood stains, possibly because they are a mixture of acidic dyes, and therefore very much affected by slight changes in technique or environment.

4. Neutral red (I%)

A group of red and colourless spherule amoebocytes were observed while neutral red was allowed to diffuse under the cover-slip. The colourless inclusions became a yellow-orange colour, while the red spherules became very dark.

These reactions indicate that the colourless spherules are basic and therefore acidophilic, whereas the red spherules are acidic and therefore basophilic. This does not agree with some previous observations, e.g. Kollmann (I908), see p. 132 , and will be discussed later.

THE COMPOSITION OF THE SPHERULES - HISTOCHEMISTRY

The composition of the spherules was investigated primarily by the use of histochemical techniques (for pigments see p. 120). The procedure already outlined, p. 135 was used for fixation except where otherwise specified; for some techniques formol fixation is inappropriate. Reference to sources of information concerning the techniques used will be made as required.

The methods and results

It would be tedious to detail the exact method used, and the theory behind each technique. I propose therefore to refer to the author whose method or modification of a standard technique has been utilised.

Some of the reactions used have been unsatisfactory in that almost no staining has resulted. Where this has occurred a detailed description of the result is

unecessary, and if destructive reagents have been used and the rather fragile coelomic cells have succumbed, this is noted rather tersely, as again, a detailed description would be superfluous.

The investigation is divided into the tests for the 'major' groups of substances, and where it becomes obvious that one particular type of inclusion responds, this fact is noted. Again, it would be tedious to record negative results each time. The summary and discussion at the end of each section should clarify the extent and significance of the results.

When the investigation was initiated coelomic cells of Diadema antillarum were utilised but unfortunately, the supply was disrupted and subsequent investigations were made with Psammechinus miliaris. Differences between the two species are noted as relevant in the text. Generally though it appeared that the cells contained similar if not identical material.

PROTEINS AND AMINOACIDS

The colours developed by standard reactions for amino acids are usually too pale for histochemical localisation. Some of these reactions have been used, with slight modification, for example, Millon's test for tyrosine, but the colours have usually remained too pale. Attempts to devise more suitable reactions, usually for specific amino acids, have produced a number of more or less specific tests, few of which, unfortunately, are entirely satisfactory. Some tests rely on elaborate reagent preparation, others incorporate cytologically destructive reagents. The principal difficulty however remains in the weak colour produced by many of the reactions, in spite of the use of devices such as coupling of products with diazonium salts.

The reactions utilised in the present study may be conveniently divided into 3 groups; those specific to amino groups, those which react with several, usually specified amino acids, and those specific to particular amino acids.

I. Reactions specific to the amino grouping

NINHYDRIN (triketo hydrindene hydrate)

Method Casselman (1959)

Reaction The presence of 'protein-bound' amino groups is indicated by a blue colouration.

Result The coelomic cells were all faintly stained, but very little cytological detail was discernable. This

result is typical of ninhydrin, for the coloured compound produced in the reaction tends to diffuse, see comments by Casselman (1959).

NINHYDRIN-SCHIFF

Method Casselman (1959), but Schiff's procedure modified as per p.149 Carnoy fixation. Diadema antillarum

Reaction Pinkish-red to magenta colouration indicates the presence of protein-bound amino groups.

Result The flagellated cells stained magenta, and some of the inclusions of the spherule amoebocytes stained a paler pink. Some of the spherule amoebocytes and the bladder and filiform amoebocytes remained unstained, but both these and the unstained nuclei remained clearly visible.

OH-NAPHTHALDEHYDE

Method Pearse (1960), after Weiss, Tsou and Seligman, (1954) Diadema antillarum

Reaction A blue colour develops in sites containing many reactive amino groups; when these are more sparse the colour is red or pink.

Result The nuclei were stained blue, while the cytoplasm of all the coelomic cells was stained a faint pink, with no discernable concentration of colour in the inclusions.

2. Reactions positive to several amino acids

COUPLED TETRAZONIUM

Method Pearse (I960), after Danielli (I947). D.antillarum

Reaction Reddish-brown colouration indicates certain amino acids: tyrosine, histidine , and possibly tryptophan, lysine and arginine.

Result Very little colouration. The reagents proved both difficult to prepare and destructive in action. It was not possible to conclude that there was any positive staining.

DINITROFLUOROBENZENE

Method Pearse (I960), after Burstone (I955) D.antillarum

Reaction Reddish-purple colouration is said to be produced with free amino acids: lysine, sulphydryl groups - cystine and cysteine, tyrosine and histidine.

Result No staining; reaction destructive and cells damaged.

MERCURIC CHLORIDE BROMPHENOL BLUE

Method Mazia, Brewer and Alfert (I953) D.antillarum

Reaction 'Proteins' are said to stain blue, see comments on specificity below.

Result Inclusions, as far as could be determined, of the colourless spherule amoebocytes, stained a deep blue. Counterstaining with eosin revealed that non-stained amoebocytes were not eosinophilic, which confirmed the conclusion that the colourless spherules were stained by the preceding procedure. The blue staining was intense,

against a fainter background staining of both nuclei and cytoplasm of all the coelomic cells. Unfortunately, this reaction is not considered to be a specific one, Baker, (I958), and may only indicate acidophily.

3. Reactions for specific amino acids

MILLON'S REACTION FOR TYROSINE

Method Pearse (I960), after Bensley and Gersh (I933)
D.antillarum

Reaction Orange-yellow colouration, or red-rose, stable for about I2 months, indicates the presence of the hydroxy-phenol group.
Result There was pale yellow colouration of the majority of the coelomic cells, and spherules of some of the amoebocytes were stained a pale orange. It was not possible to further identify the amoebocytes; unfortunately some cytological detail was destroyed, presumably by the nitric acid of the reagent.

THE DDD REACTION FOR DISULPHIDE AND SULPHYDRYL GROUPS

Method Pearse (I960), after Barnnett and Seligman (I952)
P.miliaris

Reaction Blue staining indicates a high concentration of SH groups; red staining areas contain lower concentrations.
Result The inclusions of some of the spherule amoebocytes were stained a deeper pink than the remainder of the coelomic cells. Counterstaining with Alcian blue, see p.152 stained inclusions of the previously 'unstained' amoebocytes. It is concluded that the inclusions stained by the DDD reaction are probably the colourless spherules.

SAKAGUCHI'S TEST FOR ARGININE

Method After Baker (I947); 'Milton' was used as the hypochlorite. D.antillarum Pearse (1960)

Reaction Orange-red colouration indicates the presence of arginine.

Result There was general faint orange colouration of the coelomic cells, but cytological detail was very poor. When formalin fixation was employed there was additional difficulty due to the retention of echinochrome, see p.134 This reaction is said to be highly specific, but is admitted, Pearse (I960), to be technically very difficult.

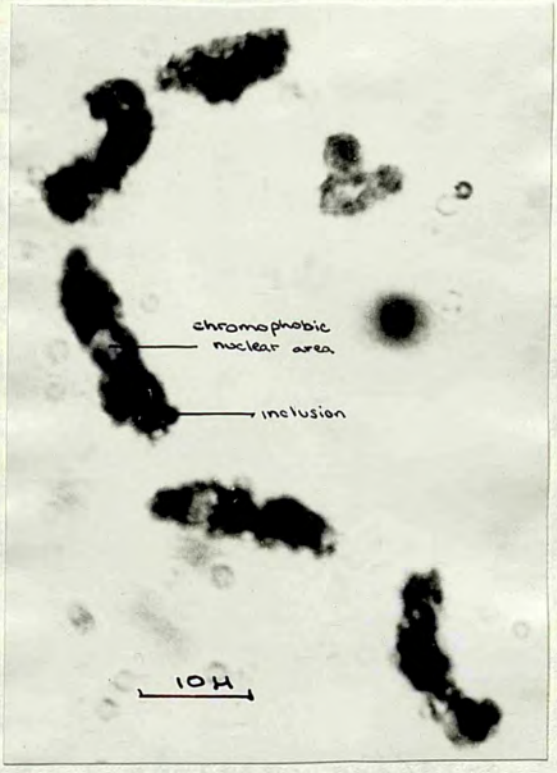
THE DMAB-NITRITE REACTION FOR THE INDOLIC NUCLEUS

Method After Adams (I957) D.antillarum P.miliaris

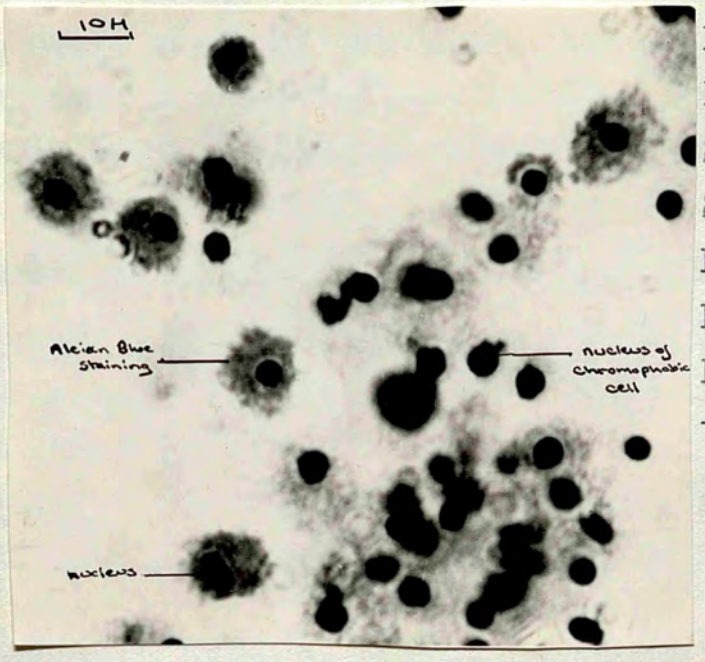
Reaction A deep blue stable colouration is produced at indole rich sites; usually indicating the presence of tryptophan and its derivatives, 5-OH tryptamine, indole etc.

Result D.antillarum: Examination under the low power of the microscope showed that a number of spherule amoebocytes were stained an intense blue, but the rest of the coelomic cells were colourless. Examination under oil immersion showed that it was only the spherules of certain amoebocytes which were stained by this procedure. The cytoplasm and nucleus of such cells was not stained at all but the spherules were stained uniformly, see Fig. 26. The method of correlating cells with specific reactions, see p. 135 , showed that it is the inclusions of the colourless spherule amoebocytes which are stained, inclusions of the red spherule amoebocytes are not affected.

FIGURE 26 A, B



A. DIADEMA ANTILLARUM INCLUSIONS OF THE COLOURLESS SPHERULE AMOEBOCYTES STAINED BY ADAM'S DMAB-NITRITE METHOD FOR INDOLES Formalin vapour fixation



B. PSAMMECHINUS MILIARIS INCLUSIONS OF THE RED (?) SPHERULE AMOEBOCYTES STAINED WITH ALCIAN BLUE, NUCLEI STAINED WITH HAIDENHAIN'S HAEMATOXYLIN, Formalin vapour fixation

Control oxidation with peracetic acid, (freshly prepared), obliterated the staining, confirming the indolic nature of the reacting substance.

P.miliaris: Staining of the colourless spherule amoebocyte inclusions showed considerable variation, see p. 173. Inclusions of some of the flagellated cells also stained faintly.

It was concluded that the inclusions of the colourless spherule amoebocytes contain a relatively high concentration of an indolic substance.

THE NAPHTHYL ETHYLENE DIAMINE REACTION FOR THE INDOLIC NUCLEUS

Method Pearse (1960), after Bruemmer, Carver and Thomas, (1957).

Reaction A positive reaction is indicated by purple colouration.

Result Most of the coelomic cells were stained a pale violet, but inclusions of some of the spherule amoebocytes were more reactive, being stained a deeper colour.

XANTHYDROL REACTION FOR THE INDOLIC NUCLEUS

Method Pearse (1960), after Lillie (1957).

Reaction Violet colouration indicates the presence of indolic groups.

Result The coelomic cells were stained a pale pinkish-violet, some amoebocytes stained more intensely.

PHOSPHORIC ACID REACTION FOR TRYPTOPHAN

Method Gurr (I958), after Romieu (I925).
Reaction Positive colouration is red or violet.
Result I was unable to detect any colour reaction.

CONCLUSIONS

Many of the histochemical tests for protein are unsatisfactory due to the pale colours produced, or the destructive reagents utilised. Negative results may not reflect total lack of the substance under investigation under these circumstances.

The inclusions of the colourless spherule amoebocytes are positive to the mercuric chloride bromphenol blue test, used histochemically by Mazia, Brewer and Alfert, (I953). This test is not necessarily specific to protein as it tends to colour basic tissue components, Baker (I958). However, there is also a weak positive reaction to the DDD test for sulphydry and disulphide groups, which implies the presence of cysteine or cystine. There is also a weak positive reaction by some of the inclusions of the spherule amoebocytes to the modified Millon's reaction for tyrosine, and a weak positive reaction to the xanthydrol and naphthyl ethylene diamine tests for indoles. There is a strong positive reaction by the colourless spherules of the amoebocytes to the DMAB-nitrite test for indoles. It has been generally assumed that, for mammalian tissues, the only indoles likely to remain in the tissues after fixation and embedding procedures, would be tryptophan,

5OH-tryptamine or serotonin, indole and skatole. However this assumption cannot be made in this study, and the preliminary conclusion can only be that the inclusions of the colourless spherule amoebocytes are associated with an indole.

The probable identity of the indole will be considered later, p. 166 . It seems probable that the colourless spherules also contain the amino acids cysteine or cystine and possibly tyrosine.

Inclusions of the flagellated cells of D.antillarum stained with the Ninhydrin-Schiff method for protein-bound amino groups, and inclusions of some flagellated cells of P.miliaris stain faintly with the DMAB-nitrite test for indoles.

The red or naphthoquinone-containing spherules do not appear to be associated with appreciable amounts of proteinsor amino acid, contrary to the assertions of Shapiro (I946), who believed the pigment to be combined with a protein. However, the pigment appears to be contained by a thin envelope, see p. 51 , which probably contains some protein.

CARBOHYDRATES

The four main groups of carbohydrate which can at present be demonstrated histochemically are:

1. Polysaccharides, e.g. glycogen.
2. Mucopolysaccharides, acids and neutral.
3. Muco and Glycoproteins.
4. Glycolipids.

Monosaccharides are not easily shown due to their extreme solubility.

FIXATION

The method used for fixation of many carbohydrates must be carefully selected because of their solubility in aqueous media.

McManus and Mowry (1958) recommend the use of very cold absolut ethanol for neutral polysaccharides, and the use of ice-cold buffered neutral formalin or cold, alcoholic formalin for acid mucopolysaccharides.

However, as I have not used embedding or sectioning techniques for these studies of the coelomic cells, it was possible to avoid aqueous media by the use of formol fixation, or Carnoy vapour where formol was unsuitable. This type of fixation was quite suitable as leaching was avoided.

THE PERIODIC ACID SCHIFF REACTION

Histochemical detection and differentiation of carbohydrate depends initially on the response to the periodic acid Schiff reaction, referred to hereafter as PAS. The reaction was introduced by Lillie (1947) and

Hotchkiss (I948). Periodic acid oxidises the I:2 glycol groupings present in carbohydrates, to dialdehydes, the presence of which can then be demonstrated by Schiff's reagent. Although the reaction was originally thought to demonstrate all the above groups of carbohydrates, it has recently been stated that not all acid mucopoly-saccharides give a positive reaction. It is proposed to discuss this later.

Method The importance of this reaction has encouraged repeated attempts to improve the method.

In the present study the following methods were used:
0.05% periodic acid was used, either in aqueous or in acid solution, McManus and Mowry (I958). The oxidation was not allowed to exceed I0 mins., otherwise nucleic acids are affected.
Barger and Delamater's Schiff reagent was used, as recommended by Pearse (I960).
3 sulphite reducing baths were used, after the Schiff reagent, as recommended by Casselman (I959), after Hotchkiss and McManus. McManus (I948) noticed that washing in running tap water enhanced the final colour. A ten minute wash was therefore incorporated in the schedule.

Reaction Magenta (rather than red) colouration indicates the presence of the I:2 glycol groupings found in carbohydrates.

Results Inclusions of some of the spherule amoebocytes, possibly those of the red spherule amoebocytes, of P.miliaris and D.antillarum, stained a faint magenta, against a very pale magenta general staining of the cytoplasm of the other coelomic cells.

ENZYME HYDROLYSIS

It is usually assumed that a PAS positive substance, removable by the action of ptyalin or diastase, and not removed in a control incubation with the enzyme medium, is glycogen.

Method Saliva was found to be unsatisfactory (D.antillarum) so I% diastase (B.D.H.) in distilled water, was used, at room temperature, for I hour, after Casselman (I959), Pearse (I960), with a control incubation in distilled water.

Reaction If the slide exposed to the enzyme shows weak or little staining as compared to the control slide, the presence of glycogen is indicated.

Results The reaction of the cells to the PAS technique was not affected by the diastase incubation (P.miliaris) and it was concluded that glycogen was not present.

DIMEDONE

Method Modified after Bulmer (I959). Cells were fixed in Carnoy vapour, and control slides treated with the PAS procedure, while others incorporated a 3 hour dimedone 'block' with the PAS procedure.

Reaction Staining of both control and dimedone slides indicates the presence of glycogen.

Results It was possible to detect very minute amounts of maganta staining in a very few cells in the dimedone slide, much less than in the control slide. It is therefore concluded that there was very little glycogen in the cells tested, (P.miliaris)

BEST'S METHOD FOR GLYCOGEN

Method Pearse (I960), and Casselman (I959), after Best, (I906).
Reaction Glycogen is stained red.
Results None of the inclusions of the spherule amoebocytes were stained, but inclusions of a few bladder amoebocytes were a brilliant red, (D.antillarum) Again, it was concluded that little glycogen was present.

METHODS FOR ACID MUCOPOLYSACCHARIDES

The methods used depend on the binding of certain substances to the acidic group.

METHYLENE BLUE EXTINCTION

Method Pearse (I960).
Reaction The capacity to bind methylene blue below pH 4 indicates the presence of nucleic acids or acid mucopolysaccharides.
Results Spherules of amoebocytes were found to retain the methylene blue under pH 4, (P.miliaris)

ALCIAN BLUE

Method Pearse (I960), after Steedman.
Reaction The presence of acid mucopolysaccharide is indicated by blue-green staining.
Results The red spherules of the amoebocytes of D.antillarum stain positively; there is no evidence that the colourless spherules stain. There is also a general 'background' staining of the cytolpasm of the other coelomocytes. Although some spherule amoebocytes of

P.miliaris stain with Alcian blue, there is not such a clear or selective correlation between staining and the position of the red spherules. Indeed, it is evident that with some individuals many of the inclusions of the red spherule amoebocytes are not stained with Alcian blue, while in the same individuals it is apparent that small cells, presumably flagellated cells, give a distinctly positive reaction.

COLLOIDAL IRON

Method Mowry's (I958) modification was used, after Muller (I955). P.miliaris
Reaction Blue staining indicates acid mucopolysaccharides.
Results Many inclusions of spherule amoebocytes were positive, but some spherules were negative. When counterstained with the PAS technique, all the inclusions of the spherule amoebocytes were Mowry positive rather than PAS positive, which showed that the substance reacting to PAS was also reacting to stains and techniques for acid mucopolysaccharide.

METACHROMASIA

Method Toluidine blue was used. P.miliaris
Reaction Pink or purple staining would substantiate the presence of acid mucopolysaccharides, Pearse (I960).
Results Some of the inclusions of the spherule amoebocytes appear to stain metachromatically, but a certain amount of pigment retention, see p. 134 , might account for this effect, which cannot therefore be considered reliable.

ALUMINIUM MORDANT METHOD FOR SULPHATED ACID MUCOPOLYSACCHARIDES

Method Methylene blue, in aluminium sulphate solution, was used as recommended, Heath (I962). P.miliaris
Reaction Dark blue staining indicates acid mucopolysaccharide which is ulphated.
Result Inclusions of the red spherule amoebocytes stained dark blue.

GLYCOLIPIDS

Method A positive staining by Sudan black B at the same site as a carbohydrate moiety may indicate a glycolipid, Pearse (I960); see p.157 for lipid staining.
Results Although there is probably some lipid associated with the colourless spherules, see p. 161 , there is no evidence that there is any associated with the red inclusions which react with stains for carbohydrates, P.miliaris, D.antillarum.

GLYCO AND MUCOPROTEINS

Method Protein associated with glyco and mucoprotein is usually demonstrated by Millon's reagent or the alkaline tetrazonium reaction, Pearse (I960).
Results Tests for protein, p. 140 , reveal that although the colourless spherules are associated with protein, the red spherules, which often, if not invariably, contain carbohydrate, do not appear to be, D.antillarum, P.miliaris.

CONCLUSIONS

The results of the above tests for carbohydrates may be summarised as follows:

All of the coelomic cells show weak positive reactions to the PAS and to the alcian blue tests.

The inclusions of some of the spherule amoebocytes, the red spherules in the case of Diadema antillarum, and possibly the red spherules of Psammechinus miliaris, stain more intensely with PAS. This staining is diastase fast, but removable by a short dimedone block. It is unlikely therefore that the substance stained is glycogen, and this is substantiated by negative staining with Best's carmine.

