THE MORPHOLOGY AND HISTOCHEMISTRY OF THE ECHINOID AXIAL ORGAN

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CONTENTS

The structure of the main portion of the axial organ of *Arbacia lixula*, *Diadema antillarum* and *Paracentrotus lividus* is described. This portion, which lies at the confluence of the perivisceral coelom, water vascular and 'haemal' systems, is pre-eminently glandular. It has an irregular central lumen containing the contractile vessel and a spongy peripheral region permeated by three systems of cavities: the embayments of the central lumen, the lacunae and the canaliculi. The lacunae communicate with the contractile vessel and the canaliculi with the perivisceral coelom. These two systems are closely associated, being separated at the most by an attenuated epithelium which in many areas breaks down, allowing the contents of the lacunae to spill or proliferate into the canaliculi.

The predominant histological features of the organ are associated with the production of secretion by the transformation of cells including amoebocytes within the lacunae, and in *Diadema* and sometimes in *Arbacia*, with the accumulation of large quantities of pigment.

The organ is permeated by connective tissue, unevenly distributed muscle fibres and by varying numbers of amoebocytes. Secretion may be discharged into the lacunae, the central lumen, the canaliculi or directly from the free surface of the organ into the perivisceral coelom.

Histochemical tests showed the presence of abundant *PAS*-positive material in the secretion, most of which is not glycogen, but the amount of glycogen is increased by carbohydrate feeding, after which it appears in the cells lining the canaliculi and some is secreted into the lacunae and canaliculi. Most of the *PAS*-positive substance proved to be acid mucopolysaccharide. The secretion also showed the reactions of lipid-bound and protein-bound reducing groups, together with other reactions of protein or amino acids and indole derivatives, at least one of which is a 3-indolyl compound, probably tryptophan. Both tyrosine and tryptophan are conspicuous in some amoebocytes.

The possible function of some of these compounds is discussed.

Tests for enzymes such as tyrosinase and alkaline phosphatase gave no clear indication of their presence.

There was no indication of any significant amount of neutral fat or phospholipid.

Variable and sometimes impressive amounts of brown and orange to red pigments occur in the

axial organ. In *Diadema* and *Arbacia* the latter appears to be echinochrome A. The reddish pigment is mostly in the lacunae where it arises from the disintegration of amoebocytes, though in *Arbacia* it may possibly arise also within other lacunar cells. A possible function of this pigment is discussed.

Brown pigment is widely distributed in the axial organ and is frequently associated with amoebocytes. In all three species this pigment proved to be melanin and an iron-containing pigment of nuclear origin. In *Diadema*, lipofuscin arising from phospholipid is also present.

Melanin is liberated into the lacunae by the degeneration of amoebocytes, some of which contain tyrosine but when in the axial organ, appear to possess no active tyrosinase. Part, at least, of the iron-containing pigment can also be traced to amoebocytes.

Introduction

The axial organ is perhaps the most enigmatic structure in echinoderms, and has been the cause of much controversy. Its structure is imperfectly understood and indeed certain of its features have been incorrectly described. Though several functions have been proposed for it none has been clearly shown. It has been considered excretory, genital, vestigial, as producing amoebocytes or pigment, as destroying effete amoebocytes and either wholly or in part as a heart. These ideas have often been based solely on considerations of its structure. Such procedure is dangerous but particularly so when structure is imperfectly understood. There have been few attempts at experimental analysis and some of these are open to objection. There is therefore much disparate information and it is clear from both the description and figures in early accounts, e.g. Hamann (1887), that the structure of the organ was not understood. To make matters worse, textbook accounts have done less than justice to some of the early work, notably that of Prouho (1887) so that a number of important findings have been forgotten. It is therefore evident that the structure and function of the axial organ has proved, and is still proving, extremely difficult to interpret. The main portion of the organ is clearly glandular. Here the situation has been clarified by recent findings (Millott 1966). Recent investigations have also emphasized three cardinal features of the organ. First, it is pervaded by an elaborate contractile structure (Boolootian & Campbell 1964). Secondly, it is a central meeting point for perivisceral coelom, the so-called haemal system and the water vascular system (Millott & Vevers 1964). Thirdly, the organ is not essential, at least on a short-term basis (Schinke 1950; Millott & Vevers 1964). These features may be related to the recent discovery that the organ is involved in an intimate mechanism of defence against injury and invading organisms (Millott 1966).

It is unlikely that this will prove to be the only function of the organ, for not only may considerable quantities of fluid be pumped through it as in the case of *Strongylocentrotus purpuratus* (Boolootian, private communication) but large quantities of pigment sometimes accumulate within its tissues. Despite the prominence of the contractile vessel and the fact that it may move large quantities of fluid, its function is still a matter of debate.

In the context of much uncertainty some knowledge of the histochemistry of the organ might prove revealing. We therefore decided to base our studies on the axial organs of three echinoid species, *Arbacia lixula* (Linnaeus), *Diadema antillarum* Philippi and *Paracentrotus lividus* (Lamarck). Where appropriate, reference will be made to the recent findings of Millott (1966) on *Arbacia punctulata* (Lamarck) and *Strongylocentrotus droebachiensis* (O. F. Muller).

Morphology

The structure of the organ in various echinoids has been described by Hofmann (1871), Koehler (1883), Perrier (1875), Hamann (1887), Prouho (1887), Leipoldt (1893) and Fedotov (1924). All these accounts are inadequate and it is evident that the essential structural features of the organ have been incompletely understood.

The main part of the organ is more or less spindle-shaped and in full-grown specimens measures approximately 3·0 mm in length in *Arbacia*, 4·5 mm in *Diadema* and 2·0 mm in *Paracentrotus*. The aboral extremity lies just below the madreporite. This constitutes the so-called head process, which with its extension into the main part of the organ forms the contractile vessel, the detailed structure of which has been described in *Strongylocentrotus purpuratus* by Boolootian & Campbell (1964). We shall not concern ourselves with this portion of the organ, but only with the main part, which is pre-eminently glandular. It occupies a position that is more or less axial in the animal and is accompanied by the stone canal which lies along one aspect. In section (figure 1, plate 9) the organ is seen to be covered by peritoneum (*p*) and to consist essentially of a variety of cells and irregular spaces supported by connective tissue. The most conspicuous of these spaces (*a*) occupies a central position, being bounded on one side by a thin wall carrying the stone canal (*sc*) and on the other by the main bulk of the organ. This space is regarded by Boolootian & Campbell (1964, 1966) as representing the axial sinus but is designated here in non-committal fashion as the central cavity. It is pervaded by the large, elaborate and irregular vessel (*v*), corresponding in this region at least, in both form and position with the pulsating vessel of Boolootian & Campbell. Although we have never observed its contractions in the three species under study, we shall, because of this correspondence, distinguish it as the contractile vessel. The nature and extent of this vessel had not been realized prior to the work of Boolootian & Campbell. Koehler (1883) described a large canal extending the length of the organ and receiving a considerable number of tributaries from the periphery. This system appears to correspond with a portion of the contractile vessel but Koehler considered that it was the duct of what he regarded as the axial gland. As will be shown later, the finer branches of the supposed duct appear to correspond with shafts of cells containing irregular spaces communicating with the contractile vessel.

The nature of the cavities in the spongy periphery of the organ has only recently been elucidated. Koehler (1883) differentiated two kinds of space in the axial organ of *Echinus*, *Sphaerechinus*, *Paracentrotus* and *Psammechinus*. Recently, however, Millott (1966) has shown the existence of three types of space permeating this region in the axial organs of *Strongylocentrotus droebachiensis* and *Arbacia punctulata*. These spaces have their counterparts in the organs of the three species described here. First, there are finger-like embayments (figure 1 *e*, plate 9) of the central cavity which penetrate the periphery. Secondly, there are irregular spaces of varying size which communicate with the surrounding perivisceral coelom by fine canals (figure 2, plate 9). These spaces, which we distinguish as the canaliculi (*c*), may be distended locally to form cavities or follicles. They are lined by flattened cells, and with the notable exceptions to be described they often appear empty, though they may contain secretion. Thirdly, around the canalicular spaces are irregular cavities which

constitute the main bulk of the periphery and by contrast are conspicuous by their content of cells, cell debris, pigment and what has often been assumed to be excretory matter. We shall distinguish them as lacunae (figure 1 *l*, plate 9). The last two types of space have hitherto been referred to collectively as haemal spaces or blood lacunae. Because of their different character and morphological relationships and since the fluid contained by the canaliculi at least, is not haemal but coelomic, we find it essential to distinguish them. In any event it is preferable to avoid the use of such terms as 'haemal' in the present state of our knowledge (Millott & Vevers 1964).

The ramifications of the canalicular system are difficult to follow but there is evidence that these spaces may communicate with the central cavity. Thus, both types of cavity are lined by similar cells and in certain circumstances both may house the characteristic cysts, already described (Millott 1966). Further, in *Arbacia* some of the canals pass directly from the surface into the central cavity of the organ.