Alcian blue stains all of the coelomic cells a very pale blue, but stains some spherule amoebocytes very intensely. The red spherules of Diadema antillarum are selectively stained by this technique. These inclusions are also stained by Mowry's colloidal iron at a low pH, and retain methylene blue staining under pH 4, (P.miliaris) They may also show metachromasia with toluidine blue, but this affect may be due to the retention of echinochrome, and is not considered reliable. However, the first three tests indicate that these spherules probably contain some acidic mucopolysaccharide.

The red spherules do not stain with Sudan black B, or with protein stains, and it is therefore unlikely that they contain much glycolipid or glycoprotein.

Pearse (I960) quotes several authors as saying that acid mucopolysaccharides do not stain by the PAS reaction. Fusaro and Goltz (I960), however, dispute this conclusion,

finding that 'all those substances which stain magenta with PAS are also alcian blue positive, while those substances which stain red are alcian blue negative.' They admit that there are a few instances where substances which stain with alcian blue do not stain with PAS.

The preliminary conclusion therefore is that the red spherules are associated with an acid mucopolysaccharide, probably sulphated, contained within a thin envelope of a different nature, (from observations of staining after the spherule have burst, see also p.51) Many of the coelomic cells are associated with a smaller concentration of acid mucopolysaccharide or a similar substance.

LIPIDS

The lipids are a rather heterogeneous group, usually described as soluble in certain 'fat solvents', but not water, and including the higher fatty acids, their compounds, and substances usually found in association with them, Bloor (1943).

As Casselman (1959) says, 'with few exceptions the histochemical characterisation of lipids is limited to determining which classses are represented.' He gives the following classification of histochemically demonstrable lipids:

Simple lipids	Derived lipids
Triglycerides	Fatty acids
Ester waxes	Fatty aldehydes
Compound lipids	Alcohols etc.
Phospholipids	Sterols and sterol esters
Glycolipids	Cholesterol and esters

Associated substances

Carotenoids

Fat soluble vitamins

Steroids

Lipid pigments

Tissue preparation

Formalin fixation was used, as recommended by Casselman (1959). Fat solvents were of course avoided except where specifically required by a method.

OIL SOLUBLE COLOURANTS

The most widely used techniques for lipids depend on the solubility of certain dyes in the fats themselves.

These dyes are usually used in alcoholic solution to facilitate penetration, but there is therefore a risk of losing some material. Alcoholic solutions also tend to precipitate. Ethylene and propylene glycols have been recommended as giving stabls solutions and good colouration, but Gomori (I952) states that the latter, at least, may be a fat solvent. He recommends 60% triethyl phosphate, which has no affect on lipids. Casselman (I959) found results obtained with this last solvent to be excellent.

The choice of the oil-soluble colourant

Sudan 3 and 4 have been largely superceded by the use of oil red O. These are all red dyes however, and are liable to be confused with echinochrome, partly retained after formalin fixation, in the present study.

OIL RED O Particularly recommended for neutral lipids, Casselman (I959).
Method after Casselman (I959), alcoholic solution.
D.antillarum
Result Not very satisfactory because the fixation procedure preserved some of the echinochrome in a semi-diffused state, and therefore confused the results. However, as far as could be ascertained, neither type of spherule amoebocyte was stained red, nor were any of the other coelomocytes.

SUDAN BLACK B

This is a slightly basic dye, which is probably responsible for its affinity for phospholipids.

Saturated solution of Sudan black B in 70% ethanol
D.antillarum
Result Faint positive general reaction

Acetylation The basic property of this dye may give rise to non-specific staining, and this is said to be reduced by acetylation of the dye, see Casselman (1959).
Method Acetylated Sudan black B in 70% ethanol, P.miliaris
Result Some spherule amoebocytes were slightly stained; most of the coelomocytes were grayish but some inclusions of the spherule amoebocytes were heavily stained black. When counterstained with alcian blue some inclusions were stained blue. In these preparations it was noted that there were minute black specks in the 'fibroblasts'. A pyridine extracted control did not stain so densely with the dye, but some staining did occur.
Method 2 Acetylated Sudan black B dissolved in 60% triethyl phosphate did not give much staining of any of the coelomocytes.

OSMIUM TETROXIDE

The reduction of osmium tetroxide in weak aqueous solution has long been used as the basis for the demonstration of lipids. Primary blackening is considered to be due to the presence of unsaturated fatty acid. However non-specific blackening also occurs, and some authorities, for example Lison (1953),consider the reaction unsatisfactory.
Method Vapour exposure. D.antillarum
Result Osmium tetroxide vapour does in fact blacken the red spherules of echinoid amoebocytes, as already recorded

by Bookhaut and Greenburg (I940), for Mellita quinquieperforata. If however some HCl/ether extract is evaporated on a slide to give a red residue of echinochrome, a rapid blackening occurs with exposure to osmium vapour. It is therefore suggested that the blackening of spherules known to contain echinochrome may be due to the reducing action of echinochrome and not to the presence of fatty acid.

Osmium vapour or solution also slightly blackens the surround of the colourless spherules. This was noted by Ohuye (I937) for Temnopleurus hardwickii.

NILE BLUE METHOD FOR DISTINGUISHING ACID AND NEUTRAL LIPIDS

Method after Casselman (I959) P.miliaris
Reaction Cain (I947) found that triglycerides coloured red in the dilute solution while lecithin and oleic acid stain blue in both solutions and other fatty acids stain blue only in the dilute solution.
Result Inclusions of some spherule amoebocytes stained blue in both solutions, which indicates the presence of lecithin or oleic acid.

ACID HAEMATIN METHOD FOR PHOSPHOLIPIDS

Method Pearse (I960), after Baker (I946). P.miliaris
Reaction Dark colouration, not present in a pyridine extracted control, indicates the presence of phospholipids.
Result Inclusions of some spherule amoebocytes were blackened, and nuclei were blueish; the control slide

also showed blackened inclusions in some amoebocytes, but no blue nuclei. However, as it is now realised that pyridine may not be entirely effective in removing phospholipids from preparations, see review on lipid extraction, Pearse (1960), it is possible that there was a positive reaction.

CONCLUSIONS

It is tentatively concluded that the colourless spherules may be associated with some lipid, possibly in the form of phospholipid or fatty acid. However, there is some variation in the results, which may not be due to the admittedly inadequate techniques available, and it seems that the amount of lipoidal material demonstrable shows some variation between both individual cells and animals.

It must be emphasized that association of the colurless spherules with lipid has been argued on the results of counterstaining with alcian blue and on the affect of osmium teroxide etc., rather than on easily reproducible correlations described on p. 135 .

NUCLEIC ACIDS

METHYL GREEN PYRONIN

Method I. after Kurnick (I955) 2. Acetate buffered, after Brachet (I942). Pearse (I960) P.miliaris
Reaction D.N.A. green, R.N.A. red.
Results The Kurnick reaction was decidedly unsatisfactory, as nuclei were stained red. With the Brachet reaction, nuclei were stained green, and all cytoplasm was stained pink. The staining did not reveal nucleoli, or any significant concentration of the stains by the inclusions of the spherule amoebocytes.

FEULGEN

Method De Thomasi's Schiff reagent was used, as recommended by Pearse (I960). P.miliaris
Reaction D.N.A. is stained magenta.
Result The nuclei of all the coelomocytes were stained by this procedure.

ENZYMES

ALKALINE PHOSPHATASE: GOMORI'S TEST

Method after Gomori (1946), from Gomori (1952).D.antillarum
Reaction Sites of phosphatase activity are shown by black deposits.
Result There were grayish granular deposits round the colourless inclusions of the amoebocytes.

CYTOCHROME OXIDASE: G-NADI REACTION

Method Gomori (1952) D.antillarum
Reaction Sites of oxidase activity are shown by blue staining.
Result No staining occurred.

DOPA OXIDASE OR TYROSINASE

Method After Pearse (1960) D.antillarum
Reaction Tyrosinase activity is indicated by black-brown colouration.
Result Some inclusions of the spherule amoebocytes were 'stained' a dark gray. However, the preparations proved difficult to mount, and it is not certain which of the amoebocytes reacted. There are a number of difficulties in interpreting results given by this method, as it is now realised that there are a number of different tyrosinases, which might require activation before their presence could be demonstrated, and the adequacy of this test has been seriously questioned, see Pearse (1960)

THE CONSTITUTION OF THE RED SPHERULES

Previous authors have stated, with little evidence to support their suggestions, that the red spherules of regular echinoid amoebocytes are associated with protein and lecithin, St.Hilaire (I897), Kollmann (I908), or have suggested that the red naphthoquinone pigment is combined with a protein, Shapiro (I946). It has also been said that the red spherules are acidophilic, Kollmann (I908), Kindred (I926).

The present investigation indicates that, contrary to the above opinions, the red spherules are basophilic, and that the red spherules of D.antillarum, and probably those of P.miliaris, are not associated with appreciable amounts of protein or lipid.

However, these inclusions are certainly associated with carbohydrate, and this is probably a sulphated acidic mucopolysaccharide. It is suggested that the naphthoquinone pigment is associated with this acidic polysaccharide, (and it should be noted that the red colour of this pigment in vivo is appropriate to an acidic environment, see p. 122), and that this complex is in the form of a viscid liquid contained within a very thin envelope of unknown constitution, see p. 156

THE CONSTITUTION OF THE COLOURLESS SPHERULES

As far as can be determined the often quoted statement that the colourless spherules are 'albuminous' has never been well substantiated. Cuénot (I891b) seems to have made the original statement, backed by general observations. Kollmann (1908) does not give details as to how he arrived at his conclusions on the composition of the spherules, except for allusions to alcohol solubility.

It seems also that in the older work differentiation between the colourless and red spherules has not always been possible, and hence some confusion has arisen.

In the present investigation the colourless spherules from amoebocytes of D.antillarum and P.miliaris have been found to stain with the mercuric chloride bromphenol blue technique. They also show a vigorous positive reaction to Adam's DMAB-nitrite test for the indole nucleus, and show weak positive reactions to other tests for the indole nucleus, and for cysteine, cystine and possible tyrosine. Other protein tests have given more or less general staining. The colourless spherules also show an affinity for Sudan black B and it seems that there is some fatty material present, as suggested by St.Hilaire (1897). This lipid material seems to be acidic, and might be fatty acid, which is notoriously difficult to demonstrate histochemically, or phospholipid; the available tests for which are not very specific. The nature of this substance cannot, in fact, be further clarified on histochemical evidence.

Extraction of a sufficient quantity of cells might provide suitable material for further analysis.

If indeed the colourless spherules are composed of a slightly basic protein, and an acidic lipid, the confusion in some previous accounts of dye affinities of these inclusions may be explained.

The positive reaction to indole tests

Adam's (1957) test for tryptophan is based on a test for indoles. He states 'although all 3-indolyl derivatives would be expected to react with this DAB-nitrite method, only tryptophan and possibly serotonin need be considered, since other soluble tissue indoles, which are not bound to protein would not survive ordinary embedding procedures.'

However such assumptions cannot be made in this study, for 'ordinary embedding procedures' were not used, and it is reasonable to suppose that echinoderms might normally contain substances not found in the mammalian tissues with which this technique was evolved. It was therefore thought advisable to check the identity of the substance which gives such a powerful reaction to indole tests in the colourless inclusions of the spherule amoebocytes. The histochemical techniques available are obviously not sufficient to determine conclusively which indole(s) might be present, so it was thought advisable to attempt extraction and chromatographic analysis.

Indole chromatography

1. Extraction Smith (1958) recommends maceration, and extraction with water, buffers or organic solvents. He does not recommend de-salting, which he considers too destructive. Burnett (personal communication) finds methanol extraction to be very useful for indoles.

Both aqueous and methanolic extraction was therefore attempted. Methanolic extraction, combined with maceration was not very successful; very little indolic material was obtained, and it seemed likely that the cells were not sufficiently lysed by this process. Extraction with glass-distilled water, which lyses the cells effectively, gave much higher yields of indole, as well as naphthoquinone, and other impurities. Fortunately, as Smith (1958) says, 'the chromatographic behaviour of indoles is little disturbed by the presence of amino-acids, salts, urea and etc., in moderate quantities'. The relatively impure extract was therefore used.

2. Chromatography The extract was analysed by ascending chromatography, using glass tanks, at a temperature of about 20° C., and allowing sufficient time for a solvent ascent of about 20 cm. Smith (1958) suggests 6 solvents suitable for indoles, and 3 were selected as being particularly relevant to the problem. These were isopropanol-ammonia, butanol-pyridine and aqueous potassium chloride. Further details are given below, p.168

Isopropanol Ammonia:	Isopropanol	200 parts
	.88 Ammonia	IO "
	Water	20 "

This solvent is said, Smith (1958) to give high reproducibility and equilibration of the paper in the solvent vapour, before solvent ascent, is unnecessary. The solvent is very little affected by inorganic salts.

Butanol Pyridine	n-Butanol	60 parts
	Pyridine	60 "
	Water	60 "

20% Potassium chloride w/v (aqueous)
This solvent is said, Smith (1958) to give very constant Rf values, with easily obtained, compact spots.

Paper Whatman's No. I paper was used, as recommended by Smith, (1958).

Location reagent Ehrlich's location reagent was used. This is composed of:

para dimethyl aminobenzaldehyde	IO% w/v,	dissolved
in hydrochloric acid	1 volume,	
acetone	1 volume.	

The paper is immersed in this reagent, and the spots outlined in pencil as they develop.

The reagent is said to detect 1 microg. of indolylacetic acid, 0.3 microg. of 5OH-indolylacetic acid, and 0.5 microg. of 5OH-tryptamine.

RESULTS The results obtained, with cells of P.miliaris

TABLE 10a

A COMPARISON BETWEEN THE RF OBTAINED WITH THE INDOLE EXTRACT FROM COELOMIC CELLS OF PSAMMECHINUS MILIARIS, MARKER TRYPTOPHAN RUN IN PARALLEL AND 'STANDARD RFS'

INDOLE	SOLVENT		
	ISOPROPANOL-AMMONIA	BUTANOL PYRIDINE	20% KCl
Coelomic cell extract, P.miliaris	20	33'	55
Marker tryptophan	22	33'	55-60
Tryptophan*	25	56	55
Tryptamine*	83	76	48
3 Indolyl acetic acid*	35	84	60
5OH-Tryptamine*	56	70	34
5OH-Indolyl acetic acid*	18	73	48

* = Standard Rfs, as given by Smith (1958)

' = Low values, in butanol pyridine, as compared with the standard Rf. However this solvent is not noted for reproducibility of results, and it is probably more significant that the same values were obtained from both coelomic cell extract and the marker tryptophan

TABLE 11b

RFS OF SOME INDOLIC COMPOUNDS (SMITH, 1958)

INDOLE	SOLVENT		
	ISOPROPANOL-AMMONIA	BUTANOL PYRIDINE	20% KCl
Anthranilic acid	34	82	73
N:N Diethyl Tryptamine	98	83	62
Ethyl Indolyl acetate	95	98	41
Hippuric acid	48	66	85
4OH Tryptamine	54	72	33
5OH Tryptophan	15	40	35
Indole	90		
Indolylacetyl-Glycine	36	65	65
Indoxyl sulphate	60	83	59

are shown in Table IIa, they are compared with marker tryptophan, and the Rfs given by Smith (1958), for ascending chromatograms of biologically important indoles, in the same solvents. Table IIb shows the Rfs of a number of indoles in the same solvents, for comparison.

CONCLUSION It is tentatively concluded that the indole extracted from the coelomic cells of Psammechinus miliaris most resembles tryptophan in its chromatographic behaviour.

THE POSSIBLE SIGNIFICANCE OF THE INDOLE

The apparently high concentration of tryptophan or a similar indole in the inclusions of some of the echinoid coelomic cells may be primarily a matter of the use of a fairly sensitive test. However, an indole is undoubtedly selectively present in the inclusions of the colourless spherule amoebocytes, and its possible significance cannot be ignored.

Tryptophan is one of the 'essential' amino acids for man's nutrition, and is present in many proteins, although not as a major constituent, by percentage weight. In vertebrate tissues tryptophan is particularly prominent in fibrin, chief cell granules, zymogen granules of the pancreas, thyroid colloid, neurokeratin and muscle fibres. It is associated with several syntheses;

1. The formation of indole acetic acid, a plant growth substance.

2. The formation of 5OH-tryptamine, a substance associated with neurotransmission.

3. The formation of nicotinic acid, associated with Coenzymes I and II.

4. The formation of certain pigments, notably ommochromes.

Tryptophan is apparently, therefore, associated with a number of 'active' substances. A further example of this is that it is present in actin and myosin, 'which transfer chemical energy into motion' although it is not found in paromyosin, which plays a passive role, in supplying a 'catch-mechanism', Szent-Györgi (1960). It has been suggested that the association of the indole nucleus with active substances is due to its properties as an electron transmitter, Szent-Györgi, (1957, 1960). He states that 'derivatives of indole seem to be among the most powerful instruments of living tissue' and 'it is tempting to think that Nature introduced this amino acid' (tryptophan) 'into the protein molecule to mediate its charge transfer reactions. This may throw light some day on the real meaning of protein.'

However, it is not possible to be sure that the indole present in the amoebocytes is involved in any such 'exciting' role, even if it is present as a constituent of protein. It is salutory to note, indeed, that some indoles, e.g. kynurenine, may be excreted in quantity, and that no active role has been assigned to such compounds.

VARIATION IN THE INDOLIC CONTENT OF INCLUSIONS

Only once has a diminution of the content of individual colourless spherules been noted from Diadema antillarum. Inclusions stained with mercuric chloride bromphenol blue, see p. 142, showed a very unusual response. The position occupied by the inclusions was faintly outlined and principally chromophobic. A small fraction of the inclusions was stained by the bromphenol blue.

In the initial studies, Adam's DMAB-nitrite test was used with cells from D.antillarum. The inclusions of the colourless spherule amoebocytes invariably gave a vigorous positive reaction. Cells from Psammechinus miliaris, obtained from Millport, see p. 10 , were first tested in May I96I, and no negative reactions were noted from this time until late March I962, although animals tested at Plymouth, in September I96I, showed a very poor reaction. It should be noted that they were in a very poor condition, as they had had no opportunity to feed for several weeks.

Cells of specimens of P.miliaris, received from Millport in February I962, were DMAB negative on 30.3.62. after total starvation. However, when control animals, received 6 weeks previously, and which had been feeding freely, were also tested, their cells were also found to be DMAB negative. Animals received in late March were also found to be 'negative'. Fresh solutions were prepared to check the results, and negative reactions were consistently obtained, with a slightly

positive reaction from the body of the flagellated cells. The coelomic cells when examined appeared quite normal, especially in the animals which had been allowed to feed, but the colourless spherule amoebocytes were less numerous and had smaller inclusions than usual. Although some cells with the usual large spherules were present, they were in the minority.

A female, received on 20.3.62., in which the coelomic cells were 'negative', was examined as follows; smears were made of the stomach and ovarian tissues, which were then fixed in formalin. The stomach gave a negative reaction to DMAB, whereas the ovarian tissue, which was, however, denser, gave a 'medium-positive' result. A fresh consignment of animals was received on I.5.62 and 2 animals were tested on 4.5.62. The first, a female, had very few colourless spherule amoebocytes in the coelomic fluid, and the inclusions of these cells were smaller than usual, I micron in diameter. The other animal was also a female; the coelomic fluid examined contained many oocytes. These were 'positive' to DMAB, although the strength of the reaction should perhaps be correlated with their relatively high density, for sections of the gonads, oesophagus and stomach, fixed in neutral formalin, did not show striking differences in their response to DMAB.

The next consignment of animals was received on I2.6.62. A few animals were tested on arrival and the coelomic cells were found to be DMAB negative. However, 3 animals tested on 2I.6.62 were found to contain cells positive to DMAB. They were therefore separated from the

other animals. The next day all the coelomic fluid from one of the animals found to give a negative reaction with the test, was removed onto a watch glass, and examined. The coelomic fluid contained some 'normal' red and colourless spherule amoebocytes, but they were not as numerous as would have been anticipated from the size of the animal. The entire contents of the watch glass was fixed in formalin vapour and dried slowly onto the glass. The watch glass was then treated with the DMAB-nitrite procedure. There was a fairly general 'background' reaction, but no indication of any staining of the inclusions of the colourless spherule amoebocytes. 4 more animals were tested and found to be DMAB positive, and a fifth animal was negative on 26.6.62. A dilute solution of potassium chloride, see below, was injected into the coelom of these 5 animals. The 'negative' animal spawned ripe ova, whereas no gametes were produced from the 4 'positive' animals. All the remaining animals were then tested for their reaction to DMAB and injected KCl. Only one of these animals was DMAB negative, and this was the only animal to produce ripe gametes on injection, in this case, ova.

It seemed therefore that the diminished response of the coelomic cells to the DMAB test, might be associated with the state of the maturity of the gonads. It was decided that this should be investigated for a further season, to see if the correlation was valid.

PROCEDURE

Each animal received, in the consignments obtained at regular intervals from Millport, was tested as soon after arrival as possible, to determine the response of the coelomic cells to DMAB and the state of the gonads.

Determination of the state of the gonads

The use of potassium chloride to determine ripeness was found to be injurious, see Costello et al., (1957). Harvey's (1956) electrical method for obtaining gametes from urchins was therefore used. Initially a 6v. A.C. supply was used, and then a 12v., and finally, the recommended 10v., when a suitable transformer was obtained. Lead electrodes were used, as recommended, placed symmetrically on the aboral surface of the urchin, a short distance from the gonopores.

When working at Millport marine laboratory, (September, 1962), it was not possible to use this method, so an alternative method was improvised, based on an observation by Fox and Partington (personal communication), who found that mature specimens were readily detected by swinging the animals, wrapped in a cloth, to simulate the affect of a low-speed centrifuge.