The lacunar system is ultimately continuous with the lumen of the contractile vessel, the communication between the two taking place by irregular spaces within the shafts of tissue (trabeculae) that cross the central lumen between the contractile vessel and the periphery (figures 1 and 3, plate 9).

The distinction between the spaces of the lacunar and canalicular systems is not absolute, for the wall bounding the canaliculae may break down, allowing the contents of the lacunae to enter the canalicular system. Even where this does not occur it will be evident that the two systems of spaces come into intimate relation for they are separated, at the most, by the attenuated epithelium mentioned above.

The precise way in which the branches of the contractile vessel communicate with the lacunar system varies. In *Strongylocentrotus* and *Paracentrotus* they open directly into the lacunar system, but in *Arbacia* and *Diadema* the communication between the two types of

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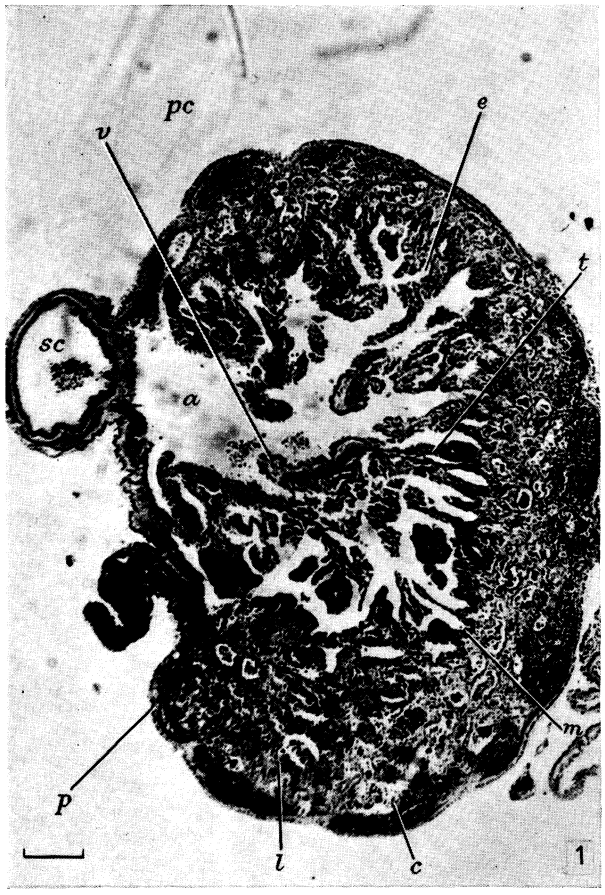
Description of plate 9

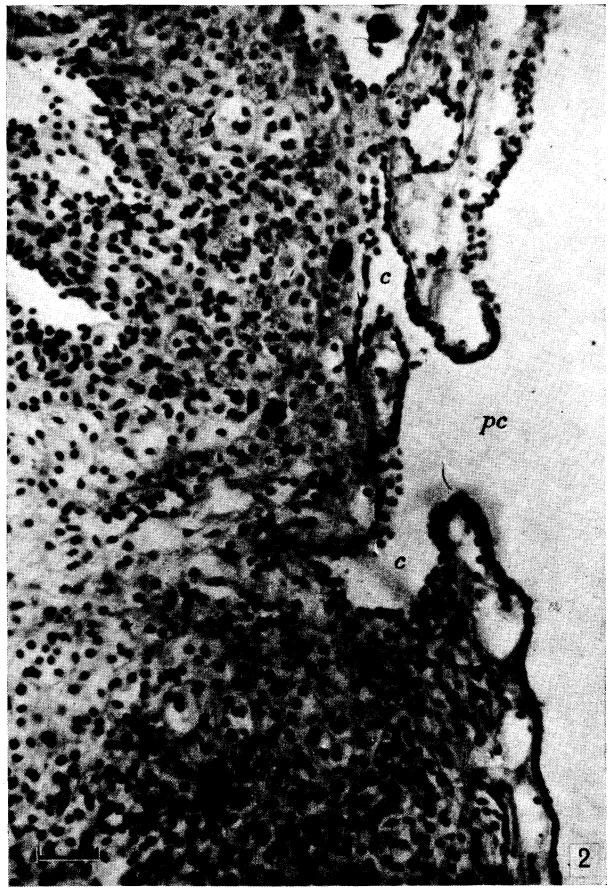
Figure 1. *Diadema antillarum.* Transverse section of the axial organ showing the main structural features. Fixed Duboscq-Brasil. Stained Heidenhain's iron haematoxylin with Ponceau-fuchsin and light green. Scale 85 μm; *a*, central lumen; *c*, follicle of canalicular system; *e*, embayment of central lumen; *l*, part of lacunar system; *m*, channel extending around lumen; *p*, peritoneum; *pc*, perivisceral coelom; *sc*, stone canal; *t*, trabecula; *v*, portion of contractile vessel.

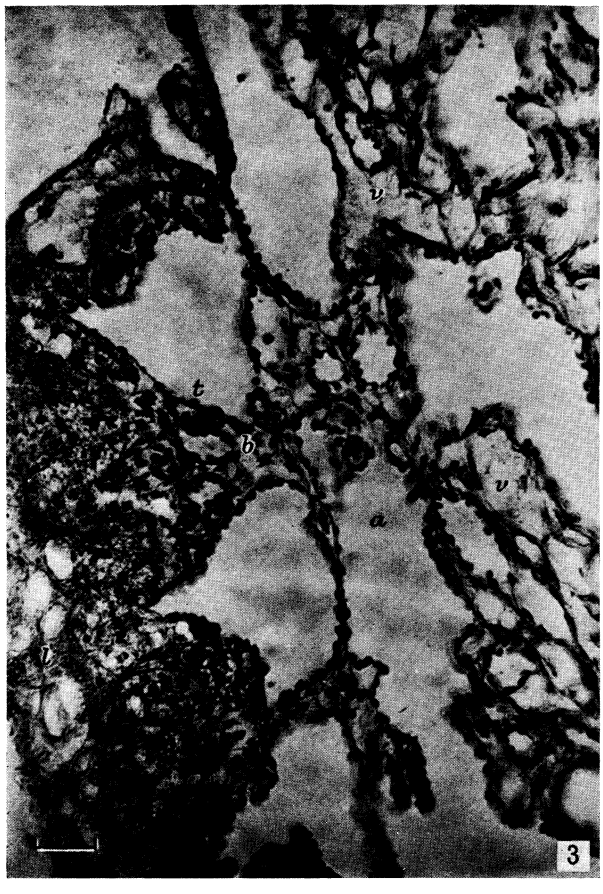
Figure 2. *Paracentrotus lividus.* Periphery of the axial organ showing portions of the canalicular system (*c*) opening into the perivisceral coelom (*pc*). Fixed Rossmann. Stained Ehrlich's haematoxylin and eosin. Scale 15 μm.

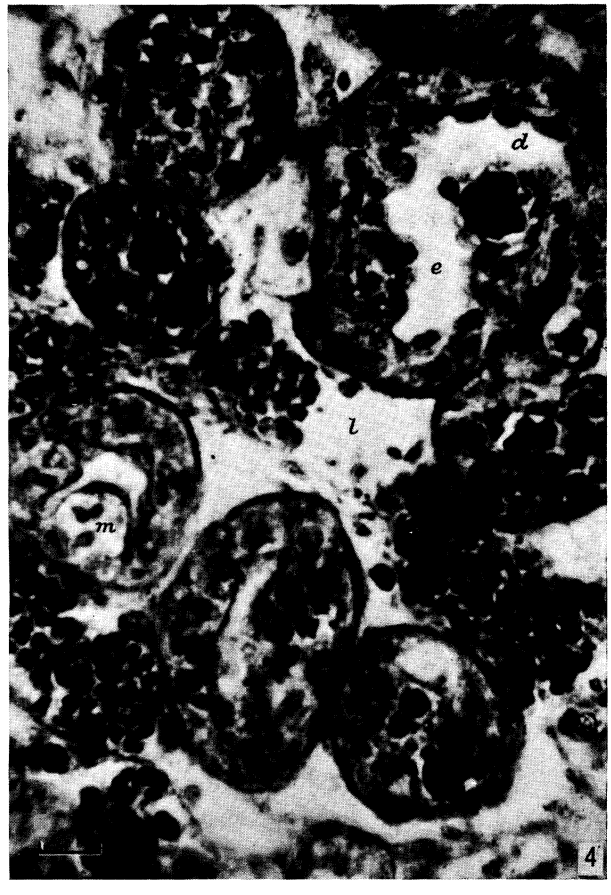
Figure 3. *Arbacia punctulata.* Portion of the axial organ showing the communication between the contractile vessel (*v*) and the lacunar system (*l*) at the periphery by means of channels (*b*) within the trabeculae (*t*). Fixed Bouin. Stained Mallory's triple stain. Scale 20 μm. *a*, portion of the central cavity; *v*, ramifications of the contractile vessel.

Figure 4. *Diadema antillarum.* Transverse section across several embayments of the central lumen of the axial organ. Note their superficial resemblance to glandular acini. Fixed Flemming's strong fluid. Stained Heidenhain's iron haematoxylin. Scale 8 μm. *d*, attenuated lining epithelium of embayment. *e*, embayment of central lumen. *l*, part of lacunar system. *m*, channel continuous with contractile vessel.









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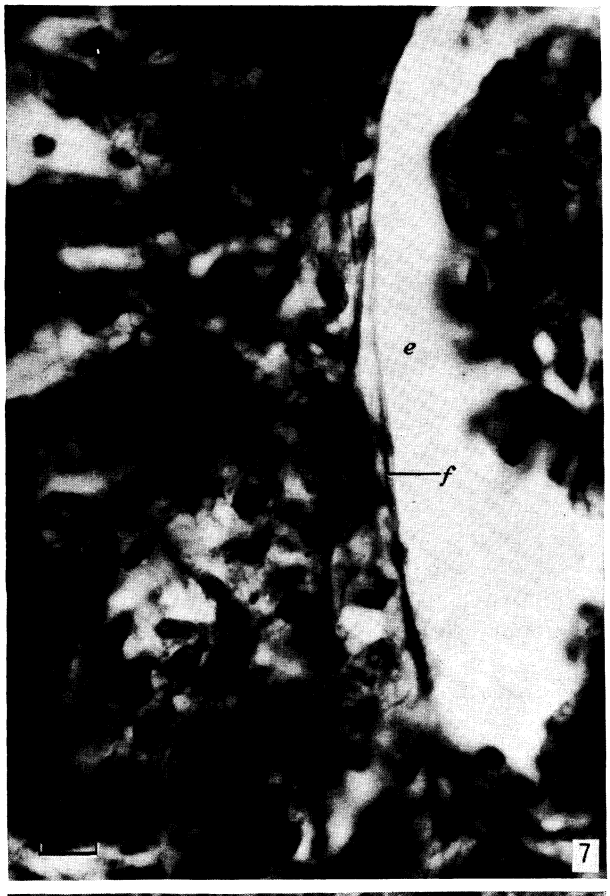
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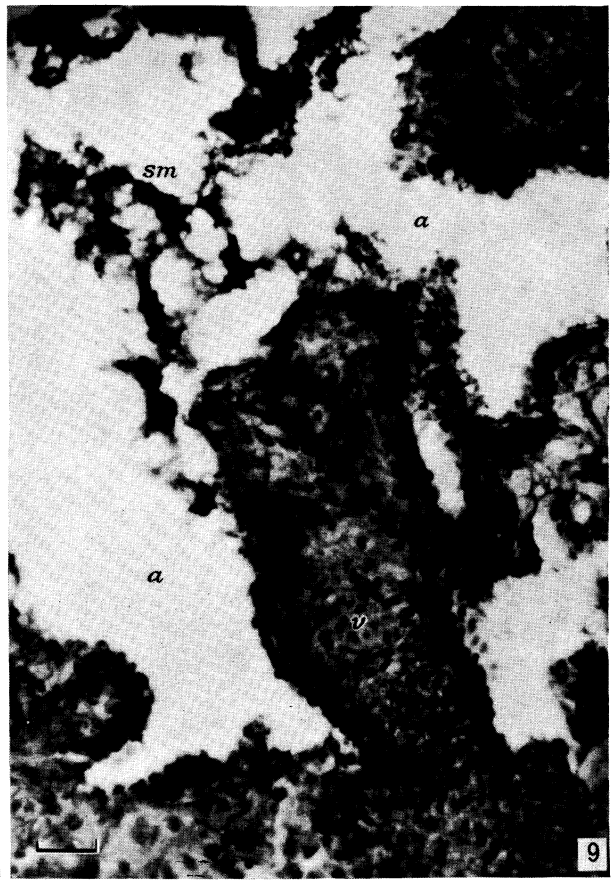
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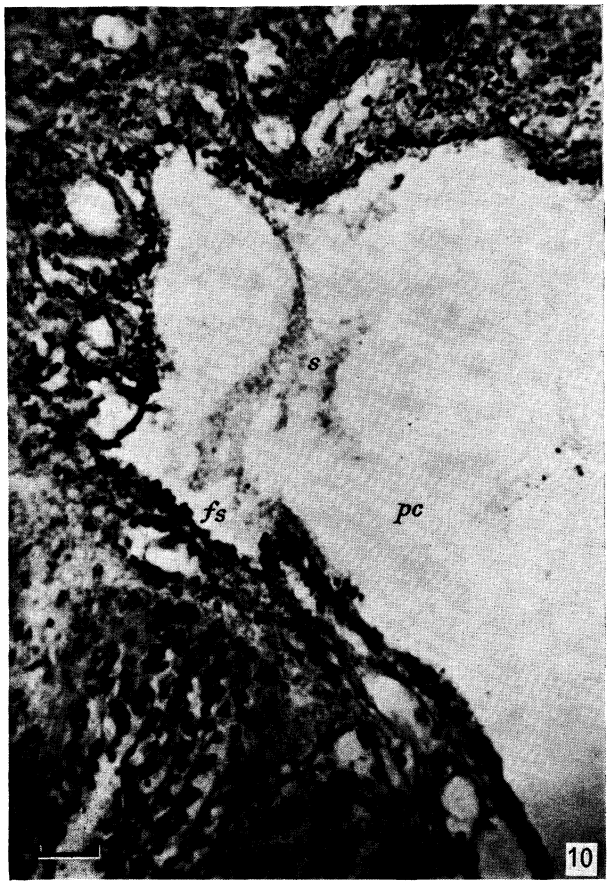
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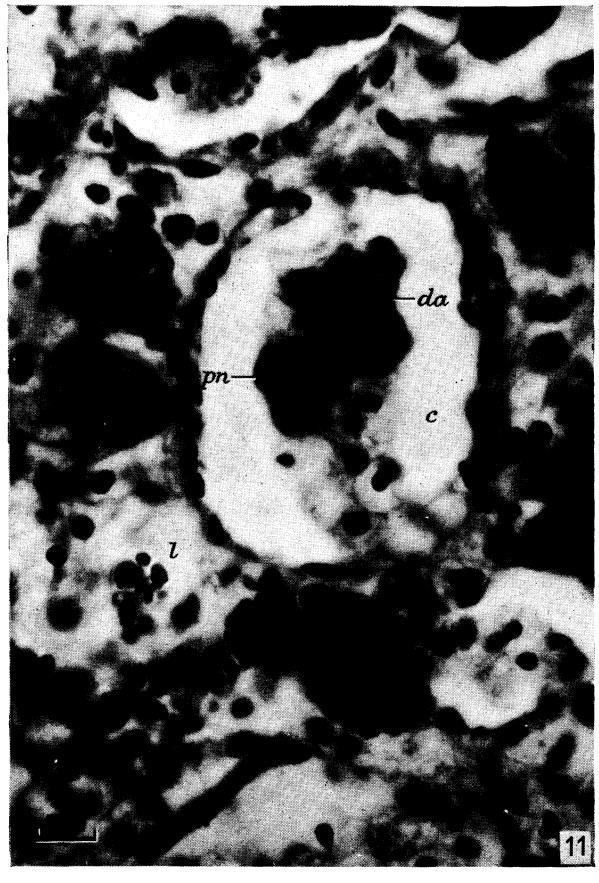
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space is indirect. In these two echinoids the channels from the contractile vessel enter a more or less continuous space (*m*) (figure 1, plate 9) that extends around the embayments of the central cavity of the axial organ, except in the area adjacent to the stone canal. Despite the apparently well-defined character of this space its walls break down in places, so that it becomes continuous with the adjacent spaces of the lacunar system.

The central cavity of the axial organ and its embayments are lined by an extremely attenuated epithelium immediately below which lies the irregular continuous space just mentioned. In transverse section the embayments and the surrounding cells may bear a superficial resemblance to glandular acini (figure 4, plate 9) especially as they may contain secretion. It must be emphasized, however, that the apparent acini are in reality lined by the extremely attenuated epithelium already mentioned, below which lies the continuous space, which is in continuity with the contractile vessel. This space corresponds with the *couches d'alvéoles* of Prouho (1887). The origin of the secretion is discussed below. In *Arbacia lixula* the peripheral extremities of the embayments are often conspicuous owing to their pigment which forms characteristic terminal caps.

Histology

Axial organs from the three species were fixed in a variety of fluids, including Bouin, Carnoy, Champy, Duboscq-Brasil, Flemming (strong formula), Helly–Zenker, Rossmann and Susa. Paraffin sections cut at 8 μm were stained in Ehrlich's haematoxylin, Mallory's triple stain, Mayer's haemalum, van Gieson or in Heidenhain's iron haematoxylin either alone or in combination with Ponceau-fuchsin and light green.

We must emphasize at the outset that the histology has proved exceedingly difficult to interpret, and although certain features have become clear a number still lack adequate explanation. These may perhaps be interpreted when more is known concerning the function of the organ.