RESULTS

Tables 12a and 12b, and the graph no.I, represent the results obtained from June 1962 to July 1963. A total of 280 animals was investigated.

The results are recorded as follows; if a

TABLE 12.

THE CORRELATION OF GONAD RIPENESS WITH 'DMAB' NEGATIVITY OF THE COELOMIC CELLS OF PSAMMECHINUS MILIARIS

DATE	%RIPE	%RIPE & -ve or ± to DMAB	%-ve DMAB	No. tested	Origin of animals
26.6.62	29	29	29	7	M*
29.6.62	12	12	12	8	M
6.7.62	20	20	16	20	M
24.7.62	6	6	29	17	M
3.9.62	24	24	9	36	M North End & Crosshouses
16.10.62	48'	8	8	13	M North End
7.11.62	-	-	-	10	M
27.11.62	-	-	6	16	M North End
8.1.63	-	-	-	14	M dredged
28.1.63	-	-	9	13	M Crosshouses
12.2.63	-	-	7	15	M
5.3.63.	-	-	-	14	M West Bay Pt.
26.3.63	-	-	8	14	M East Flats
19.4.63	18	18	9	11	M East Flats
30.4.63	-	-	15	14	M West Bay Pt
21.5.63	46	33	13	15	M Lion Rock
4.6.63	9	9	45	11	M Crosshouses
25.6.63	38	33	7	18	M Crosshouses
23.7.63	43	36	43	14	M

' = Voltage of stimulating electrodes too high, animals damaged.

* = Millport

TABLE 12.

RESULTS OF TESTS FOR GONAD RIPENESS AND 'DMAB' NEGATIVITY OF COELOMIC CELLS OF PSAMMECHINUS MILIARIS

DATE	%DMAB / %GONAD	− / −	− / +	± / +	± / −	+ / −	+ / +	% RIPE
26.6.62		−	29	−	−	71	−	29
29.6.62		−	12	−	−	88	−	12
6.7.62		4	12	8	20	50	−	20
24.7.62		23	6	−	35	35	−	6
3.9.62		3	6	18	45	42	−	24
16.10.62		8	−	−	16	38	38(?)'	38(?)'
7.11.62		−	−	−	40	60	−	−
27.11.62		6	−	−	37	56	−	−
8.1.63		−	−	−	36	64	−	−
28.1.63		9	−	−	38	53	−	−
12.2.63		7	−	−	13	80	−	−
5.3.63		−	−	−	36	64	−	−
26.3.63		8	−	−	28	64	−	−
19.4.63		−	9	9	72	9	−	18
30.4.63		15	−	−	50	35	−	−
21.5.63		−	13	20	13	40	13	46
4.6.63		*(45	−	9	36	9	−	9)*
25.6.63		6	16	22	12	38	6	44
23.7.63		14	28	14	−	44	−	43

' = Voltage of stimulator too high, animals damaged

* = Animals in very poor condition, all dead the following day

N.B. 0.5%, additional to some of the figures given, has been omitted.

proportion of the coelomic cells exhibited a positive reaction to the DMAB-nitrite the result is recorded as positive (+) to DMAB. If a very few cells were positive or some cells showed a weakly positive reaction the result is expressed as intermediate (±) to DMAB. If no cells were positive then the result is expressed as negative (-) to DMAB. The reaction of the animals to electrical stimulation is expressed as positive (+), where gametes were emitted, and negative (-), where there was no such response.

In presenting these results I have omitted reference to the size and sex of the animals. The animals tested were of the size range 23-45 mm., as a measurement of the width across the ambitus, and most consignments included a reasonable sample of size and sex, the latter being determined externally by the shape and appearance of the gonopores.

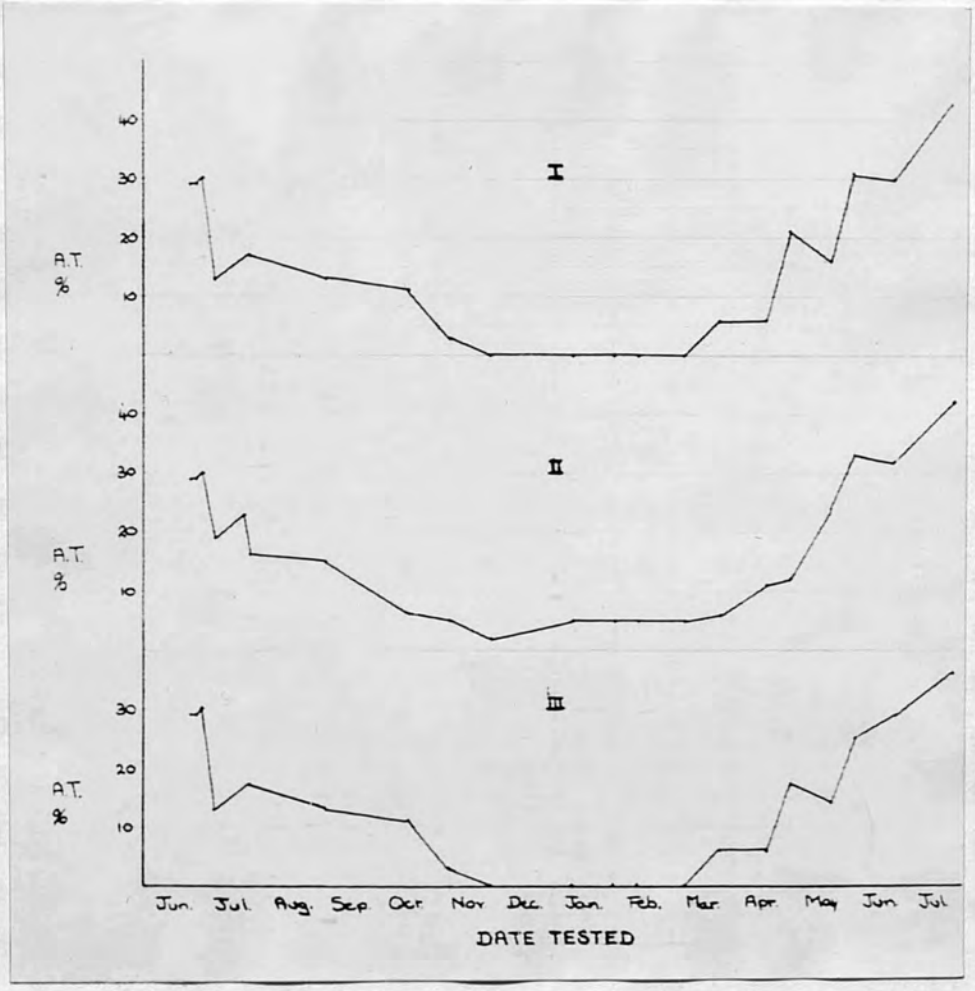
As will be seen from the graph, no. I, there appears to be a clear correlation between the incidence of DMAB negativity and gonad ripeness, when these are plotted separately. This is further emphasized by plot III, which shows the incidence of animals which are both ripe and which have coelomic cells which are DMAB -ve or ±ve.

PRELIMINARY DISCUSSION OF RESULTS

Before discussing these results in general, I would like to briefly consider 2 factors which might affect their validity.

GRAPH 1

PLOTS TO SHOW THE APPARENT CORRELATION BETWEEN GONAD RIPENESS AND 'DMAB' NEGATIVITY OF THE COELOMIC CELLS OF PSAMMECHINUS MILIARIS



AT% = Animals tested, percentage giving,

I A positive reaction to the gonad ripeness test

II A negative reaction to Adam's DMAB test for the coelomic cells

III A positive reaction to the gonad ripeness test, and also a negative or 'intermediate' reaction to the DMAB test.

I. The animals tested were always examined as soon after arrival as possible, and they had usually been collected 2 days before despatch, and were received the day following despatch. During the journey the animals were packed in wet algae and abnormal temperature conditions must be assumed to have obtained. It is assumed that such a short-time exposure to unusual conditions would be unlikely to alter the gonad condition but this cannot be assumed for longer periods. Thus at the onset of the breeding season a sustained but slight rise in temperature caused by transfer to the laboratory probably causes a 'premature maturity', (for general discussion of seasonal temperature and breeding see p. 184). The winter of I962-63 was extremely cold, and no ripe animals were received from Millport until 2I.5.63, as compared with the first 'fresh' ripe animals received in I962, at the end of April. However, animals which had been kept in the aquarium, and which gave no response to electrical stimulation on arrival, on 26.3.63., when retested 25 days later showed a total of I8% ripe, although the next fresh consignment received, 30.4.63., was still I00% negative to electrical stimulation.

It appears therefore that 'laboratory' conditions have a marked affect on the condition of the gonads, and this serves to emphasize the fact that, ideally, the data presented here, should have been obtained immediately on collection of the animals.

2. In comparing the results obtained from different consignments of animals received it is difficult to allow for the slightly different origin of the animals. The origin of most of the animals is known, and all consignments save that received in early January I963, when weather conditions were especially difficult, were littoral in origin, and collected in the vicinity of the Millport marine laboratory. However, they were not all collected from the same place, and do not represent a 'natural population'. Nevertheless the conditions in most of the collecting grounds used appear to be broadly similar.

Unfortunately, it was not possible to arrange more rigorous sampling.

However, bearing in mind the limitations of the sampling procedure, it may still be said that the apparent correlation, originally noted in April-June, I962, between the onset of gonad maturation and a negative reaction to the DMAB-nitrite test for indoles by the coelomic cells, was again noted from tests recorded for a further year. The obvious explanation would be that the coelomic cells, particularly the colourless spherule amoebocytes, release stored material to the maturing gametes. This is not a new idea; Liebman (I950) believed that amoebocytes transferred material to gametes in Arbacia punctulata, but his evidence was scanty.

Liebman (I950) based his conclusions that the

amoebocytes directly transfer material to the gonads on observations that granules, presumed to be derived from disintegrated amoebocytes, are found in close proximity to oocytes in fixed sections of the ovaries. It is interesting to recall that the red echinochrome-containing spherules found in the ova of Arbacia species are apparently identical with the inclusions of the red spherule amoebocytes, and may well be directly derived from them, although the functional significance of these inclusions to the ova remains elusive. Moreover, I have observed amoebocytes, especially the red spherule type, closely applied to the maturing ova of Arbacia lixula, in fresh preparations.

A further investigation, of the gonads of P.miliaris is obviously of interest: it was decided that if amoebocytes transferred material directly to the gametes, the process might be revealed by careful study.

THE GONADS

A survey of the vast quantity of published work on the developmental physiology of the echinoids showed that there is very little available information on the histology and gametogenesis of the gonads, although this may be rectified to some extent by Boolootian (work to be completed and published, personal communication).

Structure and development of the gonads

The latest account of the ovaries and oogenesis is provided by Raven (I96I). He states that in the echinoderms the ovaries have a wide lumen, lined with a rather flat epithelium, which is considered to be the germinal epithelium. Scattered cells of this epithelium enlarge and develop to oocytes, which further enlarge, and 'bulge' into the lumen, eventually becoming loose: the full-grown oocytes being free in the lumen. It is thought, and Caullery (I925) expressed this view, that unspawned oocytes are phagocytosed by 'amoeboid cells', which then form a syncytium in the ovarial cavity; it is believed that this syncytium transfers nutrients to the next batch of maturing oocytes. However, one may well ask whence the original supply of nutriment was derived, and it is obvious that if the syncytium does perform a 'nurse-cell' function then it must be reinforced by an external supply of nutrients from time to time.

Caullery (I925) believed that the gonads of P.miliaris unlike those of Echinocardium cordatum show several cycles of maturation in one season, during the period April-September, at Wimereux, (France). Elmhirst (I923) recorded the spawning dates of this species, at Millport (Clyde), as being June-August, and Orton (I923) recorded spawning at Plymouth between February and August.

There is little information as to what determines the onset of maturation, but is thought, Moore (I934), that commencement of spawning follows soon after the sea temperature starts to rise, with Echinus esculentus.

This, Elmhirst (1923), is a fairly general correlation with species of a certain distribution, and is possibly connected with the sunshine curve and the dependence of larvae on the diatom food supply.

The gonads of Psammechinus miliaris

Gonads of P.miliaris were examined from fresh animals as they were received to obtain further information on gametogenesis.

Gonads from Paracentrotus lividus and P.miliaris were fixed in the following, to determine the most suitable fixative for routine use:

Champy, Susa, Strong Flemming + acetic, Strong Flemming - acetic, Bouin, Formol calcium, Neutral formalin and Zenker-acetic.

It was decided that Bouin and Formol calcium would be adequate for the investigation, and testes and ovaries were fixed in these, and stained with Haidenhain's iron haematoxylin, and the DMAB-nitrite procedures respectively.

Structure of the gonads The origin of the gametes was clearly, as previously described, from the epithelial region. The interior of the gonad lobules was filled with an 'amorphous' and fatty material, containing no obvious nuclei. This was evidently the syncytium, to which the function of 'nurse-cell' has been ascribed. It was difficult to detect any cells apart from a few muscle-fibres, the epithelium and the gametes. There was no evidence, at any time, of any coelomic cells or

amoebocytes being present within the lobules, in P.miliaris, although cells may occur between the lobules.

However, it should be mentioned that the gonads examined did not give evidence of progressive maturation, from November-April, I962-63, for some animals examined in January I963 appeared to have reached a more mature state, judging from the number and size of the gametes, than some examined in March of the same year. This was probably linked with the extremely severe weather conditions obtaining from January-April of this year; it has already been noted that spawning was later in I963 than in I962. The development of the gonads may well have been abnormal in consequence, although this cannot account for the apparent lack of participation of the amoebocytes, as maturation did occur.

The above microscopic investigation of gonads of P.miliaris therefore failed to show direct transfer of material from coelomic cells to gonads by contact with gametes.

The seasonal 'weight relationship' of gonads and coelomic cells

If material of the coelomic cells is drawn upon at maturation in much quantity, as appears to occur with some polychaete annelids, Dales (I96I), this should be evident in the seasonal weight relationships of the two tissues. Unfortunately, it has not yet been

possible to make a comprehensive study, which would require quantities of fresh material, but a few observations have been made:

Thus, a spent ovary of P.miliaris is about half the wet weight of a ripe ovary.

In ripe animals the wet weight of the coelomic cells is from I/IIth to I/26th of the wet weight of the gonads, and from I/5th to I/20th of the gut.

In a spent animal the wet weight of the coelomic cells is about I/I8th of the wet weight of the gonads, and I/32nd of the wet weight of the gut.

In a maturing animal the wet weight of the coelomic cells is about I/4th of the gonads and I/2 of the gut.

The figures given above are compared with gut as well as gonad weights becausethe weight of the gut varies with total wet weight rather than with the 'breeding season', and also, to a certain extent, with the state of nutrition.

From the very scant data available therefore, it seems that there may be a variation of the total wet weight of the coelomic cells, independent of the size of the animal, such that maturing animals have more, and that the quantity decreases with maturity, being lowest in spent animals. This may substantiate the suggestion that coelomic cells transfer material to the gametes.

THE RELATIONSHIP BETWEEN THE COELOMIC CELLS AND THE GONADS

An apparent correlation between onset of gonad maturation and a negative reaction to the DMAB-nitrite test for indoles by the colourless spherule amoebocytes has been noted over two seasons. However, I cannot claim that the evidence presented is better than circumstantial, for exact biochemical micro-analyses from many individuals for another season would be necessary to substantaie the correlation, not the least because there is more individual variation of content among the coelomic cells of Psammechinus miliaris than has been found with Diadema antillarum. This might be explained by varying environmental conditions, such as availability of food-stuffs. This affect is not necessarily direct, for little dimunition is observed in urchins starved for periods of up to four weeks, and might indicate 'general storage' function of the coelomic cells, affected by varying turnover. In any event such variation complicates the issue, and necessitates adequate sampling, strict controls and cautious interpretation.

Nevertheless these observations may verify Liebman's (1950) suggestions, and those of Giese et al, (1958), that coelomic amoebocytes are concerned in supplying nutrient materials for growth and maturation of the gonads.

Bulk transfer of material from the coelomic cells to the gonads at the season of maturation has not yet been adequately shown, although Giese et al (1958)

have said that the accumulation of material by the gonads may well derive from such a source. If transfer of material, either in bulk, or as small quantities of essential constituents, does occur, it is probably not a direct transfer, at least with Psammechinus miliaris, although such a relationship may occur with Arbacia species.

This 'indirect' relationship which is postulated tends to agree with the idea that the gonadal syncytium has a 'nurse-cell' function, see p. 184 It is suggested that coelomic cells may release material into the coelomic or haemal fluids or to the syncytium at the inter-lobular level.

DIGESTION AND TRANSPORT

THE FUNCTION OF THE COELOMIC CELLS: DIGESTION AND TRANSPORT

Some authors have assumed a general inadequacy of 'normal' vascular function in echinoids, and the spherule amoebocytes in particular have been associated with digestion and absorption both because of this, and because their inclusions suggest stored food or possibly enzymes.

Many diverse opinions have indeed been expressed as to the role of the coelomic cells in digestion, absorption and subsequent transport of food material, such that a thorough review is necessitated.

NOTE: Terminology applied to the gut and haemal system

The macroscopic appearance of the gut of regular echinoids is well known. It has suffered however from a variety of terminology applied to the different parts. I shall use the terminology applied by Stott, (1955), in his recent study of the digestive tract of Echinus esculentus, that is: pharynx, oesophagus, stomach+ siphon, intestine, rectum.

The terminology applied to the haemal system will accord with that used in Section 3, p. 210 . The figure 29 , p. 220 , illustrates the major features of both systems.

HISTORICAL

Frenzel (I892) believed echinoid spherule amoebocytes to have an enzyme-secreting function, because the cells showed close connection with the gut.

He thought that these cells passed through the intestinal epithelium to penetrate the gut lumen. The idea that the inclusions of colorless spherule amoebocytes represent precursors of digestive enzymes was further explored by Oomen (I926), who however worked principally with holothurians.

Cuénot (I89I) believed that the inclusions of the colourless spherule amoebocytes of the echinoids which he studied, see p. 19 , represented food stores. Kindred (I926) saw droplets, 'apparently of digested food', in the connective tissue of the intestine, picked up by phagocytic amoebocytes (=bladder amoebocytes), and concluded that amoebocytes with spherules represent phagocytes laden with nutritive material. Stott (I955) found that iron saccharate fed to Echinus esculentus was taken up by coelomic cells, probably 'agranulocytes' (= bladder amoebocytes), in the stomach and taken to the haemal system where migration of iron granules 'associated' with (but not necessarily enclosed in?) amoebocytes occurred through the haemal canals to the radial and gonadal regions. He therefore concluded that the phagocytic bladder amoebocytes are responsible for intracellular digestion of particulate matter, whereas the spherule amoebocytes amoebocytes were envisaged as penetrating the gut lumen, and there incorporating soluble nutrients into their inclusions. He believed that nutrients are then distributed by these coelomic cells by migration through the haemal system.

Other work: Van der Heyde (I922), Lasker and Giese (I954), Farmanfarmaian and Phillips (I962), Boolootian (in the press), has shown that, contrary to previous opinion, nutrient materials pass very rapidly from the gut lumen to the perivisceral fluid. This will be discussed more fully later, p. 241 , it is mentioned here to complement the above conclusions.

ABSORPTION OR TRANSPORT FROM THE GUT LUMEN BY AMOEBOCYTES

Stott's (I955) conclusions that the coelomic cells are actually responsible for absorption, and some digestion, of nutrients, are of great interest. The implications of such a relationship, in enhancing the functional importance of the coelomic cells are obvious and of great importance. The evidence presented by Stott however, is scant, so it was decided to re-investigate the situation.

Firstly, it is essential to determine whether amoebocytes do penetrate the gut lumen.

The occurrence of coelomic cells in the gut lumen

I. Fresh living animals Specimens of Psammechinus miliaris and Echinus esculentus were examined at Millport marine laboratory immediately after collection. Guts of the animals were examined as follows:

Group I: The test was opened from the aboral side, and the perivisceral coelomic fluid removed. The viscera were then washed, in situ, with sea water. The stomach

contents were then examined, preferable after ligaturing the section to be opened, so that contents could be released onto a slide.

No coelomic cells were observed. The contents principally consisted of portions of algae, and parts of crustacea, with some ciliates.

Group 2: The viscera were not washed with sea water and the gut was not ligatured.

Under these circumstances the gut contents appear to include coelomic cells. It was interesting to note that the stomach contents are in no way harmful to the coelomic cells.

2. Preparation of gut I have never observed coelomic cells in the lumen of fixed sectioned gut, although the contents has included boli of algal fragments etc. It is important to note that neither Boolootian (personal communication) nor Giese and other workers have ever remarked the occurrence of coelomic cells in the gut lumen in their intensive studies of some aspects of the biochemistry and physiology of echinoid digestion.

Conclusions

I have never observed coelomic cells in the stomach or intestinal lumen of echinoids, even where algal fragments have been abundant, except where insufficient precautions have been taken, with dissected specimens, to prevent contamination by the coelomic fluid.

Stott based his (1955) conclusions apparently on fresh dissected material, (only actually reported in his thesis, (1952), and it is concluded that the dissections may have been contaminated by coelomic fluid, or cells adhering to the gut wall, as he reports no procedure to avoid this.

In view of these findings considerable doubt is cast on the idea that amoebocytes actively participate in absorption, and as a further check I decided to repeat experiments, with iron saccharate, of the type described by Stott.