The predominant feature of the glandular region is the production of secretion by what appears to be an extensive process of cell transformation and disintegration. The cells involved form the main mass of the periphery of the organ and occupy much of the lacunar system of spaces. Certain features of the process will be evident from figures 5 and 6.

Description of plate 10

Figure 7. *Diadema antillarum.* Presumed muscle fibres (*f*), (see p. 207) situated superficially in the wall of an embayment (*e*) of the central lumen. Fixed Bouin. Stained Mallory. Scale 8 μm.

Figure 9. *Paracentrotus lividus.* Secretion of acid mucopolysaccharide (*sm*) into the central lumen (*a*) of the axial organ (see p. 212). Fixed Rossmann. Stained Mowry's colloidal iron. Scale 20 μm. *v*, part of contractile vessel.

Figure 10. *Arbacia lixula.* Discharge of *PAS*-positive secretion (*s*) from the free surface (*fs*) of the axial organ into the surrounding perivisceral coelom (*pc*) (see p. 209). Fixed Rossmann. Stained periodic-acid-Schiff and Ehrlich's haematoxylin. Scale 20 μm.

Figure 11. *Diadema antillarum.* Invasion of a canaliculus (*c*) by a peninsula of cells (*pn*) from the surrounding lacuna (*l*) (see p. 208). Fixed Duboscq–Brasil. Stained Heidenhain's iron haematoxylin with Ponceau-fuchsin and light green. Scale 8 μm. *da*, degenerating amoebocytes.

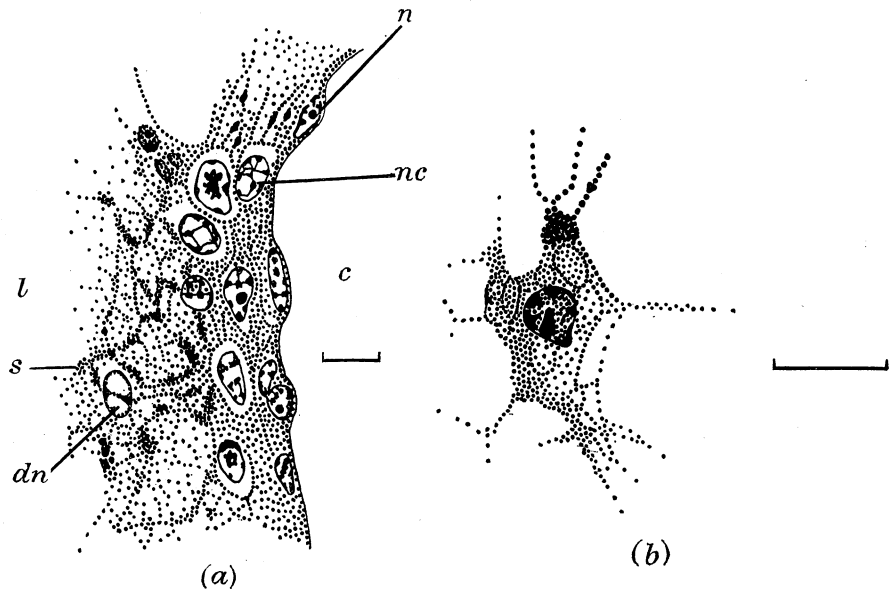

FIGURE 5. *Diadema antillarum.* Production of *PAS*-positive secretion. (*a*) Portion of the wall of a canalicular space (*c*) and an underlying portion of the lacunar system (*l*) in which cells appear to be disintegrating to produce deeply staining secretion. Note the numerous nuclei (*nc*) and the absence of cell boundaries, the nucleus in several cases being surrounded by a clear space. A gradual transformation of cytoplasm into strings of secretion (*s*) seems to be occurring from right to left. Some nuclei, such as that marked *dn*, appear to be degenerating. Fixed Rossman. Stained periodic-acid-Schiff and Delafield's haematoxylin. Scale 6 μm. *n*, nucleus of attenuated cell forming part of the lining of a canalicular space. (*b*) A cell from the lacunar system, to show the apparent transformation of cytoplasm into secretion. Fixed and stained as above. Scale 6 μm.

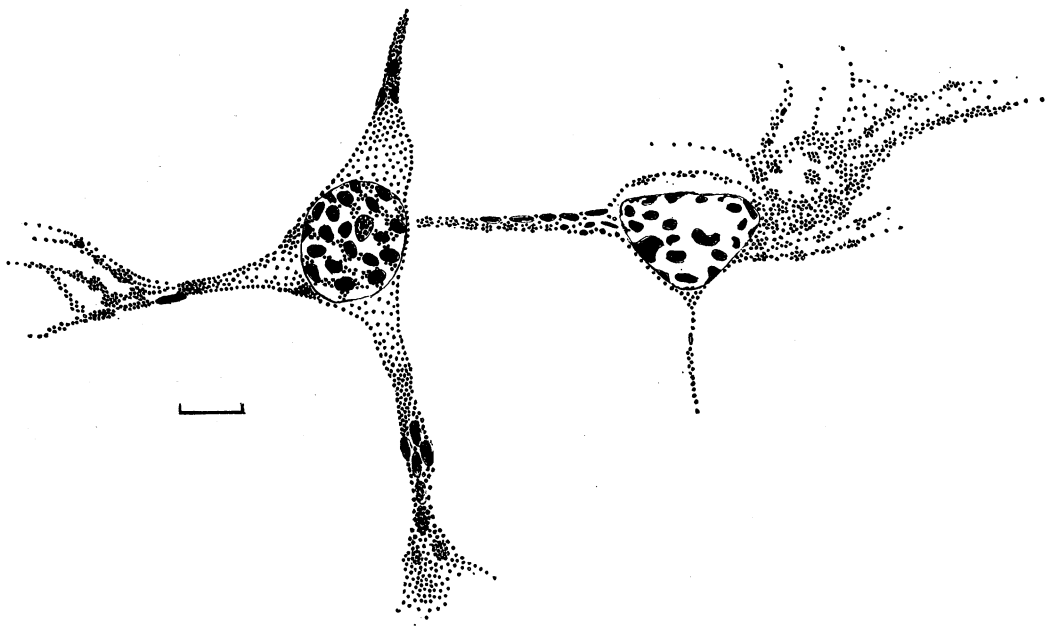

FIGURE 6. *Arbacia lixula.* Two cells from the lacunar system showing transformation of their cytoplasm into threads and nodules of acid mucopolysaccharide. Fixed ice-cold Rossmann. Stained Mowry's colloidal iron method. Scale 1·5 μm.

Within the lacunae large numbers of nuclei are evident. In some cases the nuclei appear normal and surrounded by a mass of cytoplasm that results in the appearance of a more or less normal cell. The outline of the cell, however, is often extended into fine processes which, interlacing with their fellows, impart a 'stringy' appearance to the area. In other cases the nuclei appear to be pycnotic, and lack their complement of cytoplasm, the area around them being composed of irregular interlacing strands. The general picture therefore is one of cells undergoing transformation into secretion that comes to fill the lacunar system (figure 12, plate 11). This observation is significant in relation to the contention of Boolootian & Campbell (1966) that the appearance of secretion is illusory and due to the presence of 'haemal or coelomic fluid trapped in the interstices of the organ.

The whole mass is permeated and presumably supported by a network of what appear to be connective tissue fibres. In addition, fibres of a different character are present. They have a diameter of about 2·0 μm, whereas the connective tissue fibres are finer, being about 0·5 μm in diameter. The stouter fibres appear short and in some cases fusiform, with a prominent nucleus and clear cytoplasm that stains red or pink with Mallory. They are not uniform in distribution, being most numerous in the region of the contractile vessel and stone canal; those associated with the latter are especially numerous midway along the length of the organ. Some, however, penetrate farther afield and enter the glandular region where they are sparsely distributed, occurring singly or in loose bands of few fibres, running in all directions. At times they run near to the surface especially in the walls of the embayments of the central lumen (figure 7, plate 10).

Their nucleated character and the other clear distinctions from the connective tissue fibres just mentioned, together with their association with the contractile vessel suggest that these fibres are muscular.

Amoebocytes in varying number permeate the axial organ. They are ubiquitous and often appear to be moving bodily through the walls bounding the cavities of the organ. The amoebocytes often showed signs of disintegration (figure 8) especially in the spaces of the lacunar system, into which their contents are spilled out being added to the secretion which appears in these spaces. Since many of the amoebocytes contain pigment in the form of the well-known spheroids, some of which have unfortunately been named chromatophores, the amount of pigment accumulated may become very striking, especially in *Diadema* (see below).

The fate of the secretion and other substances that accumulate in the lacunae and their function are not yet wholly clear. Some forms the highly characteristic cysts, the formation and function of which have recently been described by Millott (1966) in *Arbacia punctulata* and *Strongylocentrotus droebachiensis*. It is unlikely, however, that this will prove to be the sole function, for histological appearances indicate that significant quantities of secretion, including substances derived from the degeneration of amoebocytes, are discharged from the organ. Secretion may be discharged into the central lumen of the organ (figure 9, plate 10) or into the spaces of the canalicular system, including the channels by which this system communicates with the perivisceral coelom. Numerous amoebocytes may be found in these channels and some of them appear to degenerate there (figure 8).