METHODS

Absorption of iron saccharate was studied in Psammechinus miliaris, as this was a much more suitable laboratory animal than Echinus esculentus.

Stott's technique was used. Suspensions of saccharated iron carbonate (Flatters and Garnnett), were injected through the mouth of healthy animals, which were then kept in the aquarium, and examined after a suitable time interval. Viscera were fixed by the method used by Stott (1955), after Yonge's (1926) method: The fixative is 50% of 5% ammonium sulphide in 95% ethanol, and 50% Bouin. The presence of iron was detected by use of 10% potassium ferrocyanide, for 10 minutes.

Control experiments (not performed by Stott)

The following control experiments were performed: None of the animals used were injected or contaminated

with iron saccharate.

I. The stomach of one fresh specimen was dissected and fixed, and treated with ferrocyanide in exactly the same way as the injected experimental animals.
2. A different fixative was used with another animal, as Stott admitted the standard fixation used was not very satisfactory. Stomach was therefore fixed in formalin, and then treated with dilute ammonium sulphide for I-3 hours. The standard procedure, which incorporates a hydrochloric acid wash after the ferrocyanide treatment, removed much of the colouration. A modified reagent: 50% of 20% potassium ferrocyanide, 50% of I% hydrochloric acid was therefore used.
3. Sections prepared by the new method described were stained by the Feulgen method for nucleic acids, using de Tomasi's Schiff reagent, and then treated with the ferrocyanide reagent.
4. Stomach sections prepared by formalin fixation were tested by the dinitro-resorcinol method for iron, Pearse (I960).
5. Coelomic cells were exposed in vitro to iron saccharate.

RESULTS

A. Injection of iron saccharate

5 hours after injection iron was present in the lumen of the stomach-intestinal region. There were no cells in the lumen, and there was no iron in the coelomic cells of the sub-epithelial area of the gut or in the perivisceral coelomic fluid.

6 hours after injection of another individual iron granules, but again no coelomic or any other cells, were present in the lumen of the ligatured stomach-oesophageal region and the ligatured mid-stomach region. Very small quantities of iron were detected in the stomach epithelium, apparently in ovoid cells, each containing only one granule. This corresponded well with Stott's figure, which is shown on p. 198. No iron was detected in the coelomic fluid.

17 hours after injection no iron was detected in the stomach lumen or epithelium. The (internal?) marginal sinus* of the haemal system however, contained clumps of iron granules, and although there were no iron granules in coelomic cells large clumps of iron were found free in the coelomic fluid.

28 hours after injection of another individual a small amount of iron was found in the stomach lumen, and more in the epithelium and the haemal spaces, between the epithelium and the peritoneum. Some granules were apparently passing across the peritoneum. In the intestinal region most iron was detected in the peritoneal area. Iron granules were also found in the haemal system, mostly in the external marginal vessel, but some were seen to be passing to the internal marginal vessel, 'round' the siphon, presumably via the vessels, described later, p. 221, called the 'vessels to the stomach', Fig. 31 p. 226. A little 'iron', see comment later, p. 199 was present in the axial organ. Iron was also present in the coelomic fluid, but not in the faeces.

* doubtful identification, possibly the internal marginal vessel

FIGURE 27

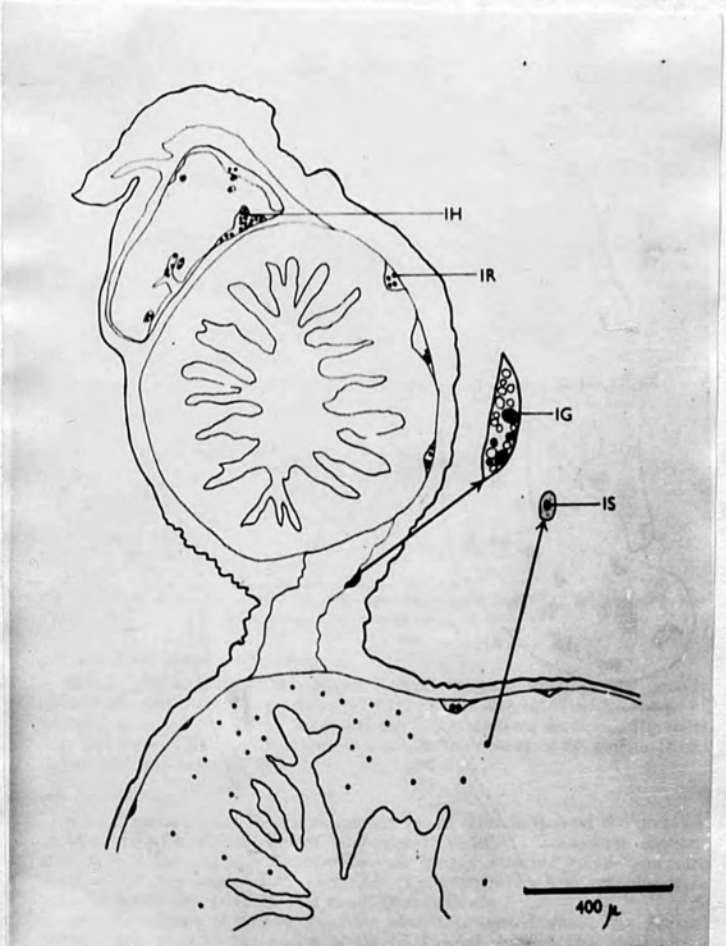


Fig.11.—T.S. stomach showing distribution of iron granules after feeding on iron saccharate (Key to lettering, p. 86.)

STOTT'S (1955) FIGURE, with a 'phagocyte' shown containing an iron granule

Control experiments

When gut sections from animals which had not had contact with iron saccharate were treated with ferrocyanide, granules in ovoid bodies in the epithelium stained blue.

When Feulgen preparations of control sections were treated with ferrocyanide it was found that 99% of the positively staining (blue) granules were singly situated in ovoid nuclei of the epithelium.

Dinitroresorcinal staining failed to produce any positive reaction for iron.

Bladder amoebocytes were found to phagocytose iron saccharate in vitro, invariably taking up several granules.

CONCLUSIONS

After injection of iron saccharate iron may be traced as passing across the stomach epithelium, after which it passes not only to the haemal system, but also to the coelomic fluid, apparently across the peritoneum. Although bladder amoebocytes will ingest iron saccharate in vitro there is no evidence that they pass to the lumen of the gut to transport iron across the epithelium. Indeed, much of the iron detected in both the perivisceral and haemal fluids after injection was not enclosed by coelomic cells.

Control experiments show that granules staining blue with ferrocyanide are present in some (effete?) nuclei of the stomach epithelium. Prof.Millott, (personal communication), has noted these granules in

the axial organ and the skin, and believes them to be connected with pigment bodies he has previously noted. Their significance and origin, interesting as it is, is not relevant to this study, except in so far as these granules apparently confused Stott's (I955) interpretation of his results. There is little doubt that the rounded bodies, containing only one granule of 'iron saccharate' per body, which he figures as amoebocytes passing across the stomach epithelium, see Fig. 27 , p. 198 , may be identified with these 'aberrant' nuclei.

It is concluded that there is no evidence to support the idea that amoebocytes transport iron saccharate from the gut lumen. The apparent passage of iron granules across the peritoneum, as well as to the haemal system, is rather remarkable; it might be explained by clumsy injections and preparation of material but sections show iron apparently passing between peritoneal cells. It is also interesting to recall Awerinzew's (I9II) observations of injections of Strongylocentrotus drobachiensis; he found that carbon and carmine particles injected into the mouth passed to the perivisceral coelom.

ABSORPTION

It was therefore considered that absorption by migration of amoebocytes has not been demonstrated in echinoids, and probably does not occur. However, although iron saccharate has been successfully used to study both digestion and excretion it is not certain that it is a very suitable substance for demonstrating

normal pathways in either function. It was therefore decided to study absorption using more modern tracer techniques.

Absorption was therefore studied by means of radioactive isotope-labelled compounds. There is limited evidence that such compounds may behave di differently from non-active compounds; thus Kitching and Padfield (I960) found that deuterium oxide - heavy water - penetrated the suctorian Discophyra collini, less rapidly than non-active water. However, provided that careful interpretation is used, there is little doubt that studies with labelled compounds can provide important information.

The absorption of C_{14} labelled tryptophan, a substance probably metabolised by the amoebocytes, see p. 166, was therefore traced autoradiographically. Later studies,using labelled sugar, were mainly concentrated on elucidating the role of the haemal system in absorption, and will therefore be considered later.

METHODS: The injection and autoradiographic tracing of labelled tryptophan

The source material, experimental animals; P.miliaris and waste container were kept in a polythene-lined 'cage' in the aquarium.

The source had an activity of 0.0I mc., and it was calculated that not less than 0.5 microcuries would be suitable for injection of an average- sized animal, (from data given by Fitzgerald, I959). The

selected animal was injected through the mouth and placed in aerated sea water in an enclosed polythene container. After a suitable time interval the animal was dissected, and viscera were fixed in ethanol.

The following procedure, adapted after Fitzgerald, (I959), Pearse (I960), was used:

I. Fix viscera etc., I-3 hours in ethanol.
2. Clear in benzene, I5 mins., benzene at 50°C., I5 mins.
3. Take thro' 4 changes of molten wax, 56°C., for 30 mins., each.
4. Embed, block and cut, at less than 8mu if possible.
5. Float sections on warm (45-48°C.) dust-free distilled water.
6. Pick up sections on slides, previously dipped in a I:I mixture of I% gelatine and I% chrome alum, and dried.
7. Dry the slides.
8. Deparaffin the slides, and either stain here, see note on staining, or after film processing.
9. In dark-room, using Wratten safe-light 5, cut stripping film (Kodak A.R.IO) into suitable squares with sharp blade, allowing for generous overlap of sections and slide.
IO. Strip one square slowly, and float - emulsion side downwards, on dust-free distilled water, at 23-25°C., until maximally expanded at that temparature, (about IO mins.). N.B. The temperature is <u>critical</u>.
II. Place a slide in the water, sections facing

upwards, and lift sotthat film covers specimens.

I2. Dry in front of fan at room temperature.

I3. Store in the dark, at room temperature, or less, for optimum time, see Note 2, below.

I4. Process film in dark-room, as before, at I7.5 - ± 0.5°C. The temperature is critical. A.R. grade chemicals are used, and all solutions filtered before use. The developer used was either Kodak's Dolmi, or DI9B., used as directed for 5-IO mins. The slides were rinsed in running tap water, and fixed for twice the time taken to clear.

I5. The slides were either mounted in aqueous media, or dehydrated, cleared and mounted in D.P.X.

Note I Staining

Fitzgerald (I959) states that because staining techniques may remove isotopes they should be postponed until after exposure, if possible. However, the gelatine and emulsion of the stripping film heavily absorb most stains and therefore obscure the underlying sections, and although special tachniques have been evolved to minimise this affect, they have not been wholly successful. Eventually an Azan schedule, used at stage 8, before the emulsion was applied, was adopted. Processing of the film removed most of the aniline blue, but the orange G and acid fuchsin survived the procedure, and were quite effective. Alternate slides were retained and mounted, without film processing, to assist interpretation.

Note 2 Time of exposure

The ideal time for exposure of radio-isotopes to sensitive fil, has been calculated to be about twice their halflife. However, as Fitzgerald (1959) comments, this would be tedious for some elements, a remark particularly applicable to the isotope of carbon used here, which has a halflife of 5000 years! The minimum suitable time of exposure for the experiments described here was 7 days.

The use of whole animals

It was felt that the analysis of dissected gut portions, although these provided some information, would be better replaced by serial sections of small whole animals. This would allow better and more complete analysis of the distribution of the active material throughout the whole animal. For this material Bouin fixation, with penetration facilitated by cutting the peristome, and subsequent decalcification in nitric acid-alcohol, was used. The material was then treated as previously.

INTERPRETATION OF RESULTS

The method was only moderately successful. Silver grains were deposited in concentrations, some of which could be distinguished as artifacts. Common causes of artifact were minute folds in the gelatine, and any minute dust particles on the slide; hence the emphasis on dust-free reagents. It was therefore suspected that some deposition might be due to interaction at the

surface of the section. However, control sections showed that this was not so, and that the major deposition of silver was due to the radio-isotope.

Grain counting, a severe strain on the eyes, was considered to be unnecessary.

RESULTS

28 hours after injection the principal concentration of active material was found to be within, or closely associated with, the nuclei of the sub-peritoneal region of the stomach. Some activity was detected in an associated haemal vessel, probably the internal marginal vessel, (which had been partly 'split-off' and displaced by fixation). Control sections showed very sparse depositionoof grains, apparently in uniform distribution. Feulgen staining confirmed that the round bodies containing concentrations of activity were the elongated and ovoid nuclei found at the base of the stomach epithelium.

50 hours after injection activity was principally found in the sub-peritoneal and intra-epithelial regions of the stomach and oesophagus. No activity was detected in the coelomic fluid.

I40 hours after injection of a small animal - diameter across ambitus about I5 mm. - activity was found to be diffused, much of it being on the coelomic surface of the peritoneum, and presumably in the coelomic fluid. Some activity was detected in haemal spaces of the gut, with more in the region of the sub-peritoneal 'vacuolated' cells of the stomach.

Experiments involving sectioning of whole animals, which it had been decided, see p.204 , would give a more complete analysis of the fate of the injected material, were unfortunately marred by difficulties in mounting. The difficulty had not occurred previously, and was possibly due to an increase in the ambient temperature, which could not be controlled. The gelatine became infiltrated with a thin layer of air, either at, or soon after, mounting, such that detailed high power analysis was impossible. Considerable thought and experiment designed to overcome this problem, by varying pre-mounting and mounting technique, was generally unsuccessful, so that these experiments were reluctantly abandoned.

In vitro exposure of coelomic cells to labelled tryptophan

Fluid and cells were exposed to the tryptophan for 30-45 mins., then fixed in formalin vapour, see p. 12 , and treated as per p. 202 . Nuclei and cytoplasmic inclusions were readily distinguished. Concentrations of activity were found to be sited in the nuclei of some cells, round the peripheral inclusions of the flagellated cells, and in the inclusions of the colourless spherule amoebocytes.

CONCLUSIONS

It was possible to conclude that there was no evidence that coelomic cells were directly involved in the absorption of injected tryptophan, even though the exposure of coelomic cells to labelled tryptophan, see

above, showed that they will absorb it.

Labelled tryptophan appeared to pass fairly rapidly across the epithelium of the stomach, to the sub-peritoneal region, becoming concentrated in the sub-epithelial nuclear and haemal areas. It was subsequently detected in other haemal areas and in the coelomic fluid, but not concentrated in the coelomic cells. It is, of course, important to recall that this amino acid is an important constituent of protein and might be retained at sites of nutritional deficiency. This partly explains its 'diffusion' after passage through the gut wall.

DISCUSSION: THE ROLE OF AMOEBOCYTES IN DIGESTION AND ABSORPTION

The concept of amoebocytic digestion and absorption in invertebrates may be traced originally to the work of Yonge: he first described (1926) amoebocytic digestion of particulate matter in the gut of the lamellibranch, Ostrea edulis. Oomen (1926) found free amoebocytes in the gut of holothuria, and this, combined with previous observations on asteroids, led him to conclude that amoebocytes play an important part in the absorption and transport of nutrient materials. Takatsuki (1934) made some observations on the amoebocytes of O.edulis, which were thought to confirm Yonge's (1926) conclusions, and he (1937) included a section on 'amoebocytic digestion' in his

review of the metazoan digestive system. He then referred to echinoderms, citing various authors, including Oomen, and stated that 'In the Echinoidea and Holothuroidea, amoebocytes occur in the epithelium of the gut and also free in the lumen, and it is possible that these may be the principal agents of absorption and some intracellular digestion.' As far as I can ascertain however none of these papers which he cites state that amoebocytes occur in the gut lumen of echinoids.

It appears that Stott (1955) was partly influenced by this opinion, for he cites Yonge, and the other authors mentioned. Unfortunately, his experiments, which led him to confirm Yonge's suggestion, were not controlled, and his conclusions are therefore at least partly invalidated. T

The investigations described above show no evidence to support the idea that amoebocytes are responsible for intracellular digestion of food, or its absorption.

Firstly, there is no evidence that coelomic cells penetrate the gut lumen.

Secondly, experiments with iron saccharate, previously performed by Stott (1955), which purported to demonstrate passage of iron granules across the stomach epithelium, were found, as stated above, to have been erroneously interpreted. In fact, there is every reason to suppose that the ovoid bodies carrying one 'iron' granule, which Stott found in his preparations, and which he thought represented migrating

amoebocytes, and which can be shown to occur in control sections, are epithelial nuclei, albeit rather strange ones.

Further, iron granules may be shown to pass across the epithelium, and can be traced to the haemal and perivisceral fluids, independent of any association with coelomic cells.

Moreover, absorption of labelled tryptophan occurs without participation of coelomic cells.

It is interesting to note that the concept of amoebocytic digestion in lamellibranchs has recently undergone extensive attack and revision; thus, Mansour and Mansour-Bek (1946) queried the value of the original observations, and recent reviews, Owen (1958), Morton (1960) have given emphasis to the role of the intracellular digestion by cells of the digestive diverticula.

In conclusion, therefore, I would emphasize that coelomic cells are not concerned in the initial absorption and transport of nutrients, although they may well store nutrients, and to some extent act as transport agents, see p. 189 , in the echinoids studied.

THE HAEMAL SYSTEM

THE HAEMAL SYSTEM

There is little evidence to justify the name of the 'haemal system', for its function has never been established, nor has its extent been conclusively determined. Indeed, as Farmanfarmaian and Phillips (I962) record, the first description of the haemal system, Tiedemann (I8I6) was apparently in response to the prize offered by the French Institute in I809 for a description of the 'circulatory' system of asteroids, echinoids and holothuroids. The system was subsequently the subject of much research and discussion in the nineteenth century, and yet even the doyens of comparative anatomy failed to agree on its structure, or establish its function as deserving the description 'haemal'. Nevertheless it is described as such in textbooks, although the descriptions, even those deriving from the same source, tend to vary considerably.

The earlier authors, for example, Perrier (I875), Bonnet (I925), assumed that the system carried absorbed material from the gut. However, in a more recent paper, Farmanfarmaian and Phillips (I962), have dismissed the system as enigmatic and of no importance in digestion and transport of nutrients. Conversely, Stott (I955), confidently asserted that amoebocytes migrated through the haemal system to distribute nutrients.

The study of the haemal system presented here was initiated for two reasone. First, because it was understood that function attributed to it were relevant to the study

of the functional importance of the coelomic cells, and many amoebocytes are found in it, and secondly, because so many accounts of the haemal system disagree or contain unsubstantiated data.

HISTORICAL

As already mentioned, Tiedemann (I8I6) first described a 'circulatory' system in echinoids, and noticed contraction and 'expansion' of some vessels. As Koehler (I883) says, 'La circulation chez les Oursins a été l'objet d'un grand nombre de récherches', for Tiedemann's paper was elaborated by a number of authors, whose work is now difficult to trace. They include the following, quoted by Koehler (I883) and Hamann (I887); Valentin (I84I), Gegenbaur, Hoffmann, Milne-Edwards, Teuscher, Muller, Agassiz (I874) and Perrier (I875). The careful investigations of the last-named, Perrier, in I875, settled much of the controversy surrounding the previous papers, which had disagreed on some fundamental points, such as the actual numbers of vessels present, in a given species. Perrier studied the anatomy and circulation of the haemal and water vascular systems of Echinus esculentus. Perhaps his most important conclusion was that the organ previously described as the heart, supposedly pumping fluid round the haemal system, and now known as the axial organ, had no such function. His detailed observations will be referred to as appropriate later, his concluding remarks are of interest here;

'L'appareil circulatoire, dépourvu de coeur, se compose:

a. D'une partie absorbante comprenant les deux troncs

marginaux de l'intestin, le vaisseau collateral et le réseau capillaire limité à la première courbure de l'intestin;

b. D'une partie respiratoire comprenant le canal du sable, les vaisseaux ambulacraires terminés en cul-de-sac et les tentacles on tubes ambulacraires. Ces derniers et les vesicules aplaties dont ils dependent constituent le veritable appareil locomoteur.'

This study was impressive, containing some beautiful illustrations, and although he did not resolve all the difficulties, his masterly work apparently discouraged further investigations. Since that time there have been only two detailed studies of the haemal system; Hamann (I887) and Bonnet (I925). Hamann studied Sphaerechinus granularis and Bonnet made a comparative study of the 'appareil digestif et absorbant' of Echinus esculentus, Sphaerechinus granularis, Arbacia lixula and Paracentrotus lividus.

Interpretation of this literature has been extremely liberal and there is confusion even surrounding the basic anatomy of the haemal system of Echinus esculentus. Thus Chadwick (I900); 'Both vessels'(intestinal)'have been described as opening into a circular vessel, which is said to surround the oesophagus in close proximity to, yet quite distinct from, the water vascular ring canal. The existence of such a separate circular vessel is, however, open to doubt; and this remark applies with greater force to the blood-vessels which have been described as radiating from it, and traversing the ambulacra between the water vascular and pseudhaemal canals. Such vessels are not evident in carefully prepared serial

sections of the ambulacra of the present species.' Perrier (I875) did describe a peri-oesophageal haemal ring, but did not describe any radial ambulacral haemal vessels. Neither could he find evidence for a peri-anal haemal ring, which had previously been described.