A somewhat striking variant of these events has occasionally been observed in which the peritoneum investing the whole axial organ breaks down in places allowing the contents

of the lacunar system (especially secretion) to enter the perivisceral coelom directly (figure 10, plate 10). More often it is the attenuated lining of the canalicular system which disappears in localized areas, especially in the follicles and in the channels communicating with the perivisceral coelom (figure 8), so that the canalicular and lacunar systems become confluent, allowing the contents of the latter to spill out into the former. It will be evident that although the histological picture presented in these various cases is rather different, the result is the same, in that substances eventually find their way from the axial organ into the perivisceral coelom either directly, or indirectly via the canalicular system and its openings on the surface of the organ.

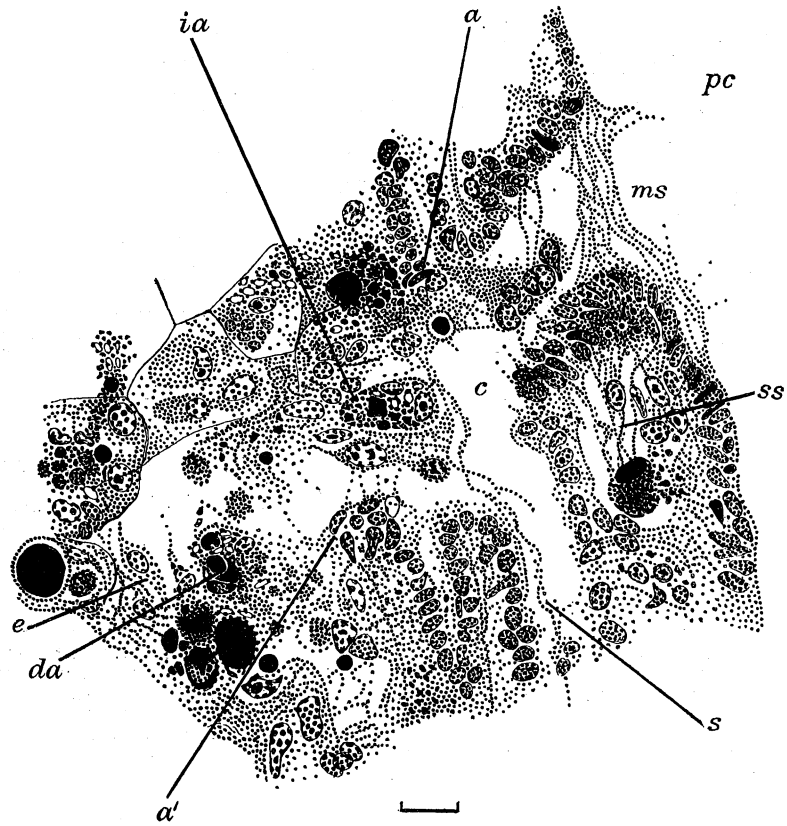

FIGURE 8. *Paracentrotus lividus*. Portion of the peripheral region of the axial organ in the vicinity of a canal (*c*) opening into the perivisceral coelom (*pc*). Note that in the deeper portion of the canal (to the left of *a* and *a'*) its lining epithelium has disappeared. Secretion (*s*), effete cells (*e*), intact (*ia*) and degenerating (*da*) amoebocytes have entered the canal from the surrounding spaces of the lacunar system. A mass of secretion (*ms*) spans the opening of the canal as it enters the perivisceral coelom. Note also the strings of secretion (*ss*) arising from cellular disintegration within the lacunar system. Fixed Duboscq–Brasil. Stained Heidenhain's iron haematoxylin. Scale 6 μm.

Reference may now be made to the appearances shown in figure 11, plate 10. In addition to secretion, cells may enter the canaliculi from the lacunar system. These cells include those whose degeneration appears to produce secretion, together with whole or degenerating amoebocytes and the effect is to produce a peninsula of cells that extends into the lumen of the follicle. The number of cells involved may be considerable and appearances suggest that cellular proliferation may sometimes occur in the region where the wall of the follicle

has broken down, but it is difficult to be sure on this point. Invasion of a follicle may occur at several points. A somewhat similar process occurs in the canals which discharge into the perivisceral coelom (figure 8).

The full significance of the secretion and added substances has therefore yet to be discovered. A valuable index to its importance might be forthcoming from some knowledge of their chemistry. It was therefore decided to examine the axial organ histochemically.

Histochemistry

Methods devised to reveal various classes of compounds were employed as indicated below. Most of them were the routine or special methods specified by Casselman (1959), Gomori (1952), Lison (1953) and Pearse (1960), but in a few cases they were slightly modified. The results will be described in sections devoted to the main classes of compound considered.

1. *Carbohydrate*

The distribution of carbohydrate was studied by means of the periodic-acid-Schiff technique (*PAS*) following fixation in Rossman's fluid or in absolute alcohol at about −20 °C, as recommended by McManus & Mowry (1958). Ehrlich's haematoxylin or Mayer's haemalum was used as counterstain. The reaction was checked against rat liver controls.

In all three species, much of the axial organ stained, including the basement membranes and some fibres within the connective tissues, but particularly the abundant masses of secretion (*s*) in the lacunae (figure 12, plate 11). The lining of the canalicular spaces also stained prominently but owing to the attenuated character of the cells lining these spaces they are difficult to distinguish, so that it was not possible to be sure whether the more intense staining was in fact attributable to their cytoplasm. Subsequent observations (p. 210) are relevant in this connexion. The secretion in the central lumen and in the canalicular spaces, including that seen passing into the perivisceral coelom from their openings as well as that discharged directly from the lacunar system via the outer surface of the organ (p. 208), was also *PAS*-positive. Many of the amoebocytes stained intensely and their disintegration added its quota of positive-staining material to the secretion in the lacunae and canaliculi.

There appeared to be a relationship between material staining in this fashion and the brown or black pigment. Thus, the aggregations of pigment forming the terminal caps in *Arbacia* stained intensely. Again, pigment granules in other situations are mixed with granules of the same size and form that also take up the stain, suggesting that the two types of body are in some way related. Lastly, granules packed densely in some of the amoebocytes show both brown and *PAS*-positive areas. Such granules may therefore originate in the amoebocytes, a possibility that will be examined when the origin of pigment is discussed.

Tests based on the dimedone blocking method (Bulmer 1959) combined with salivary digestion indicated that most of this positive staining material is not glycogen. Thus, after dimedone treatment alone, staining is abolished except in some amoebocytes and in a few fibrous structures within the connective tissue. Salivary digestion of preparations blocked by dimedone abolished all staining by the *PAS* technique.

The reaction of fibrous structures within the connective tissue deserves comment. Some of the stained areas resembled irregular droplets which here and there were associated with the fibrillar structures making them appear like knotted cords. The nature of the droplets is difficult to discern; they could be artifacts. Their association with fibres is suggestive and is especially clear in the vicinity of the stone canal. This suggests that they may represent glycogen that has diffused from these fibres which resemble those that have already been interpreted as muscle (see p. 207) and which are particularly numerous in this region. This idea is substantiated by an inverse relationship between the extent of the fibres stained and the number of droplets. Though the effect may appear after using the standard *PAS* technique, it is more evident after the use of a blocking agent.

In all three species therefore the greater part of the abundant *PAS*-positive material does not appear to be glycogen. However, the tests employed may not reveal all the glycogen present in the sections, for according to Kugler & Wilkinson (1961), they would show only acid-soluble or lyoglycogen.

Bearing in mind that the echinoids studied were all captive and that their feeding habits under such conditions are unknown, it is possible that the small amount of glycogen and its spasmodic appearance may be a reflexion of their nutrition. It is well known that echinoids in captivity can survive prolonged starvation, which might account for the small amounts observed. To test this idea, animals were fed on carbohydrate and its effect on the occurrence and distribution of glycogen in the axial organ was determined. In the initial experiments, individuals of each species were offered plates of agar mixed with glucose and starch or with triturated seaweed (*Fucus*). The urchins did not feed during the 4 or 5 days they were watched and indeed appeared to avoid the plates. Accordingly, other urchins were force-fed for 4 days on a mixture of glucose and starch introduced into the mouth by hypodermic syringe. Their axial organs were removed and fixed in ice-cold ethanol. Paraffin sections were floated in 50 to 95% alcohol and then stained as previously using similar controls of rat liver. Alternatively, the modification suggested by McManus & Mowry (1958) was adopted, in which sections were oxidized using 0·5% periodic acid in glacial acetic acid. Blocking by dimedone, with and without salivary or diastatic digestion, was employed as before.

In *Arbacia*, the response to *PAS* staining was significantly different after the force-feeding, in that the cells lining the canalicular spaces, normally much attenuated and showing no marked response to the *PAS* stain, now appeared somewhat swollen and stained deeply. This appearance persisted after treatment with dimedone (figure 13, plate 11), but the staining disappeared completely after treatment with diastase. The lining cells therefore appear capable of accumulating glycogen. So far as can be judged from the density of staining, the amount of glycogen in these cells is of the same order as that found in the rat liver controls. A further significant feature revealed was the presence of some glycogen within the spaces of the lacunar and canalicular systems. This appeared like secretion mixed with that normally found in these spaces (see above), but the latter secretion did not retain its stain following treatment with dimedone. Only a proportion of the carbohydrate appearing as secretion in these spaces is therefore glycogen. This is discharged into the canalicular system by the breakdown of the lining cells which appear to accumulate glycogen, indeed some of these cells are cast off into the canalicular spaces before they

break down. Occasionally, further glycogen is added to this secretion from the underlying lacunae.

The situation revealed in *Diadema* and *Paracentrotus* is essentially similar though less striking. In *Paracentrotus*, also, there were clear signs that some of the lining cells containing glycogen are cast off into the canalicular lumen and others into the central cavity of the axial organ.

The positively staining material which is not glycogen may now be considered. The properties of mucins and allied substances have received much attention and their histochemistry has been extensively investigated (Mowry 1963). Two notably useful tests have been devised for acid mucopolysaccharide, namely the Alcian blue and the colloidal iron (Mowry 1958) reactions. With the latter method appropriate controls were prepared in which sections were similarly treated except that they were not exposed to colloidal iron. The results of both methods may usefully be combined with those of the *PAS* reaction, for the Alcian blue and colloidal iron tests reveal carboxyl groups, whereas the *PAS* technique shows reactive vicinal hydroxyl groups (Mowry 1963).

In *Arbacia* the use of Alcian blue showed the existence of positively staining material in the lacunar and canalicular spaces including the central lumen of the organ, in the channels penetrating the trabeculae and in the canals opening on the surface of the organ. Some of this appeared to be secretion lying free within these spaces. Although some of the staining was attributable to connective tissue, most of the stained material appeared within the lacunar system. A notable feature was the staining of some of the pigment spheroids which are deposited in the lacunar system and which in this species form the characteristic terminal caps at the ends of the embayments of the central lumen already referred to (p. 203).

In *Diadema* and *Paracentrotus*, the distribution of positively staining substance is essentially the same. In *Diadema*, the staining of some of the amoebocytes is noteworthy.

It is most significant that in all three species much of the material that stained with Alcian blue appeared to arise as a cytoplasmic transformation product in branched cells lying within the lacunar system.

In *Arbacia*, *Diadema* and *Paracentrotus*, Mowry's method showed clear positive reactions for the characteristic intense blue colour was totally absent from the control sections. Moreover, the positive reactions appeared in the branched cells and in secretion within the lacunar system. It was also shown by secretion lying within the canalicular system, including its follicles, in the canals opening on the surface of the organ and passing off from its outer surface.

The powerful, sharp staining which results from this method enabled us to confirm that some of the secretion produced by branched cells arises as a transformation product, the cell processes becoming changed into irregular, nodulated threads (figure 6), which stain intensely blue. Among them may be seen scattered and apparently isolated nuclei, similar to those shown in figure 5*a* and presumably left behind by the cell disintegration.

The presence of positively staining secretion in the channels penetrating the trabeculae is noteworthy because a substance staining similarly appears in the contractile vessel. It would be premature to assess the significance of this, but it certainly suggests that the vessel might be receiving the secretion from the lacunar system via channels in the

trabeculae. Histological appearances suggest that some of this secretion may escape through the walls of the vessel (figure 9, plate 10) into the central lumen of the organ. Incidentally, considerable quantities of secretion with similar appearance and staining properties often occur in the stone canal.

In connexion with the origin of secretion it must be borne in mind that some of it arises from amoebocytes, in which it is often associated with brown pigment (see p. 220). Such cells showing a positive reaction may appear in varying numbers scattered throughout the axial organ, in which many of them disintegrate, liberating their contents including both pigmented and colourless spheroids. Large numbers of such disintegrating cells may often be seen in the spaces of the lacunar system and occasionally also in those of the canalicular system.

The staining of the pigment masses is a notable feature in *Arbacia* and *Diadema*, in which as a consequence some of it appears deep blue or purple. The distribution and properties of the pigment are discussed below.

It is clear that the Alcian blue and Mowry's colloidal iron techniques are confirmatory, showing an essentially similar distribution of staining which is due to the presence of acid mucopolysaccharide. Of the two, the results of Mowry's test are more striking because of the intensity of the blue colour produced.

The *PAS* reaction, with blocking and digestion, showed that the greater part of the carbohydrate present was not glycogen, which is largely confined to the cells lining the canaliculi. The correspondence in the histological distribution of the remaining *PAS*-positive substance and that which responds to the Alcian blue and Mowry's techniques indicates the property of staining with both. This implies that the substance produced by the transformation and disintegration of cells within the lacunar system resembles epithelial mucin rather than connective tissue mucin (Mowry 1963).

Despite our clear histochemical demonstration of the discharge of acid mucopolysaccharide into the perivisceral coelom, it is worth recalling that Messina's (1957) analysis of the perivisceral fluid in *Arbacia lixula* does not mention acid mucopolysaccharide or acid mucoprotein, though she records the presence of glycoprotein.

2. *Protein*

The distribution of proteins and amino acids was revealed by general reactions as well as by those for specific amino acids. Protein-bound amino groups were identified by Yasuma & Ichikawa's (1953) ninhydrin-Schiff method, using Mayer's haemalum as a counter-stain. Organs were fixed in Carnoy, as recommended by Pearse (1960), and rat pancreas was used as a control.

In *Arbacia* the axial organ stained generally red, but the lacunar system coloured prominently, due to the positive reaction of abundant secretion. This appeared to arise as a transformation product of cells similar in position and appearance to those already shown to produce acid mucopolysaccharide. Much secretion staining similarly was also seen in the central cavity and on the outside surface of the organ. A variety of fibres stained prominently. Smaller pervasive fibres, somewhat irregular in outline, reacted with differing intensities. They appeared to be connective tissue fibres. Larger fibres coloured conspicuously. Many of them, especially those immediately below the surface in the walls of

the canaliculi and the embayments of the central cavity, resembled the fibres previously interpreted as muscle. Pigment in the characteristic terminal caps of the embayments showed a slight positive reaction.

The situation in *Diadema* is essentially similar, but the conspicuous reaction of groups of the attenuated cells lining the canalicular spaces made them difficult to distinguish from the deeply staining fibres, presumed to be muscle, lying immediately beneath. The pigment, which is particularly abundant in the canalicular spaces of *Diadema*, reacted variably. Some spheroids forming aggregates that strongly resembled degenerated fragments of amoebocytes stained markedly.

The reaction of the axial organ of *Paracentrotus* was generally the same, but here it was noted that some of the cells in the peritoneum investing the organ were strongly positive. The cytoplasm of some amoebocytes also stained prominently.

Although there is now fairly general agreement that Yasuma & Ichikawa's ninhydrin method is specific for protein, it is also highly sensitive to metallic impurities in the ninhydrin solution, as emphasized by Rappay (1963). Accordingly the tests were repeated using alloxan instead of ninhydrin, followed by Schiff's reagent.

In general, confirmatory results were obtained. Staining showed the same overall distribution, but the attenuated cells lining the canalicular spaces or the central lumen as well as the fibres running through the connective tissue stained more intensely.

The results of the ninhydrin-Schiff and alloxan-Schiff tests revealed in the axial organ of each species the presence of widely distributed reactive aldehyde groups, presumably arising from the oxidative deamination of protein or at least of amino acids.

The conspicuous reaction of some of the cells lining the canalicular spaces is noteworthy. These cells form part of the layer that may at times be rich in glycogen (p. 210), but we cannot be sure that the same individual cells are responsible for both reactions.

The presence of protein or amino acids within the central lumen and on the outside surface suggests that they are being secreted within the organ and that some are discharged into the perivisceral coelom possibly by the openings of the canalicular system. The conspicuously positive reaction of the lining of the canaliculi suggests that at least some of the cells forming the lining produce the secretion. At the same time indications of the origin of protein (or amino acids) from cell transformation should not be overlooked for this could account for their presence in the lacunar secretion.

The reactions of the pigment are difficult to assess because its natural reddish-brown colour obscures the result of histochemical tests.

Confirmatory evidence was sought from the hydroxynaphthaldehyde method of Weiss, Tsou & Seligman (1954) following fixation in Rossmann's fluid, and using rat pancreas as control. This method has the advantage of differentiating sites with few reactive amino groups (red staining) from those with many such groups (blue staining). Shortage of material compelled us to limit the test to two of the species (*Diadema* and *Paracentrotus*) and to use a fixative containing formalin which may be somewhat deleterious to the reaction (see Pearse 1960, p. 88).