Cuénot (I948) affirms the existence of the radial haemal strands but admits that they cannot be demonstrated by injection. A figure, see Fig. 28, which he uses shows the internal marginal vessel of the stomach as occurring between the stomach and the siphon, whereas it normally occurs internal to the siphon, see Fig. 30, across which smaller vessels branch to the stomach.

Perrier (I875) based much of his conclusions on the facility with which dyes could be injected, to prove existence of definite vessels. However, Parker and Haswell (I943) state that 'whatever be its functions, this system is not a system of blood vessels. It is made up of a series of strands of a kind of connective tissue, with many leucocytes, permeated in a very irregular way by minute lacunae without definite walls.' This view is reiterated by Farmanfarmaian and Phillips (I962); 'in general this system consists of poorly defined sinuses often filled with red coelomocytes.

The following observations on the contractility of the haemal vessels round the stomach show some disagreement;

Perrier (I875) 'La contractilité de ces vaisseaux ne m'a paru avoir rien de rhythmique, et l'on ne peut considerer aucun d'eux comme propre à jouer le rôle de coeur; il me parait même probable que la direction du

FIGURE 28

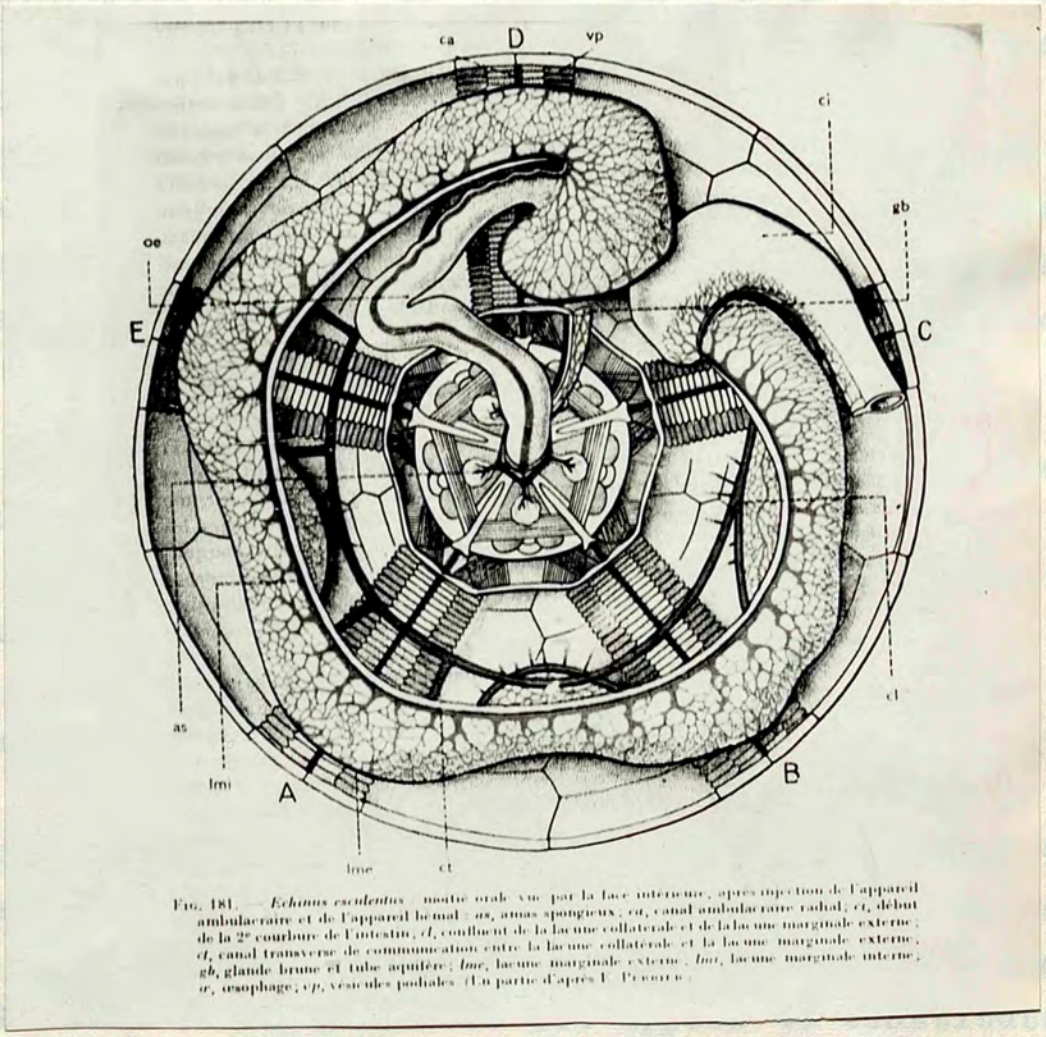


FIG. 181. — *Echinus esculentus* : moitié orale vue par la face intérieure, après injection de l'appareil ambulacraire et de l'appareil hémal : *as*, amas spongieux ; *ca*, canal ambulacraire radial ; *ct*, début de la 2e courbure de l'intestin ; *cl*, confluent de la lacune collatérale et de la lacune marginale externe ; *ct*, canal transverse de communication entre la lacune collatérale et la lacune marginale externe ; *gb*, glande brune et tube aquifère ; *lmi*, lacune marginale externe ; *lmi*, lacune marginale interne ; *or*, œsophage ; *vp*, vésicules podiales. (Un partie d'après H. Prouho.)

FIGURE USED BY CUÉNOT (1948) WHICH SHOWS THE INTERNAL MARGINAL VESSEL BETWEEN THE SIPHON AND THE STOMACH

courant sanguin intestinal n'est pas constante.'

Parker and Haswell (I943) 'Neither of these systems' (the peri-haemal and haemal) 'comprises vessels with contractile walls.'

Hyman (I959) 'Stott (I955) concludes that the use of coelomocytes for intracellular digestion is associated with a poorly developed haemal system lacking a pumping mechanism.'

Farmanfarmaian and Phillips (I962) state that 'no movement of fluid within any part of the system has been observed even though a rhythmic beat may be seen in the outer sinus of the stomach and its collateral vessel.'

On circulation the following opinions have been expressed;

Parker and Haswell (I943) 'there is no definite circulation.'

Cuénot (I948) figures a circulation, but does not comment on it.

Stott (I955) also figures the same pathway of circulation, but from the text it becomes obvious that this must refer to the pathway of migrating amoebocytes.

Farmanfarmaian and Phillips (I962) comment that 'this collection of sinuses does not appear to constitute a circuituous system, nor does it appear to have, functionally speaking, a point of origin or terminus.'

It is, in fact, almost possible to find a contradiction to every averred fact. Opposing statements regarding the structure and composition of the haemal system are summarised in Table 13 , p. 217

TABLE 13

THE STRUCTURE OF THE ECHINOID HAEMAL SYSTEM

AUTHORITY	Per--oesoph. ring	Peri-anal ring	Radial strands	Position of p.o. ring
Perrier* 1875	1	-	-	above w-v ring
Hamann* L887	2	?		
Chadwick* 1900	?	?	?	?
Jammes** 1904	1		+	below w-v ring
Sedgewick* S1909			+	
Shipley & MacBride* 1915			-	
Parker & Haswell* 1943			+	
Cuenot* 1948	1	?	+	above w-v ring
Hyman* 1950	1	?	+	above w-v ring
Stott* 1955	1	+	+	
Borradaile et al * 1958	1	+	+	below w-v ring

* Echinus esculentus ** Paracentrotus lividus

Peri-oesoph., p.o., = peri-oesophageal haemal ring
w-v ring = water vascular ring

Much of the discrepancy might be attributed to the difficulty of detecting the haemal vessels in fixed preparations, and also the great difference in prominence and distribution of the system in different species. However, as already noted, much of the original work has been performed on Echinus esculentus, which, in my experience, shows little individual variation in structure.

I describe below the anatomy of the system which I have dissected and observed in Psammechinus miliaris and Echinus esculentus, two very similar species. The pattern of the haemal system, which undoubtedly exists in these species, is not the same as that of, for example Paracentrotus lividus and Diadema antillarum. I do not intend to include a comparative survey throughout these types but will concentrate on the two species first named, as the system is more clearly visible in these.

THE ANATOMY OF THE HAEMAL SYSTEM OF PSAMMECHINUS MILIARIS AND ECHINUS ESCULENTUS

The two animals are very similar and the following remarks apply to both.

The anatomy of the haemal system is best studied by dissection of fresh non-fixed specimens. Dissection from the aboral surface is most suitable for display of the main vessels.

The haemal system is usually said to comprise the vessels round the gut, the oral and aboral ring areas and the radial vessels. However, as already shown, the existence

FIGURE 29

AO + SC = Axial organ and stone canal, connection with madreporite cut.

CV = Collateral vessel, with branches extending to the external margin of the stomach.

EMV = External marginal vessel, this is continuous with the collateral vessel.

I = Intestine, this includes all the 'unstippled' second loop of the gut in this figure.

IMV = Internal marginal vessel.

M = Mesentery. The mesenteric connections from the external margins of the gut have been cut. The narrow region of the internal margin of the intestine here labelled 'M', may also contain a haemal vessel. Injected material, however, only passes as far as a separate vessel is indicated.

O = Oesophagus.

R = Rectum, cut and displaced.

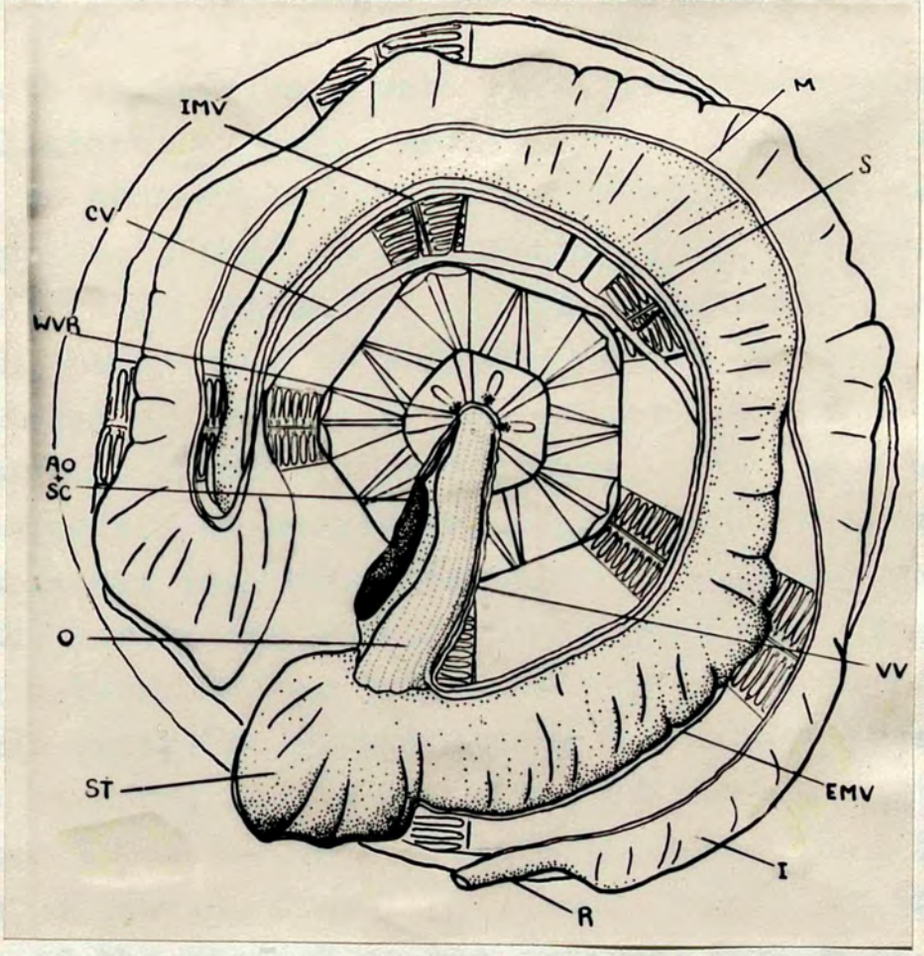
S = Siphon, see also Fig. 31.

ST = Stomach.

VV = 'Ventral' vessel of the oesophagus.

WVR = Water vascular ring, and spongy bodies, see also Fig. 30.

FIGURE 29



DRAWING OF A FRESH DISSECTION OF PSAMMECHINUS MILIARIS: DISSECTED FROM THE ABORAL SURFACE TO SHOW THE 'HAEMAL SYSTEM' AND GUT x 3

of some of these parts has been disputed.

However, in the two species investigated the existence of the vessels round the stomach region cannot be disputed, for though transparent they are readily detected. I propose therefore to consider these vessels first. Fig. 29 shows the general disposition of the area in P.miliaris.

Both species have well developed internal marginal and collateral vessels, see Fig.29, a term which is more suited to such distinct tubular entities than the description 'sinus' which has been used. The internal marginal vessel extends along the inner margin of the stomach, connecting at the oesophageal junction with a thin strand of a slightly convoluted and opaque tissue, the 'ventral vessel', which passes up the oesophagus to the region of the peri-oesophageal water vascular ring.

Branches pass from the internal marginal vessel of the stomach, across the siphon, to the stomach, where they form a network on the wall, see Fig. 30. A thin external marginal vessel is present along the entire external margin of the stomach, and is augmented at its extremities by the collateral vessel. This collateral vessel lies in the coelom, extending from the external margin of the mid-region of the stomach to the external margin of the stomach-intestinal junction. There are usually 7 connectives passing from the free collateral vessel across to the parallel external marginal region, Fig. 29. Bonnet, (1925) describes the relationship between the external marginal vessel and collateral vessel in Echinus species as follows; 'le canal marginal externe est double - par un canal collateral libre.' The general impression is that the external marginal vessel has given rise to this parallel

FIGURE 30

AV = Haemal vessel connecting with the axial organ.

HR = Haemal ring (?).

R = Radial strand (?)

SB = Spongy body.

SC = Stone canal.

VV = Ventral vessel (haemal) of the oesophagus

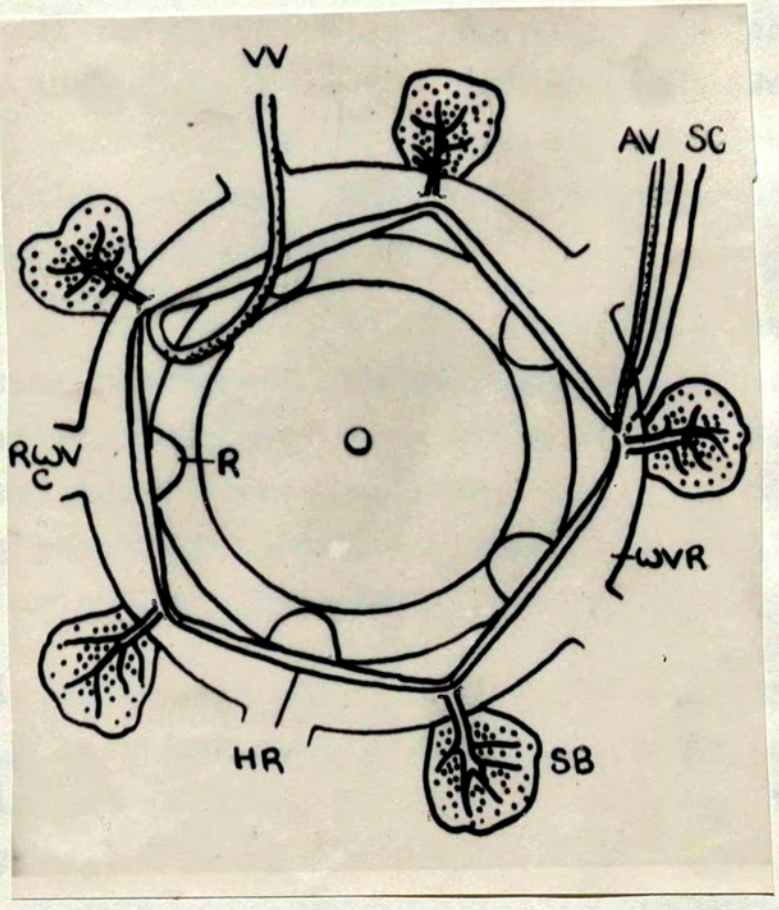
WVR = Water vascular ring.

RWVC = Radial water vascular canal.

N.B.

1. There may be 'haemal' vessels to the spongy bodies.
2. The ventral vessel is partly occluded by spongy material.
3. The stone canal enters the water vascular ring on its oral aspect.

FIGURE 30



ECHINUS ESCULENTUS: DIAGRAM OF STRUCTURES ASSOCIATED WITH THE WATER VASCULAR RING, redrawn from injected preparations ×7

vessel which remains in close connection with it and eventually rejoins it.

It is very difficult to ascertain how far the internal and external marginal vessels extend onto the intestine. The internal marginal vessel appears to become lacunar at the intestinal junction, and extends some way along the mesentery, but becomes very thin and difficult to detect. Similarly, the external marginal vessel becomes very small beyond the junction, Fig. 29.

The ventral vessel of the oesophagus may be traced towards the pharyngeal region where it appears to enter a narrow peri-oesophageal ring lying aboral to the peri-oesophageal water vascular ring, Fig. 30. A strand from this ring passes to the axial organ in parallel course with the origin of the stone canal.

It is extremely difficult to detect any radial haemal strands. I have not seen them in sections, nor have I been able to inject or dissect them. However it is possible that they collapse very easily.

I have not been able to determine the connections of the 'haemal' strands passing to the gonads. It is apparent that they may have connection with the axial supply or with the intestinal vessels but these are very small, if indeed they exist, and ordinary dissection technique cannot distinguish them from mesentery, and they collapse in section. It seems that the gonadal strands originate from a peri-anal region enclosed by mesentery, and again difficult to dissect. A study of these strands is to be published by Boolootian (personal communication).

The walls of the haemal vessels

The walls of the haemal vessels, when fixed and sectioned after ligaturing so that they are not in the collapsed condition, are seen to be relatively thin. They seem to consist of the external peritoneum, collagen fibres, scattered muscle fibres, and internally a few nuclei, which may represent the endothelium. This latter is very similar to the 'discontinuous endothelium' Hanson (1949) described from blood vessels of annelids.

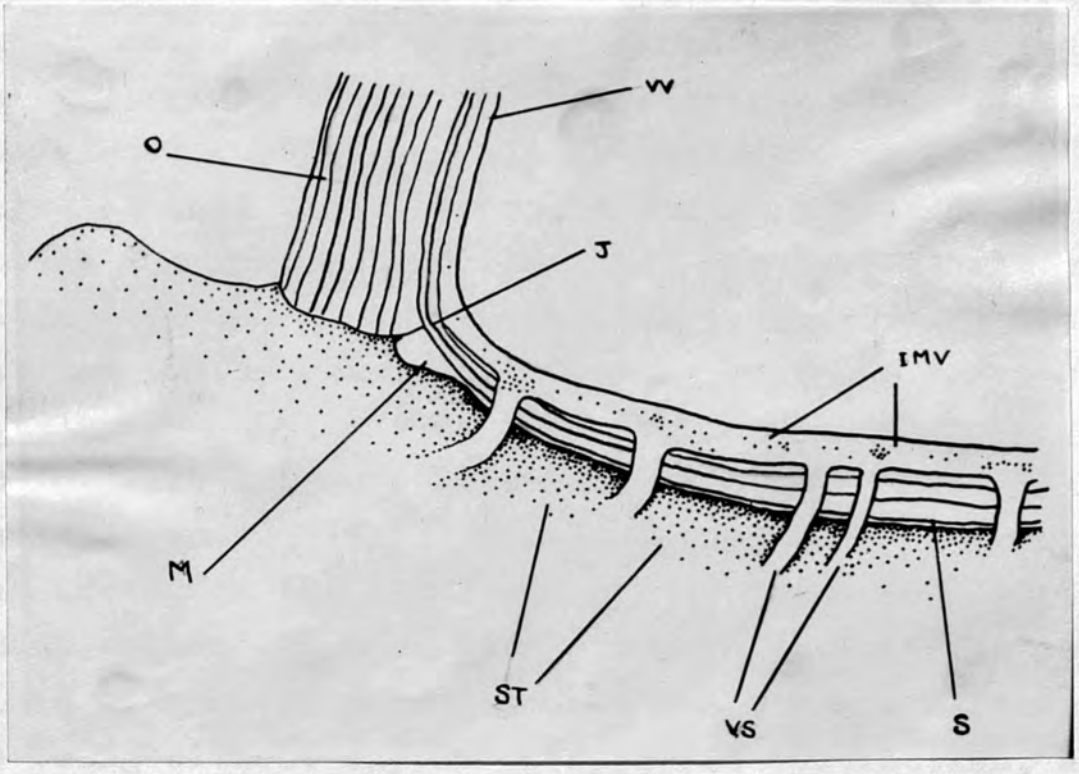
THE CONTENTS OF THE HAEMAL SYSTEM

Hamann (1887) described the content of the 'Darmlakunen' as being a yellowish fluid, containing 'Blutzellen.' The fluid is sometimes referred to as albuminous, and the fluid emitted from the cut internal marginal vessel of E.esculentus is usually thick and milky in appearance, not mixing very readily with coelomic fluid.

Small clumps of material, coloured red, are usually observed through the transparent walls of the internal marginal and collateral vessels of freshly dissected animals. As the preparation ages, the clumps become larger and may occlude the vessels.

Fluid withdrawn by a very fine hypodermic inserted into the internal marginal vessel of E.esculentus contains red and colourless spherule amoebocytes, and some, not many, bladder amoebocytes. Amoebocytes of both types were clumped around greenish granular material, and this is presumably what constitutes the visible particles of the vessels. There were no flagellated cells.