The results for both animals confirmed those previously obtained, insofar as some of the cells lining the canaliculi appeared distinctly red or blue, while those within the lacunae appeared somewhat reddish. Similarly the coagulum in the lacunae stained red or blue.

Again, some cells of the peritoneum investing the organ reacted positively, showing a slightly more prominent red or blue colour than the remainder of the organ. This clearly complicates the issue because these cells could be the source of some at least of the secretion, especially that adhering to the outside of the organ.

These indications of the distribution of protein were extended by the use of the tetrazolium reaction for S=S and SH linkages. Material from all three species was fixed in ethanol, Carnoy or Rossman's fluid, and rat liver, kidney and pancreas were used as controls.

Positive reactions indicating the presence of reducing groups associated with protein (dark blue staining) were observed in all three species. In *Arbacia* the red-staining indicative of lipid-bound reducing groups was also seen. The relative amounts of each varied considerably. Again, the reactions were manifest in the lacunae as well as in the cells lining the canaliculi, from which some of the secretion appeared to arise.

The tests for proteins or amino acids in general were supplemented by those for specific amino acids in the free or combined states. The classical Millon reaction, performed with material fixed in Carnoy or Rossmann and using rat pancreas as control, was unsatisfactory, yielding only pale colours which soon faded.

Accordingly, the improved form of the Morel–Sisley diazotization method (Glenner & Lillie 1959) was employed. This proved more satisfactory. The axial organs of all three species showed diffuse violet staining, indicating a wide distribution of tyrosine in small amounts. More pronounced purple staining was seen in the connective tissue fibres pervading the organ. In *Paracentrotus*, staining was very marked in the fibres immediately beneath the cells lining the canaliculi, whereas the lining cells themselves showed no significant amounts of tyrosine. A clear but less conspicuous reaction was given by some only of the branched cells within the lacunae. Whether this was due to exhaustion following secretory activity or to a more permanent differentiation among such cells remains undecided.

Selective staining was shown to a varying extent by the amoebocytes of *Diadema* and *Paracentrotus*. In some instances the ground cytoplasm stained prominently. In others, spheroids which appeared to be of the colourless variety also stained but the brown or yellowish spheroids did not. This indication of the presence of tyrosine in amoebocytes is confirmed by Burton (1964) who used the method of Bensley & Gersh (1933). A few of the amoebocytes with intensely staining cytoplasm showed signs of egesting brown pigment in the peripheral lacunae.

In general the test indicates that there are no significant amounts of free or combined tyrosine in the axial organ, apart from that in the connective tissue and in certain amoebocytes. With this we may associate the facts that secretion adhering to the outside of the organ gives no reaction for tyrosine and that some amoebocytes are known to contain tyrosinase and to form black or brown pigments in a wide variety of situations, including the coelomic fluid (Jacobson & Millott 1953).

Evidence for indolic compounds, particularly tryptophan, was sought by two methods of differing specificity. The first was the post-coupled *para*-dimethylaminobenzylidene (*PC-DMAB*) method (Glenner & Lillie 1957). Here the rationale rests on the fact that although indole derivatives are ubiquitous in tissues, sites with an abundance of these substances are clearly revealed. The method of fixation used, which is important (Pearse

1960), was that recommended by Glenner & Lillie, namely 10% calcium acetate formalin for $3\frac{1}{2}$ h. As anticipated, indole derivatives were revealed in the axial organs of all three species of echinoid. Though widely distributed, by far the most conspicuous concentrations occurred in the lacunae (figure 14, plate 11) especially in those within the trabeculae crossing the central lumen. Some of the deeply staining areas consisted of amoebocytes in which the spheroids stained selectively. Often the deeply stained areas of the lacunae corresponded with those in which cells were apparently being transformed into secretion. Moreover, they sometimes extended into the canaliculi as the characteristic peninsulas described on p. 208. The histochemical picture therefore suggests that the indolic compounds are incorporated into the coagulum which is eventually liberated into the canaliculi by disintegration of the peninsulas of cells or by overflow from the lacunae following breakdown of the canalicular walls (p. 204).

The second method was the more specific *para*-dimethylaminobenzylidene nitrite (*DMAB*-nitrite) technique of Adams, (1957) using rat pancreas as a control. Material was fixed in either absolute ethanol or in Rossmann's fluid.

The axial organs of the three species showed a suffused, feebly positive reaction. The amoebocytes, however, stained deeply blue, so as to become conspicuous in the lacunae, in the canaliculi and in the canals opening from these channels on to the surface of the organ (figure 15, plate 11). Moreover, amoebocytes were prominently revealed passing through the investing peritoneum. They often appeared amid blue-staining coagulum which, bearing in mind the selective character of the staining, suggests that some of the coagulum arises from these cells, possibly by their breakdown. There was no evidence of selective staining of the pigment.

Taken together, the two methods indicate the presence of indole derivatives in amoebocytes as well as in the coagulum within the lacunae and canaliculi. However, the two tests used differ in their specificity: the *PC-DMAB* reaction is claimed to be specific only for indole derivatives in general, whereas the *DMAB*-nitrite appears to be specific for 3-indolyl compounds. The enhanced staining of the amoebocytes with *DMAB*-nitrite therefore suggests that the indole derivatives in these cells are predominantly of the 3-indolyl type. By analogy with the situation in other animals. these positive reactions might be due to the presence of tryptophan. It may be noted that Burton (1964) has already described the positive reactions of amoebocytes to the *DMAB*, naphthyl ethylene diamine and xanthydrol reactions, as indicating the presence of indolic compounds in these cells.

In general, therefore, there are indications that protein or at least amino acids may be produced in the axial organ. They may arise as secretion from the cells lining the canaliculi and central lumen as well as by transformation of lacunar cells and from the degeneration of amoebocytes. Indications of their origin from the cells of the investing peritoneum should also be borne in mind. This is not surprising in the sense that the peritoneum is continuous with the lining of the canaliculi at their openings into the perivisceral coelom.

The tests were inadequate to show the precise nature of the substances produced, but there was clear indication that at least one is a 3-indolyl derivative, perhaps tryptophan.

It is significant that amoebocytes containing 3-indolyl compounds appear to pass between the axial organ and the perivisceral coelom. From the evidence available it is not of course possible to determine the direction in which they were moving.

Enzymic activity

In view of the obvious glandular character of the organ in all three species and the evidence of protein secretion, it is reasonable to seek signs of enzymic activity. The small amount of material available at any one time offered only severely limited prospects for the use of extraction methods. We therefore restricted our tests for enzymes to histochemical methods.

Phosphokinases and transphosphorylases have already been demonstrated in echinoids by Griffiths, Morrison & Ennor (1957) and in view of the ubiquity and fundamental importance in intermediary metabolism of phosphatases it is appropriate to seek evidence for their occurrence in a glandular structure such as the axial organ. We have not sought for acid phosphatase but have confined our attention to alkaline phosphatase.

Much has been argued concerning the validity of histochemical demonstrations of this enzyme and a variety of methods have been proposed. Lacking abundant fresh material we concentrated our attention on two methods using paraffin sections.

The first of these was Gomori's method as modified by Danielli (1946) in which the behaviour of sections of axial organ when incubated with glycerophosphate was compared with that of control sections of the same axial organ treated in precisely the same way except that they were not exposed to the phosphate. As a further check the behaviour of these experimental and control sections was compared with that of sections of rat kidney treated in exactly similar fashion. In the case of the sections of axial organ, neither experiments nor controls showed the blackening that indicates active alkaline phosphatase, whereas with the rat kidney the experimental sections but not the controls, showed pronounced blackening. There is thus no evidence of significant amounts of active alkaline phosphatase in the axial organ.

The second method used was the modified coupling azo dye technique for alkaline phosphatase recommended by Pearse (1960, p. 872) after cold acetone fixation. The

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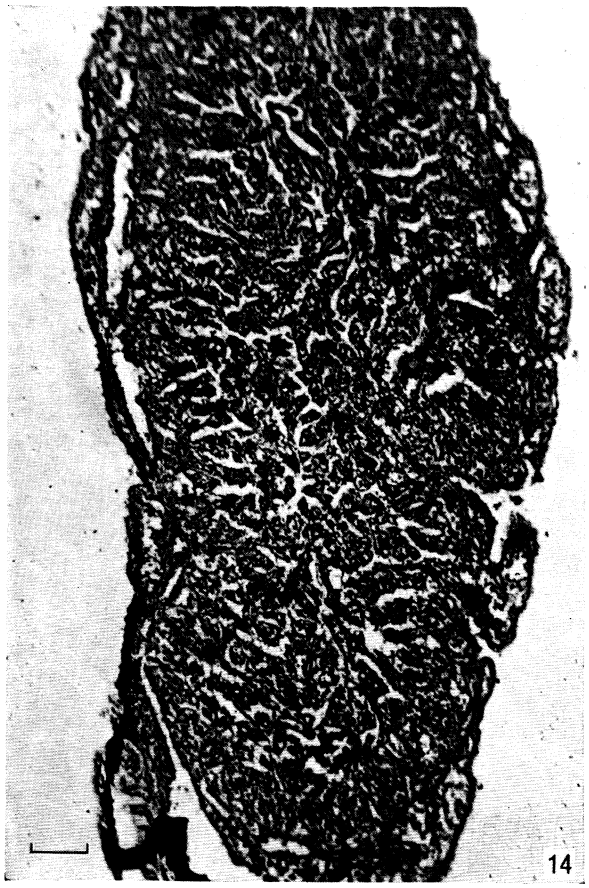
DESCRIPTION OF PLATE 11

FIGURE 12. *Arbacia lixula.* The accumulation of *PAS*-positive secretion (*s*) in the lacunae. Fixed Rossmann. Stained periodic-acid-Schiff and Ehrlich's haematoxylin. Scale 8 μm. *c*, canalicular space.