FIGURE 31



PSAMMECHINUS MILIARIS: REDRAWN FROM A LIVING PREPARATION OF THE STOMACH-OESOPHAGEAL REGION, to show the junction of the internal marginal and ventral vessels. x 6

IMV + Internal marginal vessel ST = Stomach
J = Junction of oesophagus with siphon
M = Mesentery VV = Ventral vessel
O = Oesophagus VS = Vessels to stomach
S = Siphon

CONTRACTION OF THE HAEMAL VESSELS

Active and rapid contractions of the internal marginal and collateral vessela are observed in freshly opened specimens. This was observed first in Echinus esculentus in the present study. The contractions were vigorous and stimulated further enquiry. Investigations of the literature revealed that this phenomonen has been repeatedly observed, denied and ignored. It was first recorded by Tiedemann (I8I6), as quoted by Hamann (I887). As already noted, p.214 Perrier (I875) noted contractions of these vessels in E.esculentus but stated that they could not be regarded as acting like a heart. He thought that the direction of flow varied. Hamann (I887) repeats Tiedemann's observations, see above, on the contraction and 'expansion' of the 'Blutlakunen'. Bonnet (I925) speaks of the slow movement of fluid within the internal marginal vessel. He describes it as being without rhythm or apparent contraction, although he found the vessel to respond with a strong contraction to stimulation by touch. As already mentioned, Parker and Haswell (I943) affirm that the system has no vessels with contractile walls, and Farmanfarmaian and Phillips (I962) although admitting the existence of contraction, deny that any fluid movement occurs. Stott (I955) states that movement through the haemal system depends on migration of amoebocytes and believed that the system lacked any pumping mechanism.

RECORDED OBSERVATIONS: CONTRACTIONS OF THE HAEMAL VESSELS OF PSAMMECHINUS MILIARIS AND ECHINUS ESCULENTUS

These observations were recorded from animals dissected from the aboral surface.

As noted later, the haemal vessels respond to some stimuli, p.231 , and mechanical or similar stimulation was avoided as far as possible. Sea water inhibits the vessels and also initiates deterioration of the tissues so contamination was therefore reduced to the minimum possible. As coagulation of coelomic cells round the vessels interferes greatly with their freedom of movement, much of the coelomic fluid was carefully removed on dissection, and the coelomic fluid minus the cells was replaced round the vessels, disturbing them as little as possible.

The collateral vessel usually shows more extensive contractions than the internal marginal vessel, perhaps because it is less restricted, being principally 'anchored' at its ends. As it is hanging 'free' in the coelomic fluid, it can both shorten to some extent, and constrict, to partially occlude the lumen.

Whereas the contraction of the collateral vessel is a series of propagated contractions, each contracted portion occupying about 3-5 mm. of the vessel, the internal marginal vessel contracts more or less simultaneously along its length.

The following rates of contraction were recorded from freshly dissected animals;

P.miliaris

I. Vessels not disturbed, except by original removal of coelomic cells to prevent coagulation impedance. The cell-free coelomic fluid was replaced and surrounded the vessels.

Internal marginal vessel	IO contractions	/ 90 secs.
Collateral vessel	IO "	/ I80 secs.

2. Vessels disturbed in removing products of ripe ovaries, which impeded observations. The collateral vessel was also obscured by the stomach, which was therefore displaced slightly out of its normal position.

Internal marginal vessel	IO contractions	/ 58-69 secs.
Collateral vessels	IO "	/ 45 secs.

E.esculentus

Rates of contraction of 4-8/minute were recorded for the collateral vessel, = IO contractions/ 75-I50 secs.

It is interesting to compare these rates of contraction with the rates observed for the contractile dorsal vessel of holothurians;

Henri (I903) found a rate of IO/I2 per minute, Kawamoto (I927) a rate of I-2/minute, and Prosser and Judson (I952) found that the excised vessel, 5 cm. long, would beat 4-5 times per minute when suspended in a moist chamber.

THE COLLATERAL VESSEL

The contractions pass from the intestinal area towards the oesophagus. The contracted area occupies from 3-5 mm. of the vessel, and the contraction is completed in 2-3 secs;

there is then an active expansion of the diameter of the vessel for about 3-4 seconds. There may then be a period of quiescence, for 3-10 seconds, followed by another contraction. The lumen of the vessel is temporarily occluded during contraction, when the diameter of the vessel is 0.1 - 0.3 mm., while it expands to about 1 mm. The movement of particles, principally clumped amoebocytes, see p.225, within the vessel, gives some indication of the affect of the contraction on the contents of the vessel. As the preparation ages and deteriorates the amoebocytes tend to form abnormally large clumps which block the vessels. The particles which are observed under the microscope to move freely may not therefore reflect the rate of movement possible under normal conditions; however they show some interesting features.

On contraction, the particles move very rapidly away from the source of contraction, at a rate of about 3 mm./second for about 6-8 mm. On relaxation the particles rebound in the opposite direction, but do not usually regain their former position. It was not possible to obtain very accurate measurements, but the net transfer in position was 1-4 mm.; at a rate of 6-8 contractions per minute, the particles may be calculated to move 6-32 mm. per minute.

THE INTERNAL MARGINAL VESSEL

As already noted, the internal marginal vessel does not show waves of propagated contractions, but contracts more or less simultaneously along its length; the oesophageal end slightly in advance of the intestinal end. The diameter of the internal marginal vessel is about 1.2 mm. at rest, and it contracts to about 6-8 mm., so that the lumen is

is restricted rather than occluded. The flow of particles in the vessel therefore tends to be more continuous than in the collateral vessel. The rate and extent of the movement is therefore less easy to measure, but the net movement for each contraction may be as great as 6 mm., and as little as I mm.; a net transfer rate of 6-60 mm. per minute.

STIMULATION OF THE VESSELS

Local mechanical stimulation of both vessels gives powerful local contractions, whereas general agitation of the surrounding fluid tends to increase the rate of contraction of the vessels.

As there has been so much doubt about the activity of the haemal vessels it was felt that increased rates of contraction were of much greater interest than inhibited conditions, which are more easily obtained and less informative.

Wyman and Lutz, (I930) found that adrenaline chloride at a concentration of I in I00,000, gave a marked acceleration of the intestinal blood vessel of Stichopus californicus, whereas a concentration of I in 50,000 gave initially a strong contraction. The effect of adrenaline on echinoid haemal vessels was therefore investigated.

2 drops of I in I0,000 adrenaline chloride, in sea water, were added to the coelomic fluid near the internal marginal and collateral vessels of an aborally dissected Psammechinus miliaris. This resulted in a strong contraction of the collateral vessel, followed by an increased rate and decreased amplitude of contraction of

this vessel, with little change in the internal marginal vessel.

The results, expressed as rates of contraction, were as follows;

Temperature; c. I8° C.

Normal contractions vessels immersed in coelomic fluid minus cells.

Internal marginal vessel	IO contractions	/ 9I secs.
Collateral vessel	IO "	/ I80 secs.

Control a small amount of sea water was added to the coelomic fluid near the two vessels, to determine the effect of agitation and the addition of this medium.

Internal marginal vessel	IO contractions	/ 90 secs.
Collateral vessel	IO "	/ I20 secs.

Effect of adrenaline added in the same manner as the sea water above, near the two vessels; 2 drops of I in IO,000 concentration.

Internal marginal vessel	IO contractions	/ I03 secs.
Collateral vessel	IO "	/ 55 secs.

The collateral vessel is therefore apparently accelerated by adrenaline, and the internal marginal vessel slightly inhibited. This compares with the response of the dorsal vessel of S.californicus, Wyman and Lutz, (I930), which was stimulated by adrenaline.

Although local mechanical stimulation of the internal marginal vessel causes a local contraction of the vessel, general stimulation appears to have little accelerating effect, unlike stimulation of the collateral vessel.

DIRECTION OF FLOW AND POSSIBLE CIRCULATION

I. Direction of flow

As already noted particles in the haemal vessels may be observed to move in two directions; initially on contraction, with a rebound on relaxation. There is usually a net transfer in the direction of the initial contraction movement, but this is not invariably so.

The net movement in the collateral vessel is often towards the oesophagus, whereas the concurrent movement in the internal marginal vessel is away from the oesophagus, but the exact reverse has also been observed; with flow in the collateral towards the intestine and flow in the internal marginal towards the oesophagus. I have never observed flow in the collateral towards the oesophagus concurrent with flow in the internal marginal towards the oesophagus, or concurrent flow in both vessels towards the intestine.

It is particularly difficult to determine the final direction of flow when the particles in the haemal fluid are small and therefore difficult to keep under observation, and the flow is rapid in both directions, as occurs in really healthy preparations. It is possible that very little transfer actually occurs under these conditions, and that there is merely rapid movement equally in both directions, as implied by Perrier, (1875), see p.٢١٥-٦.

When the direction of flow in the collateral vessel is towards the oesophagus, flow of particles in the collateral bracches linking with the external marginal vessel appears to be principally towards the stomach,

although there is considerable back-flow, and a pair of adjacent vessels observed had a flow from the collateral up one to the external marginal vessel, whereas at least some of the material passed almost immediately back down the other vessel to the collateral again. It is very difficult to detect flow from or to the branches of the internal marginal vessel.

II Pathways available to injected material

Injections were performed with 'Record' hypodermic syringes, using the smallest needle size available; with a diameter of 0.3 mm. As already noted the diameter of the two vessels, internal marginal and collateral, is I to I.2 mm. when fully relaxed, but the mechanical stimulation of an unsuccessful insertion causes contraction and further difficulty. In order to inject the haemal vessels of the stomach in an average specimen of Psammechinus miliaris much of the intestine and test had to be removed, to give access for the needle. Prof.G.H.Foxon uses an Agla syringe and plastic connectives to increase manoevrability, but the length of the actual needle relative to the incurving test is the limiting factor with these small echinoids.

It was not possible to inject the collateral vessel, due to its rather delicate suspension and extreme contractility. Attempts at injecting the ventral 'vessel' of the oesophagus and the peri-oesophageal ring were also unsuccessful, not only because of the small diameter involved. It was indeed possible to insert the needle in these vessels', but it seemed that these tissues deserved the description lacunar and spongy, terms that have also been used, apparently undeservedly, for the collateral and

internal marginal vessels. If a true lumen was present it was never successfully located for direct injection. It is relevant to mention that sections of this 'vessel' show a small and sometimes indistinct central cavity.

Injections of the intestinal area were also difficult because of the small diameter involved.

Prof.G.H.Foxon very kindly assisted with the injection of X-ray opaque substances into the internal marginal vessel of Psammechinus miliaris. Unfortunately however it was not possible to obtain satisfactory plates from these animals, with organs in situ, because of the heavily calcified test. It was also observed that injected 'Thorotrast' appeared to diffuse, (see note on the nature of the walls, p. 225, and see also p. 200) rather rapidly from the internal marginal vessel. This substance is used as a colloidal suspension, with a particle size of about 10 Å, Maxfield and Mortensen (1941), so that such diffusion is understandable. 'Chlorostab', an X-ray opaque barium preparation, tended to block the finer vessels.

Appropriate dyes, e.g. aqueous eosin, and methylene blue, injected into the haemal system with a fine hypodermic needle, gave some information about the pathways available within the system.

Injection I

Into the oesophageal end of the internal marginal vessel, towards the intestine.

The dye penetrates the internal marginal vessel until just after the stomach-intestinal junction. There is simultaneous passage across the siphon, via the small branches, to the stomach wall, thence into the collateral

vessel via the external margin and the connecting collateral branches, and a small way along the external marginal vessel of the intestine.

Injection 2

Into the oesophageal end of the internal marginal vessel, towards the oesophagus.

The dye passed along the first part of the ventral 'vessel' of the oesophagus, but did not reach the peri-oesophageal ring region. With another injection it was found to pass to the stomach branches, across the siphon, and did not pass into the ventral 'vessel'. When the injection was made very near to the oesophagus and the dye passed into the stomach vessels, across the siphon, see Fig. 30, it did not reach the collateral vessel.

Injection 3

Into the intestinal end of the internal marginal vessel, towards the oesophagus.

The dye encountered strong resistance and was forced back along the internal marginal vessel by active contractions; it did not, therefore, reach the oesophageal end of the vessel. Some of the dye did however penetrate the collateral vessel.

CONCLUSIONS AND DISCUSSION: PATHWAYS AVAILABLE TO MATERIAL

Dyed fluid, therefore, passes quite readily into the collateral vessel, from the internal marginal vessel, when injected from either end of the vessel towards the other, but there is resistance to injection towards the oesophagus, which tends to confirm that the principal flow in the

in the internal marginal vessel is towards the intestine. There is no evidence to suggest that the normal pathway of material would be from the internal marginal vessel to the collateral, and as the only observations on branch-flow shows that the collateral branches may show a stomach-directed bias, the reverse flow may well occur; that is, flow from the collateral to the internal marginal vessel.

It may well be that reversal of flow pattern occurs, and that mere agitation of fluid is the net result under normal conditions. However, although it is not yet clear what may be the undisturbed pattern of movement, and no method can be envisaged which would provide complete analysis for such a spherical and looped system, it is obvious that considerable transfer of material could take place.

As previously calculated, the collateral vessel can transfer particles at the rate of 6-32 mm. per minute, whereas the internal marginal vessel could transfer at the rate of 6-60 mm. per minute. The animals measured were slightly larger than the average, as they were easier to observe, with a diameter of about 30 mm., and a circumference of about 94 mm. From a transfer rate of 6-32 mm. per minute it may be calculated that the material from the oesophageal end of the internal marginal vessel might be transferred to the intestinal end in I-I2 minutes.

I have very little information on the possible extent of vessels negotiable beyond the stomach. It is possible that there is an available pathway to the peri-oesophageal ring via the ventral 'vessel', but no movement has been observed in this vessel, and injections have not

successfully penetrated it. Similarly I have no evidence that there is unrestricted passage along the intestine.

Cuenot (I948) and Stott (I955) both figure a circulation which has a wider distribution. Neither comment adequately on the derivation of the figures, which show the same circulatory pathways, although it must be assumed from Stott's comments that the pathway is principally that of migratory amoebocytes, as he does not believe the system to have a 'pumping mechanism'. Stott's figure is reproduced, Fig. 32, p. 239 . The pathway is as follows; material passes along the internal marginal region of the intestine and stomach to the ventral vessel of the oesophagus, and thence to the peri-oesophageal haemal ring. It then passes to the radii or to the axial sinus and thence to the gonads. This is therefore a 'one-way' system, passing material outwards from the absorption areas, with no feed-back into the system.

The only other published information on circulation in the haemal system of echinoderms relates to the circulation described by Kawamoto (I927) for the holothuria Paracaudina species. In these animals the 'blood' is said to run from the intestine through the haemal network into the dorsal sinus, which is contractile. Contraction of this vessel drives fluid to the ventral sinus, whence it passes to the haemal network and the haemal ring from which it is said to be distributed to the body wall via the radial haemal vessels.

SUMMARY: CIRCULATION

My observations indicate that active circulation is restricted to the vessels of the stomach, which may form a

FIGURE 32

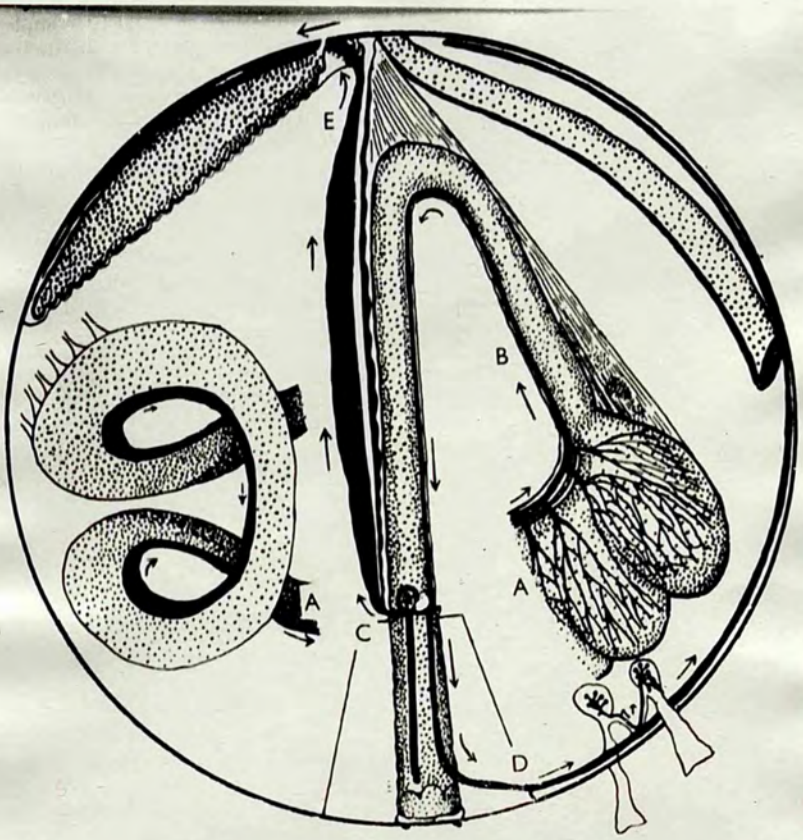


Fig. 17.—Diagrammatic figure illustrating routes taken by iron granules after feeding with iron saccharate. A, agranulocytes carrying iron through wall of stomach; B, route of iron along inner haemal canal and ventral oesophageal canal; C, perioesophageal haemal ring leading to axial organ; D, route along radial haemal strands to ampullae; E, route into aboral haemal channels to the gonads.

'CIRCULATION' OF MATERIAL THROUGH THE HAEMAL SYSTEM: FROM STOTT (1955)

closed system. If the observations were valid, then the most usual pathway is for material to pass down the internal marginal vessel from the oesophageal end towards the intestine at an average rate of about I5 mm/ second, and to pass along the collateral vessel towards the oesophagus at a similar rate. The two vessels are connected via the external margin and the collateral branches, and the predominant flow may be from the collateral to the internal marginal vessel. The possible functional significance of this activity will be discussed later, p. 243.

THE FUNCTION OF THE HAEMAL VESSELS

As already noted, the original concept, Perrier (I875), Bonnet (I925), that the haemal vessels are responsible for transport of the products of digestion, has not been substantiated or refuted. Stott's (I955) theory, the evidence for which has been critically reviewed, p.\9\- , that amoebocytes carry the products of digestion from the gut and actively migrate through the haemal system to the periphery, is not substantiated.

Recent work tends to suggest that the coelomic fluid acts as principal transport agent. Thus Lasker and Giese, (I954) found an increase in sugars in the coelomic fluid soon after introduction of carbohydrates to the gut. Farmanfarmaian and Phillips (I962) extended this work by studying the pathway of products of labelled algae, fed to Strongylocentrotus purpuratus. They found that the products of digestion, mainly galactose, diffused into the 'plasma' of the coelomic fluid (perivisceral), reaching a peak level about 6 hours after feeding. Their conclusion that the haemal system is unimportant in nutrient translocation was based on an experiment in which they severed the main 'haemal' strand supplying one of the gonads. They found no difference in accumulation of labelled products. However, this work has been partly discredited by Boolootian (personal communication), who has studied the haemal supply to the gonads in these urchins, and finds that the statement made by these authors that 'each gonad is penetrated by only one sinus from the aboral haemal ring' to be untrue, so that an alternative pathway exists.

It seems possible indeed that the haemal vessels round the stomach transfer nutrients from the gut; these then

diffusing rapidly to the coelomic fluid. I have already noted that, with iron saccharate, particles appear able to pass directly through the stomach wall to the coelomic fluid, as well as into the haemal system, but this is an unphysiological substance of large particle size, which might act as an irritant.

The possiblity that the haemal vessels round the gut act as intermediaries was therefore investigated with a more appropriate substance.

EXPERIMENTAL PROCEDURE

Psammechinus miliaris was used in these experiments. Individuals rarely exceed 4 cms. in diameter, and although the small size was convenient, in that small quantities of material are sufficient for injection, the small diameter of the haemal vessels was found to cause some difficulties, and access to them was sometimes limited by the proximity of other organs.

Solutions containing 2-6 microcuries of C_{14} labelled invert sugar (mainly sucrose), were injected into the mouth of suitable animals. After a short time interval, the suitable range having been calculated from the available data on diffusion of carbohydrates into the coelomic fluid, the injected animal was carefully dissected and appropriate samples of organs, haemal vessels and coelomic fluid, were removed for examination. These samples were placed on weighed planchettes, which were then reweighed. The samples were then dried, and examined under a Geiger-Muller or Scintillation counter, operated at pre-determined suitable voltages. The dry weights of the samples were of the order 5-10 mg., or less. They were not homogenised as it was felt

that with the very small samples, such as the haemal vessels, this procedure would cause excessive loss of material. Background counts were determined for the planchettes used. 3 minute counts were suitable for the Geiger-Muller counter, whereas 40 second counts were adequate for the scintillation counter.

All results were ultimatelt expressed as counts/ unit time/ unit wet weight or dry weight, with the background count first deducted. It was particularly important to allow for the background count where low counts were registered.

RESULTS

The results obtained are summarised in Tables 14, p. 244, and in the Graph 2 , p. 245 . Corrections for self-absorption by material of the emitted electrons, calculated from data supplied by Hevesy (1948) were applied to the results.

DISCUSSION

It is evident that the ratios calculated for activity/ unit time/ unit wet weight, p. 244 , show that both the internal marginal, and particularly the collateral vessel, have consistently higher activities than the stomach. The collateral vessel always has higher activity/unit wet weight than the surrounding coelomic fluid, in the time range investigated, whereas the internal marginal vessel usually has a similar or higher activity than the coelomic fluid.

The graph, p. 245 , shows that collateral and coelomic fluids have close correspondence in attaining peaks, whereas the internal marginal vessel appears to be 'out of

TABLE 14

PSAMMECHINUS MILIARIS: A COMPARISON OF THE ACTIVITY OF MAJOR ORGANS AND COELOMIC FLUID WITH HAEMAL VESSELS, AFTER INJECTION INTO THE MOUTH OF LABELLED INVERT SUGAR

The activities are expressed as ratios to the activity of the stomach, and are estimated as per unit time, per unit wet weight.

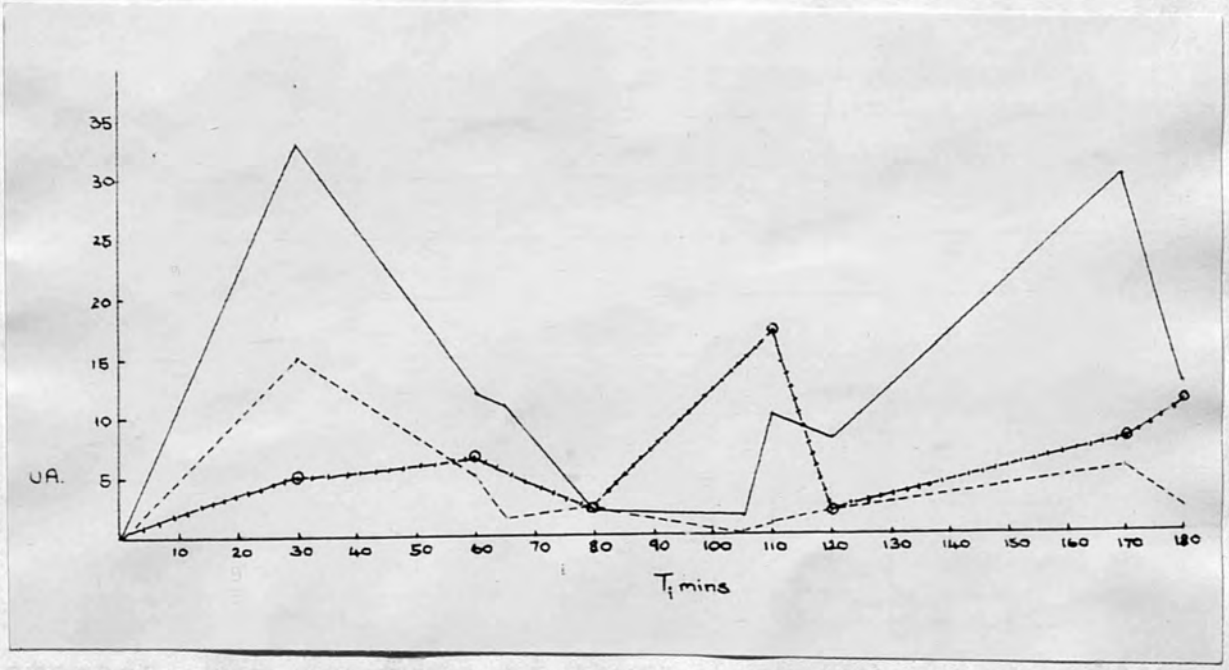
TIME AFTER INJECTION, mins.	Stomach	Intestine	Gonad	Coelomic fluid	CV	IMV
30-45	1	2.3	6	3-27	33	5
60	1	1.8	2.6	1-10	4-20'	7
65*	1		2	1-2	2-20	10
80	1	2	0.75	2.25	2	2.5
105	1	0.8	0.6	0.45	2	
110	1	2.3	1.6	1	9.7	10
120	1	0.2	0.3	2	8'	2
170	1	2.5	14	3-10	30	8
180	1	2	1.8	2	12.5	11

CV= Collateral vessel LMV = Internal marginal vessel

*= control experiment, injected through the oesophagus as a living preparation.
'= control, by ligaturing the vessels.

GRAPH 2

PLOT FROM TABLE 14 To show the relationship between the activity of the collateral and internal marginal vessels and the coelomic fluid



UA = Unit activity T = Time in minutes

—— = Collateral vessel ++++ = Internal marginal vessel

- - - = Coelomic fluid

phase'. The figures however, were, of course, obtained from different individuals.

Although these results must be regarded as of a preliminary nature, it is suggested that they indicate that the haemal vessels may be actively concerned in absorbing and transferring material from the stomach. The small size and volume of the vessels presented great difficulty in interpretation, and wet weight comparison was obligatory. Pure haemal fluid should have been compared with similar weights of coelomic and gut fluid. However this was impossible with the urchins available.

THE FUNCTION OF THE HAEMAL SYSTEM

I tentatively conclude that it is unlikely that the haemal system functions as a complete circulating system. Indeed I have been unable to locate the radial haemal strands and find the aboral and peri-oesophageal regions very difficult to elucidate structurally. However, there is undoubtedly active movement, and possibly a more restricted circulation, within the vessels surrounding the stomach. The function of these vessels, which contain a thick fluid, may well be as intermediaries in absorption, passing extracted nutrients to the coelomic fluid, both 'extraction' and diffusion being possibly aided by an imperfect circulation and its attendant agitation.

DISCUSSION

DISCUSSION: THE FUNCTIONAL IMPORTANCE OF THE COELOMIC CELLS

The copious literature shows that it has been easy to suggest functions for the coelomic cells of echinoids and other echinoderms, but that it has not been easy to establish them. The very ubiquity of the amoebocytes indeed provokes the concept that they have an important but not necessarily localised function.

The present study has been concentrated on an analysis of the coelomic cells in an attempt to elucidate their importance. Of the three main cell-types which I have described, the flagellated cells are apparently confined to coelomic spaces. They may have the function of encouraging circulation of the perivisceral coelomic fluid, by 'agitation', as Cuénot (I89I*b*) suggested. The concept that these cells are parasites cannot be ignored, but I believe that it can at least be partially discredited, see pp. 82-6 In this connection it is important to recall Geddes' (I880) idea that these cells are derived from the ciliated cells of the peritoneum, and to repeat that the flagellated cells are not found elsewhere than in the coelom, - lined with these ciliated cells. It is difficult to accept the idea that the perivisceral and peri-pharyngeal coeloms of nearly every urchin are parasitised.

The bladder amoebocytes are clearly phagocytic, and by a 'phase-change' to the filiform and 'fibroblast' condition, are responsible for coagulation. A deeper significance, relating to excretory and nutritive function, see p. 191 , has been attached to the phagocytic capacity, but it is clear that neither true excretion, (as opposed to deposition of inert substances), nor nutrition depends on

these cells, see discussion, p.207 . The importance of coagulation in echinoids might be queried, but it is certain that sea water is injurious to the internal tissues, and that small test injuries would be effectively and quickly blocked by these coagulating cells. Urchins which have obviously sustained and repaired such injuries, presumably caused by heavy seas, on rocky shores, are quite often encountered.

The function of the amoebocytes with inclusions has never been established, but has usually been considered to be connected with nutrition. Thus the name 'eleocyte' or oil-cell, for the red spherule amoebocytes, McClendon (I9I2), Boolootian and Giese (I958), and the name 'trephocyte', Liebman (I950). Cuénot, (I89I, ~~I897~~) considered these spherules to represent stored food, and the following authors concur; St.Hilaire (I897); Kollmann (I908); Kindred (I926); Liebman (I950); Stott (I955).

The principal constituents of the spherules, as I have determined them, namely carbohydrate and pigment for the red spherule amoebocytes, protein and lipid for the colourless spherule amoebocytes, do not preclude a nutritive function. They are unlikely to act as primary agents in nutrient transport; this function is probably fulfilled by the haemal vessels, see p.246, and the coelomic fluid, Farmanfarmaian and Phillips (I962), Boolootian, (in the press). However, these cells may well act as stores, possibly releasing nutrients to the tissues, and particularly the gonads, see p. 188 , (compare Dales, I96I), as need arises.

The importance of spherule amoebocytes in pigment metabolism, distribution and deposition has already been

partly analysed, Jacobson and Millott (1953). Further studies by Millott (personal communication) show that much pigmentation is found in the same form in the tissues as in the amoebocytes, and there is some evidence that amoebocytes may deposit pigment by extrusion of inclusions. Tyrosinase and a tyrosinase inhibitor have been described from the cells and coelomic fluid respectively, of Diadema antillarum, and appear to be significant in melanogenesis in this animal, Jacobson and Millott (1953). Millott has recently (private communication) suggested that there may be a relation between the indolic compounds of the colourless spherule amoebocytes, see p. 165-6, and melanogenesis. Raper has shown, in his classical studies of melanogenesis, that indoles are involved. There are difficulties however, for the red amoebocytes are known to darken on exposure, and second, the indolic compounds envisaged by Raper, are hydroxylated in the 5;6 positions whereas the indoles shown by Adam's reaction are 3 indolyl compounds.

Nevertheless, the possible significance of the very active quinone and indole groups now known to be associated with the red and colourless spherule amoebocytes respectively, see p. 120 & p. 165-6, is of great interest, and has exciting potentialities. These substances may indeed be metabolic wastes, but such groupings are often associated with the 'nucleus' of biochemical activity, Szent-Györgi (1960), as is emphasized by the recognition of the potentiality of the recently discovered substance known as ubiquinone or Coenzyme Q.

It is becoming increasingly evident that the study of echinoid, and indeed any other coelomic or 'blood' cell,

implicitly involves an attempt at understanding the functional significance of the fluid in which the cells are suspended, and the dynamic 'partition' of functions of transport, and functions subsidiary to an effective transport system, such as haemostasis and phagocytosis, between cells and fluid. Dales (I96I), has already initiated studies of this broader nature on polychaetes, and Boolootian (personal communication) has explored some aspects of this important problem in echinoids.

As has been shown, however, knowledge of transport in echinoids is both disseminated and controversial. The brief synthesis which follows may elucidate the relationships.

Invertebrate vascular systems are traditionally divided into 'open' and 'closed, a morphological and embryological, rather than a physiological distinction.

In echinoids it is obvious that there are two 'closed' systems; the peripheral water vascular system, and the internal haemal vessels round the stomach, neither of which are truly circulatory as the description is ideally applied. Although fluid movement may be uni-directional in some of the vessels it is likely that the total fluid movement is of the 'ebb-and-flow' nature, originally attributed to the vertebrate blood system.

Movement of fluid in the water vascular system is achieved both by agitation on locomotion and by the more regular ciliary activity of the peritoneum, whereas movement of fluid in the haemal vessels depends on muscular contraction of vessel walls. Both these 'systems' are in intimate association with the perivisceral coelomic fluid over much of their surface area, and movement of this fluid

is dependent on movement of viscera and the ciliary activity of the peritoneum.

The peripheral water vascular fluid, as well as playing a very important and well recognised part in locomotion, is respiratory, Farmanfarmaian (I959, I960?). The internal haemal vessels are probably concerned with absorption of nutrients from the gut, p. 246. It has already been amply demonstrated that nutrients pass to the perivisceral coelomic fluid, Lasker and Giese (I954) Farmanfarmaian and Phillips (I962), as does oxygen, Farmanfarmaian, (I959, I960?). It seems that this fluid acts as an intermediary and accessory in the essential transport functions, comparable perhaps to the role of tissue fluid in vertebrates.

A consideration of the coelomic cells in relation to the 'transport' system outlined above, reveals some interesting facts. Thus, the water vascular and perivisceral fluids, which are nearer to the exterior than the haemal vessels, contain more bladder amoebocytes, important in injury by virtue of both their phagocytic and haemostatic (coagulative) properties. The haemal vessels are actively contractile; and contain no ciliated or flagellated cells. All of the fluids contain a proportion of spherule amoebocytes, here considered to have some nutrient storage function. The concept that active migration of amoebocytes is responsible for transport either of excretory or nutrient material, over considerable distances, is seen to be an unnecessarily complex interpretation of function, for where these cells do act (if indeed they do) as transport agents, they would be transported by fluid

movement, and migration would only be of importance in moving in or between tissues.

The transport system envisaged here is different from that only recently attributed to the group, Ramsay, (I952). He states that there is no evidence that the water vascular or any other system in echinoderms performs any of the functions normally associated with the circulatory systems of other animals. Such views are not supported here, and it is pertinent to contemplate his idea of 'sluggish' echinoderms with their'lack of circulation', against the background of less active metazoa which he recognises as having a 'true' circulatory system. In contrast with his attitude is Nicol's (I960) recognition of the holothurian 'closed vascular system'! Indeed, Ramsay (I952) later admits that much so-called circulation in the invertebrates he recognizes as having a bona fide 'circulatory' system, is of an imperfect nature, and it is salutory to note that the rate of flow I have measured in echinoid haemal vessels is faster than the rate of flow in animals with 'accredited' circulations.

Unfortunately, the concept of 'sluggish' echinoderms has been over-emphasized and too readily accepted. Certainly anyone who has watched Diadema antillarum gets a different impression. Hyman's (I955) reflections on the group, as a noble one, 'especially designed to puzzle the zoologist', is nearer the truth, even though it appears to admit defeat. As far as the coelomic cells, coelomic fluid and the 'vascular' systems are concerned, the puzzle may be at least partially resolved by recognition of a very

delicate relationship between fluid and cells, even although many of the latter are apparently autonomous, and may also function in an autonomous capacity.

ACKNOWLEDGEMENTS

I would like to record my sincere appreciation of the stimulating advice given by my supervisor, Prof. N Millott, who first introduced me to the study of the Echinoidea.

I am also indebted to members of the Zoology and Botany Departments at Bedford College, to Dr. H.G.Vevers of the Zoological Society of London, and to Staff and workers at the Marine Laboratories at Plymouth and Millport.

Prof. G.H.Foxon kindly advised me on injection techniques and Dr. Spanner and Miss H. Griffiths introduced me to electron microscopy.

This investigation was undertaken while I was in receipt of a Research Studentship awarded by the Department of Scientific and Industrial Research.

SUMMARY

SUMMARY OF CONTRIBUTIONS TO THE STUDY OF THE COELOMIC CELLS AND HAEMAL SYSTEM OF SOME ECHINOIDS

1. The coelomic cells occurring in the perivisceral coelom of the regular echinoids Diadema antillarum, Arbacia lixula, Paracentrotus lividus, Echinus esculentus and Psammechinus miliaris have been described.

2. Similarly the perivisceral coelomic cells of the irregular echinoids Spatangus purpureus, Brissopsis lyrifera and Echinocardium cordatum have been investigated.

3. The coelomic cells observed have been compared with previous descriptions and a simplification of previous nomenclature and classification has been adopted. The perivisceral coelomic cells are considered to be of three types; the phagocytic bladder amoebocytes, the spherule amoebocytes and the flagellated cells. Inclusions of the spherule amoebocytes are pigmented or colourless.

4. Preliminary observations on the fine structure of these cells, including their appearance under the low power electron microscope, amplify previous descriptions of these cells.

5. The flagellated cells move at a rate of about 60/micra/sec., with rotation. The posterior flagellum is

unusual and the locomotion is more nearly comparable with spermatozoan movement than with protozoan-flagellate movement.

6. Bladder amoebocytes do not show active locomotion. The spherule amoebocytes however are truly amoeboid. Some features of their movement under a cover-slip do not wholly conform with Allen's (1961) description of events accompanying movement of amoebae.

7. Information concerning the distribution, incidence and origin of the coelomic cells has been collated and discussed. It is concluded that the flagellated cells are probably not parasitic.

8. The phagocytic bladder amoebocytes become filiform as a pre-coagulation stage. Inhibition of coagulation may be variable and must be cautiously interpreted. The coagulation change of previously uncoagulated cells in vitro may be initiated by potassium ions or centrifugation.

9. Information concerning the pigments of spherule amoebocytes is briefly reviewed. The development of pigmentation of Psammechinus miliaris is similar to that in Lytechinus variegatus as described by Young (1957).

10. The composition of the inclusions of the spherule amoebocytes was further investigated. Histochemical tests combined with vapour methods of fixation and a new method of identifying cells after staining revealed:

a). Contrary to previous opinion the red spherules are basophilic and not associated with appreciable amounts of protein or lipid in the regular echinoids investigated. The spherules are however associated with carbohydrate, probably a sulphated mucopolysaccharide.
b). The often quoted statement that colourless spherules are albuminous is now substantiated by reactions to some protein tests, particularly Adams (1957) test for indoles. Chromatography showed that the reactant is probably tryptophan.

11. There is an apparent correlation between reduction of the indole content of the colourless spherules of Psammechinus miliaris and the onset of gonad maturation. An indirect relationship is suggested.

12. A re-examination of Stott's (1955) work led to the conclusion that absorption by migration of amoebocytes from the stomach of regular echinoids has not been demonstrated, and probably does not occur.

13. Information concerning the echinoid haemal system revealed discrepancies in reported observation and opinion. Studies on Psammechinus miliaris and Echinus esculentus showed the radial and aboral haemal areas to be absent or ill-defined. However haemal vessels in the stomach region were both contractile and effective in moving contained fluid, contrary to recent observations on Strongylocentrotus purpuratus (1962) by Farmanfarmaian and Phillips. Preliminary studies on absorption suggest

that the haemal vessels round the stomach, which may comprise a circulatory system, may be concerned in facilitating the passage of absorbed material from the stomach to the perivisceral coelomic fluid.

14. Transport, and the possible role of the coelomic cells and the haemal system in echinoids, is discussed.

APPENDIX I

Oikomonas echinorum Cuénot (1912)

'C'est un petit Flagellé, dont le corps arrondi, mésurant de 6 a 9 mu, renfermé un noyau place à peu près à l'oppose du point d'attache du flagellum, qui est très long et très grêle; tantôt (Monade de Parechinus*miliaris) le cytoplasme est assez clair pour laisser voir le noyau, tantôt il est rempli de petits granules renfringents qui cachent celui-ci. De temps à autre le Monade reste immobile, le corps présentent de petits mouvements de rotation sur lui-même, mais le plus souvent elle se déplace vivement dans le liquide coelomique, le flagellum en arrière, en bousculent sur son passage les cellules flottantes.

Oikomonas Saville Kent(1880-1)

'Animalcules exceedingly minute, plastic and unstable in form, ovate globular or elongate, sometimes freeswimming and sometimes attached by a temporarily developed thread-like prolongation of the posterior extremity of the body; flagellum single, anteriorly located, subservient when swimming to the purpose of locomotion and in the attached condition to bringing food particles within reach, these incepted at any portion of the periphery; contractile vesicle and endoplast usually conspicuous.

* now = Psammechinus miliaris

Oicomonas Hollande (1948)

Les Oicomonas, catalogués parmi les Protomastigines, sont des Chrysomonadines, aplastidiées (Kyste endogène siliceux, Martin) qui ont en commun la possession d'un unique fouét antérieur, généralement plus long que le corps. Ils mênent un vie libre mais sont susceptibles parfois de se fixer temporairement au substrat par un fin pedoncle protoplasmique. Certaines espèces sont coloniales. Leur corps est nu et porte parfois un stigma.

REFERENCES

REFERENCES

ADAMS, C.W.M., 1957 A p-dimethylaminobenzaldehyde nitrite method for the histochemical demonstration of tryptophan and related compounds. J.clin.Path., 10, 56-62.

ALLEN, R.D., 1960 in The Cell Vol.2, ed. Brachet,J., & Mirsky,A.E., New York:Academic Press.

ALLEN, R.D., 1961 A new theory of protoplasmic streaming. Exptl. Cell Research Suppl. 8, 17-31.

AWERINZEW, S., 1911 Uber die Pigment von S.drobachiensis. Arch.Zool.exp.gén., ser.5, 8, i-viii.

BAKER, J.R., 1958 Note on the use of bromphenol blue for the histochemical recognition of protein. Quart.J.micr.Sci., 99, 459-460.

BALDWIN, E., 1948 An introduction to comparative biochemistry. Cambridge: University Press.

BEHRE, E., 1932 A preliminary notice on the histology of the body fluid of Mellita quinquiesperforata Anat.Rec., 54 (suppl.) 92

BHANADWAJ, T.P., & LOVE, R., 1959 Staining mitochondria with haematoxylin after formol-sublimate fixation; a rapid method.
Stain Tech., 34, 331-334.

BISHOP,D.W., 1958 Motility of the sperm flagellum.
Nature, Lond., 182, 1638-40.

BLOOR, W.R., 1943 Biochemistry of the fatty acids and their compounds the lipids.
New York: Reinhold.

BOLIEK, M., 1935 Syncytial structures in sponge larvae and lymph plasmodia of sea urchins.
J.Elisha Mitchell Sci.Soc., 51, 252-288.

BONNET, A., 1925 Récherches sur l'appareil digestif et absorbant de quelques échinides réguliers.
Ann.Inst.océanogr.Monaco, Ser.2, 209-232.

BOOKHAUT, C.G., & GREENBURG, N.D., 1940 Cell types and clotting reactions in the echinoid, Mellita quinquiesperforata.
Biol.Bull.,Wood's Hole, 79, 309-320.

BOOLOOTIAN, R.A., 1961 The physical properties and chemical composition of perivisceral fluid, Echinodermata.
Biological Handbook: Blood and other body fluids.
U.S.A., Federation of American Societies for Experimental Biology.

BOOLOOTIAN, R.A., 1962 The perivisceral elements of echinoderm coelomic fluid.
American Zoologist, 2, 275-284.

BOOLOOTIAN, R.A., & GIESE, A.C., 1958 Coelomic corpuscles of echinoderms.
Biol.Bull., Wood's Hole, 115, 53-63.

BOOLOOTIAN, R.A., & GIESE, A.C., 1959 Clotting of echinoderm coelomic fluid.
J.exp.Zool., 140, 207-229.

BORRADAILE, L.A., & POTTS, F.A., 1958 The Invertebrates revised Kerkut, G.A.
Cambridge: University Press.

BRACHET, J., 1950 Chemical embryology.
New York: Interscience.

BULMER, D., 1959 Dimedone as an aldehyde blocking reagent to facilitate histochemical demonstration of glycogen.
Stain Tech., 34, 95-98.

CAIN, A.J., 1947 The use of Nile blue in the examination of lipoids.
Quart.J.micr.Sci., 88, 383-392.

CANNAN, R.K., 1927 Echinochrome.
Biochem.J., 21, 184-189.

CASSELMAN, W.G.BRUCE, 1959 Histochemical technique.
London: Methuen.

CATTANEO 1891 Gli amoebociti dei Cefalopodi e loro confronto con quelli d'altri Invertebrati.
Atti Univ.Genova.

CAULLERY, M., 1925 Sur la structure et la fonctionnement des gonades chez les echinides.
Trav.Sta.zool.Wimereux, 9, 21-35.

CERNOVODEANU, P., & HENRI, V., 1906 Phagocytose chez les oursins.
C.Rend.Soc.Biol.,Paris, 60, 882-884.

CHADWICK, H.C., 1900 Echinus
Liverpool Marine Biology Committee Memoirs III.

COOK, S.F., 1928 The action of potassium cyanide and potassium ferricyanide on certain respiratory pigments.
J.gen.Physiol., 11, 339-348.

COSTELLO, D.P., DAVIDSON, M.E., EGGERS, A., FOX, M.H., & HENLEY, C., 1957 Methods for obtaining and handling marine eggs and embryos.
Marine Biological Laboratory, Wood's Hole, Mass.

CUÉNOT, L., 1889 Études sur le sang, son rôle et sa formation dans la serie animale. Partie 2 Invertébrés, Note preliminaire.
Arch.Zool.exp.gén., 2me ser., 97, 1-9.

CUÉNOT, L., 1891a Études morphologiques sur les echinodermes.
Arch.Biol., Paris, 11, 313-680.

CUÉNOT, L., 1891b Études sur le sang et les glandes lymphatiques, Invertébrés.
Arch.Zool.exp.gen., 9, 365-475, 593-670.

CUÉNOT, L., 1897 Les globules sanguins et les organes lymphoides des Invertébrés.
Arch.Anat.micr., 1, 153-192.

CUÉNOT, L., 1900 Zoologie déscriptive des Invertébrés.
Paris: Doin.

CUÉNOT, L., 1912 Contributions a la faune du Bassin d'Archachon 5, Echinodermes.
Bull.Soc.sci.Archachon, 14, 2-116.

CUÉNOT, L., 1948 Anatomie, éthologie et systématique des Echinodermes.
in Traite de Zoologie, ed. Grassé, P.P., v.XI
Paris: Masson.

DALES, R.P., 1957 Preliminary observations on the role of the coelomic cells in food storage and transport in certain polychaetes.
J.Mar.biol.Ass., 36, 91-110.

DALES, R.P. 1961 The coelomic and peritoneal cells of some sabellid polychaetes. Quart.J.micr.Sci., 102, 327-346.

DAVIDSON, E., 1953 Clotting of the perivisceral fluid of the sand dollar, Echinarachnius parma. Biol.Bull.,Wood's Hole, 105, 372.

DEKHUYSEN, M.C., 1901 Ueber der Thrombocyten (Blutplattchen) Anat.Anz., 19, 529.

DONNELLON, J., 1938 An experimental study of the clot formation in the perivisceral fluid of Arbacia. Physiol.Zool., 11, 389-397.

DURHAM, H.E., 1888 The emigration of amoeboid corpuscles in the starfish. Proc.roy.Soc., Ser.B, 43, 327-330.

DURHAM, H.E., 1892 On wandering cells in echinoderms. Quart.J.micr.Sci., 33, 81-121.

ELMHIRST, R.E., 1923 Notes on the breeding and growth of marine animals in the Clyde sea area. Sco.Mar.Biol.Ass.Ann.Rep. for 1922, 19-43.

ENDEAN, R., 1958 The coelomocytes of Holothuria leucospilota. Quart.J.micr.Sci., 99, 47-60.

ENDEAN, R., 1960 The blood-cells of the ascidian, Phallusia mammillata.
Quart.J.micr.Sci., 101, 177-197.

ERDL 1842 Uber den Bau der Organe, welche an der ausseren Oberflache der Seeigel sichtbar sind.
Wiegmann's Archiv., 8, 1.

FARMANFARMAIAN, A., 1959 The respiratory surface of the purple sea urchin (Strongylocentrotus purpuratus)
Doctoral Dissertation, Stanford University, California.

FARMANFARMAIAN, A., 1960(?) The respiratory surface of the purple sea urchin (Strongylocentrotus purpuratus)
Anat.Rec., 134, no.3, 561.

FARMANFARMAIAN, A., & PHILLIPS, J.H., 1962 Digestion, storage and translocation of nutrients in the purple sea urchin (Strongylocentrotus purpuratus)
Biol.Bull.,Wood's Hole, 123, 105-120.

FAURÉ-FREMIET, E., 1927 Les amibocytes des invertébrés à l'état quiescent et a l'état actif.
Arch.Anat.micr., 23, 99-173.

FITZGERALD, J.F., 1959 Autoradiography in cytology. in Analytical cytology, ed., Mellors, R.C., U.S.A., McGraw-Hill Book Co., Inc.

FOX, D.L. 1953 Animal biochromes and structural colours: Physical, chemical, distributional and physiological features of coloured bodies in the animal world.
Cambridge: University Press.

FOX, H.M., & VEVERS, G., 1960 Animal colours.
London: Sidgwick and Jackson.

FRENZEL, J., 1892 Beitrage zur vergleichenden Physiologie und Histologie der Verdauung I.
Arch.Anat.Physiol.,Lpz., (Physiol.Abst.) 81-114.

FRETTER, V., 1952 Experiments with P and I on species of Helix, Arion and Agriolimax.
Quart.J.micr.Sci., 93, 133-146.

FUSARO, M., & GOLTZ, R.W., 1960 A comparative study of the periodic acid-Schiff and Alcian blue stains.
J.invest.Dermat., 35, 305-307.

GEDDES, P., 1880 Observations sur le fluid périvisceral des oursins.
Arch.Zool.exp.gén., 8, 483-496.

GEDDES, P., 1880 On the coalescence of amoeboid cells into plasmodia and on the so-called coagulation of invertebrate fluids.
Proc.roy.Soc., Ser.B., 30, 252-255.

GEORGE, W.C., 1941 Comparative haematology and the function of the leucocytes.
Quart.Rev.Bio., 16, 426-439.

GIESE,A.C., GREENFIELD, L., HUANG, H., FARMANFARMAIAN, A., BOOLOOTIAN, R., & LASKER,R., 1958 Organic productivity in the reproductive cycle of the purple sea urchin Strongylocentrotus purpuratus.
Biol.Bull., Wood's Hole, 116, 49-58.

GOMORI, G., 1952 Microscopic histochemistry: Principles and practice.
Chicago: University Press.

GOODRICH, E., 1919 Pseudopodia of the leucocytes of invertebrates.
Quart.J.micr.Sci., 64, 19-26.

GOODWIN, T.W., & SRISUKH, S., 1951 Carotenoids in Echinocaridium cordatum.
Nature, Lond., 167, 358.

GRAY, J., 1955 The movement of sea urchin spermatozoa.
J.exp.Biol., 32, 775-801.

GRAY, J., 1958 The movement of the spermatozoa of the bull.
J.exp.Biol., 35, 96-108.

GURR, E., 1958 Methods of analytical histology and histochemistry.
London: Leonard Hill.

HAMANN, O., 1887 Anatomie und Histologie der Echinidien und Spatangiden.
Jena Z.Naturw., 21, 87-266.

HANCOCK, D.A., 1957 The feeding behaviour of the sea urchin Psammechinus miliaris (Gmelin) in the laboratory.
Proc.Zool.Soc., 129, 255-262.

HANSON, J., 1949 The histology of the blood system in Oligochaeta and Polychaeta.
Biol.Rev., 24, 127-273.

HARRIS, H., 1961 Chemotaxis and phagocytosis.
in Functions of the blood, ed. Mcfarlane, R.G., & Robb-Smith, A.H.T.
Oxford: Blackwell.

HARVEY, E.B., 1953 Electrical method of determining the sex of sea urchins and other marine animals.
Biol.Bull., Wood's Hole, 105, 365.

HEATH, I.D., 1962 Observations on a highly specific method for the histochemical detection of sulphated mucopolysaccharides and its possible mechanisms.
Quart.J.micr.Sci., 103, 457-476.

HENRI, V., 1906 Etude du liquide perivisceral des oursins.
C.Rend.Soc.Biol.,Paris, 58, 880-882.

HEVESY, G., 1948 Radioactive indicators.
New York: Interscience Publishers Inc.

HOFFMANN, 1850 Zur Anatomie der Echinden und Spatangen
Nieder Archiv., I.

HOLLANDE, A., 1948 in Phylogenie protozoaires: généralites, flagellés. Tome 1:1, Traité de Zoologie, ed. Grassé, P.P.
Paris: Masson.

HOLMES, W., 1961 Evolution of the leucocytes.
in Functions of the blood, ed., Mcfarlane, R.G., & Robb-Smith, A.H.T.
Oxford: Blackwell.

HYMAN, L.H., 1955 The Invertebrates 4, Echinodermata.
U.S.A., McGraw-Hill.

HYMAN, L.H., 1959 The Invertebrates 5, Smaller coelomate groups, Retrospect.
U.S.A., McGraw-Hill.

JACOBSON, F.W., & MILLOTT, N., 1953 Phenolases and melanogenesis in the coelomic fluid of the echinoid Diadema antillarum (Philippi)
Proc.roy.Soc., Ser.B, 141, 231-247.

JAMMES, L., 1904 Zoologie pratique.
Paris: Masson.

KAWAGUTI, S., & YAMASU, T., 1954 Pigment cells in the perivisceral fluid of the Echinoidea.
Biol.J.Okayama Univ., 1, 249-264.

KAWAMOTO, N., 1927 Anatomy of Caudina chilensis.
Sci.Rep.Tohoku Univ., Ser.4, Biol., 2.

KINDRED, J., 1921 Phagocytosis and clotting in the perivisceral fluid of echinoderms.
Biol.Bull.,Wood's Hole, 41, 144-152.

KINDRED, J., 1924 The cellular elements in the perivisceral fluid of echinoderms.
Biol.Bull.,Wood's Hole, 46, 228-251.

KINDRED, J., 1926 A study of the genetic relationship of the amoebocytes with spherules in Arbacia.
Biol.Bull., Wood's Hole, 50, 147-154.

KITCHING, J.A., & PADFIELD, J.E., 1960 The physiology of contractile vacuoles, 9. Effects of heavy water on the water balance of a suctorian.
J.exp.Biol., 37, 73-82.

KOEHLER, M., 1883 Récherches sur les Échinides des côtes de Provence.
Marseilles.

KOLLMANN, M., 1908 Recherches sur les leucocytes et le tissue lymphoide des invertebres.
Ann.Sci.nat.Zool., 8, 1-240.

KOWALEVSKY 1889 Ein beitrag zur Kenntnis der Excretionsorgane.
Biol.Central 9.

KUHL, W., 1937 Die Zellelemente in der Liebeshohlenflussigkeit der Seeigel Psammechinus miliaris und iht Bewegung physiologisches Verhalten.
Z.Zellforsch. 27, 1-13.

LASKER, R., & GIESE, A.C., 1954 Nutrition of the sea urchin Strongylocentrotus purpuratus.
Biol.Bull., Wood's Hole, 106, 328-340.

LIEBMAN, E., 1946 On trephocytes and trephocytosis.
Growth, 10, 291-329.

LIEBMAN, E., 1950 The leucocytes of Arbacia punctulata.
Biol.Bull., Wood's Hole, 98, 46-59.

LISON, L., 1930 Recherches histophysiologiques sur les amibocytes des Echinodermes.
Arch.Biol., Liege, 40, 175-203.

LISON, L., 1953, 1960 Histochimie et cytochimie animales; principes et methodes.
Paris: Gauthier-Villars.

LOWNDES, A.G., 1941 On flagellar movement in unicellular organisms.
Proc.Zool.Soc., London, 111, 111-134.

LUFT, J.H., 1956 Permanganate - A new fixative for electron microscopy.
J.Biophys.Biochem.Cytol., 2, 799.

MACBRIDE, E.W., 1914 Textbook of embryology I Invertebrates.
London: Macmillan.

MACMUNN, C.A., 1885 On the chromatology of the blood of some invertebrates.
Quart.J.micr.Sci., 25, 469-490.

MACKINNON, D.L., & HAWES, R.S.J., 1961 An introduction to the study of the protozoa.
Oxford: Clarendon Press.

MANSOUR, K., 1946 Food and the digestive processes of the lamellibranchs.
Nature, Lond., 157, 482.

MANSOUR, K., 1946b Food and digestive organs of lamellibranchs.
Nature, Lond., 158, 378.

MANSOUR-BEK, J.J., 1946 Extracellular proteolytic and lipolytic enzymes of some lamellibranchs.
Nature, Lond., 148, 378.

MANTON, I., & PARKE, M., 1960 Further observations on small green flagellates.
J.Mar.biol.Ass., 39, 275-298.

MAXFIELD, F.A., & MORTENSEN, O.A., 1941 The use of the radioactive properties of thorium for a quantitative study of phagocytosis.
J.appl.Phys., 12, 197-202.

MAZIA, D., BREWER, P.A., & ALFERT, M., 1953 Detection of proteins using mercuric chloride bromphenol blue.
Biol.Bull.Wood's Hole, 104, 57-67.

McCLENDON, J.F., 1912 Echinochrome, a red substance in sea urchins.
J.biol.Chem., 11, 435-441.

McMANUS, J.F.A., & MOWRY, R.W., 1958 Effects of fixation on carbohydrate histochemistry.
J.Histochem.Cytochem., 6, 309-316.

MICHEL,A., 1888 Sur la prétendu fusion des cellules lymphatiques en plasmodes.
C.Rend.Soc.Biol., Paris, 106, ex Kindred (1921).

MILLOTT,N., 1957 Naphthoquinone pigments in the tropical sea urchin, Diadema antillarum Philippi.
Proc.Zool.Soc., London, 129, 263-272.

MILLOTT, N., & YOSHIDA, M., 1957 The spectral sensitivity of the echinoid Diadema antillarum Philippi.
J.exp.Biol., 34, 394-401.

MOORE, H.B., 1934 A comparison of the biology of Echinus esculentus in different habitats, I. J.Mar.biol.Ass., 19, 869-881.

MORTON, J.E., 1960 The functions of the gut in ciliary feeders. Biol.Rev., 35, 92-140.

MOWRY, R.W., 1958 Improved procedure for staining of acid mucopolysaccharides by Muller's colloidal ferric oxide. Lab.Invest., 7, 566-576.

NICOL, J.A.C., 1960 The biology of marine animals. London: Pitman.

OHUYE, T., 1936 On the coelomic corpuscles in the body fluid of some invertebrates 5, Reaction of the coelomic corpuscles of an echinid, Temnopleurus Hardwickii to vital dyes and some chemical reagents. Sci.Rep.Tohoku Univ., Biol., 11, 223-238.

OOMEN, H.A.C., 1926 Verdauungsphysiologische Studieren Holothurien. Pubbl.Sta.zool.Napoli. 7, 215

ORTON, J.H., 1923 Breeding period of Psammechinus miliaris. Nature, Lond., 111, 878.

OWEN, G., 1958 Observations on the stomach and digestive gland of Scutus breviculus.
Proc.Mal.Soc., London, 33, 103-114.

PALADE, G.E., 1952 A study of fixation for electron microscopy.
J.exp.Med., 95, 285-298.

PARKER, T.J., & HASWELL, W.A., 1943 A textbook of zoology, I.
London: Macmillan.

PEARSE, A.G.E., 1960 Histochemistry; Theoretical and applied.
London: Churchill.

PERRIER, E., 1875 Appareil circulatoire des oursins.
Arch.Zool.exp.gén., ser.2, 4, 605-643.

PITTAM, M.D., 1963 Studies of an amoebo-flagellate, Naegleria gruberi.
Quart.J.micr.Sci., 104, 513-529.

PROSSER, C.L., & JUDSON, C.L., 1952 Pharmacology of haemal vessels of Stichopus californicus.
Biol.Bull., Wood's Hole, 102, 249-251.

PROUHO,H., 1887 Récherches sur le Dorocidaris papillata et quelques autres échinides de la Mediterranee.
Arch.Zool.exp.gén., ser.2, 5, 214-380.

RAMSAY, J.A., 1952 A physiological approach to the lower animals.
Cambridge: University Press.

RAVEN, C.P., 1961 Oogenesis.
U.S.A., Pergamon Press.

St.HILAIRE, C., 1897 Title not known.
Trav.Soc.Nat.,St.Petersb., 27, 221-248. Not available in England.

SAVILLE KENT, W., 1880 A manual of the infusoria.
London: Bogue.

SCHAFER, E.A., 1883 Preliminary notice of an investigation into the coagulation of the perivisceral fluid of the sea urchin.
Proc.roy.Soc., Ser.B, 34, 370-371.

SCHINKE, H., 1950 Bildung und Ersatz der Zellelemente der Liebeshöhlenflussigkeit von Psammechinus miliaris.
Z.Zellforsch, 35, 311-331.

SEAMAN, C.R., 1955 Metabolism of free living ciliates.
in Biochemistry & physiology of Protozoa II, ed.
Hutner, S.H., & Lwoff, A.,
New York: Academic Press.

SEDGEWICK, A., 1898, 1909 A students textbook of zoology 3.
London: Allen & Unwin.

'SERVALL' 1959 Thin sectioning and associated technics for electron microscopy.
Norvalk, Conn., Servall Inc.

SHAPIRO, H., 1946 The extracellular release of echinochrome.
J.Gen.Physiol., 29, 267-275.

SHIPLEY, A.E., & MACBRIDE, E.W., 1904 Zoology.
Cambridge: University Press.

SLEIGH, M.A., 1962 The biology of cilia and flagella.
Oxford: Pergamon Press.

SMITH, I., 1958 Chromatographic techniques.
London: Heinemann.

STOTT, F.C., 1952 Thesis for the degree of Doctor of Philosophy.
London: Senate House.

STOTT, F.C., 1955 The food canal of the echinoid, Echinus esculentus.
Proc.Zool.Soc., London, 125, 63-86.

SZENT-GYORGI, A., 1957 Bioenergetics.
New York: Academic Press.

SZENT-GYORGI, A., 1960 Introduction to a sub-molecular biology.
New York: Academic Press.

TAKATSUKI, S., 1934 On the nature and functions of the amoebocytes of Ostrea edulis.
Quart.J.micr.Sci., 76, 379-431.

TARTAR, V., 1950 in Studies honoring Trevor Kincaird, ed., Hatch, M.H.
Seattle: Univ., Washington Press.

TEUSCHER 1876 Beitrage zur Anatomie der Echinodermen, Echiniden.
Jena Ztschr.Naturw., 10.

THÉEL, H., 1896 Remarks on the activity of amoeboid cells in the echinoderms.
Festsch. Lilljeborg, Uppsala, 47-58.

THOMSON, R.H., 1957 Naturally occurring quinones.
London: Butterworth's Scientific Publications.

THOMSON, R.H., 1962 Quinones- Structure and distribution, in Comparative Biochemistry, Vol.3, Consituents of life, A. ed., Florkin, M., & Mason, H.S.
New York: Academic Press.

TIEDEMANN, F., 1816 Anatomie der Rohren-Holothurie des pomeranzenf. Seesternes und Steinseeigels.
Landshut, Joseph Thomannschen Buchdruckerei.

VALENTIN 1842 Anatomie du genre Echinus
Neuchatel: Livraison des Monographies des Echinodermes.

VAN DER HEYDE, H., 1922 On the physiology of digestion, respiration and excretion in echinoderms.
ref. ex Hyman, L.H. 1955.

WILLMER, E.N., 1960 Cytology and evolution.
New York: Academic Press.

WYMAN, L.C., & LUTZ, B., 1930 The action of adrenalin and certain drugs on the isolated holothurian cloaca.
J.exp.Zool., 57, 441-453.

YEAGER, J.F., & TAUBER, O.E., 1935 On the haemolymph cell counts of some marine invertebrates.
Biol.Bull., Wood's Hole, 69, 66-70.

YONGE, C.M., 1926 Structure and physiology of the organs of feeding and digestion in Ostrea edulis.
J.Mar.biol.Ass., 14, 295-386.

YONGE, C.M., 1937 The digestive system of the metazoa.
Biol.Rev., 12, 86-115.

YOSHIDA, M., 1959 Naphthaquinone pigments in Psammechinus miliaris (Gmelin)
J.Mar.biol.Ass., 38, 455-460.

YOUNG, R.S., 1958 Development of pigment in the larva of the sea urchin Lytechinus variegatus.
Biol.Bull.,Wood's Hole, 114, 394-403.