FIGURE 13. *Arbacia lixula.* Periphery of the axial organ showing the accumulation of glycogen in the cells lining the canaliculi (*c*). The *PAS* technique renders the lining conspicuous (see p. 210). Fixed, ice-cold ethanol. Stained, periodic-acid-Schiff followed by dimedone. Scale 20 μm.

FIGURE 14. *Diadema antillarum.* Longitudinal section of the axial organ showing the wide distribution of indole derivatives (indicated by the scattered darkly staining areas), particularly in the lacunae. Fixed 10 % calcium acetate-formalin. Stained post-coupled *para*-dimethylaminobenzylidene and Mayer's carmalum. Scale 70 μm.

FIGURE 15. *Diadema antillarum.* Portion of the periphery of the axial organ showing amoebocytes rendered prominent by a positive reaction for tryptophan. The cells are aggregated in a follicle of the canalicular system, near to a place where it opens on the surface. The actual opening is blocked by an amoebocyte (*ac*). Fixed ice-cold ethanol. Stained *para*-dimethylaminobenzylidene nitrite and Mayer's carmalum. Scale 18 μm.



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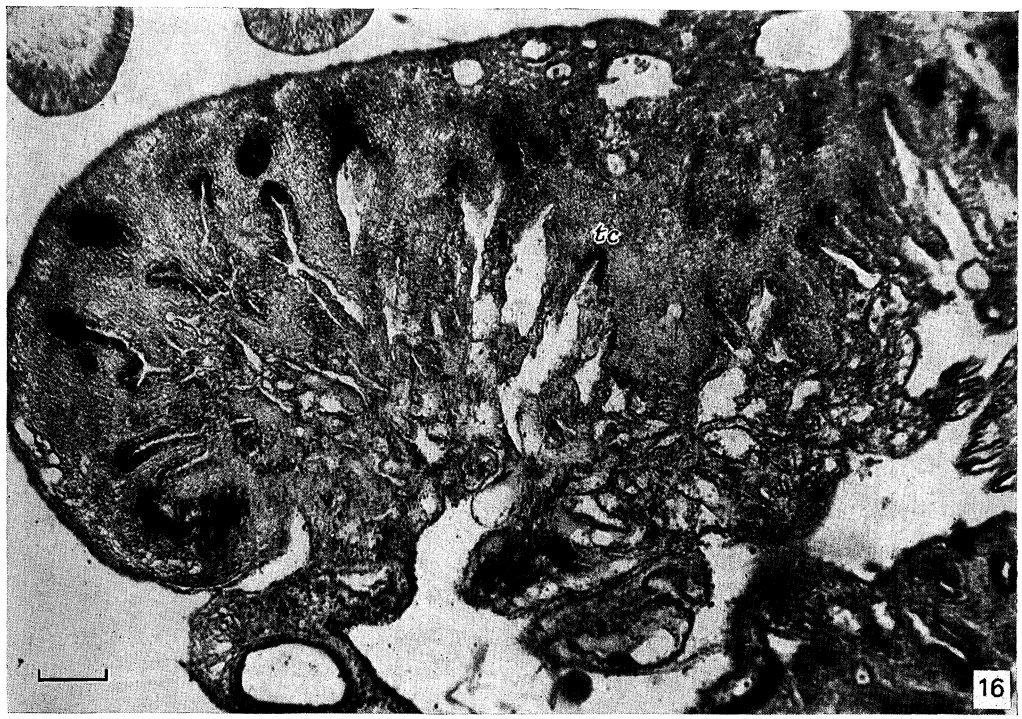
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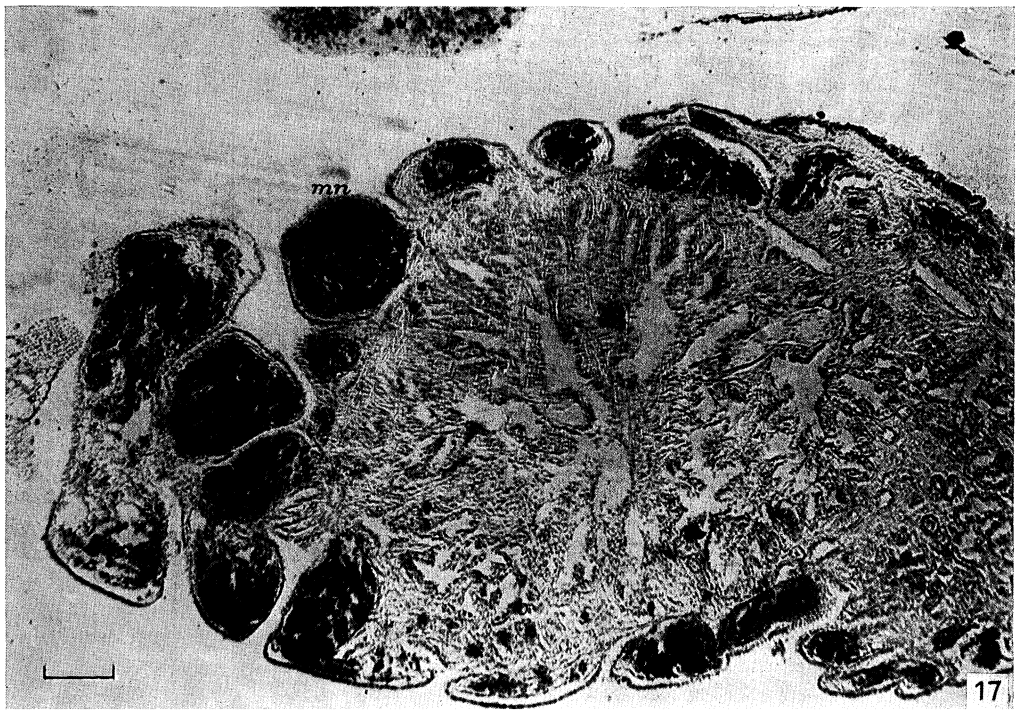
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coupling agent used required careful consideration for several reasons. In the first place it should not inhibit the enzyme, since it is likely that much activity will already have been destroyed by the fixation and paraffin embedding. Secondly, it should be as stable as possible so as to avoid any non-specific staining. Thirdly, the colour produced at any active site present should be clearly distinguishable from the naturally occurring reddish, brown or black pigments. With such considerations in mind none of the coupling agents listed by Pearse (1960, p. 407) was completely satisfactory. We therefore attempted to compromise, by using in separate experiments, 4-chloro-*o*-anisidine and 5-chloro-*o*-toluidine.

In neither case was there any clear evidence of alkaline phosphatase in the axial organ of any of the species. If, therefore, the enzyme occurs it must be in amounts too small to be detected by these means, or in some way inhibited.

The presence of melanin in the axial organ sometimes in impressive quantities as in *Diadema*, coupled with the presence of tyrosine (p. 214) suggests that tyrosinase may be present. Unfortunately histochemical detection of the enzyme by the convenient and widely used tests based on the *DOPA* reaction has dubious value. The method developed by Becker, Praver & Thatcher (1935) was tried but yielded no unequivocal evidence of the presence of the enzyme in the axial organs of the three species studied.

This was unexpected in view of the clear demonstration of the enzyme in the amoebocytes of the perivisceral coelomic fluid of *Diadema* by Jacobson & Millott (1953) who performed their tests *in vivo* and *in vitro*.

The apparent anomaly could mean that the active enzyme has limited life and has disappeared by the time the amoebocytes have developed their complement of melanin before entering the axial organ. Such a limited period of activity has been described for the tyrosinase in the melanin granules of chick retinal pigment epithelium (Seiji & Fitzpatrick 1958), and in the so-called 'melanosomes' of mammals (Seiji, Fitzpatrick & Birbeck 1961).

Again it must be borne in mind that the tyrosinase system is not only complex, but extremely sensitive to its environment (Mason 1956) and the factors governing its activity *in situ* are imperfectly understood. It would therefore be premature to deny the existence of the enzyme in the axial organ and for the moment we are inclined to suspect the reliability of the test, particularly when applied in the relatively untried field of echinoderm material.

Description of plate 12

Distribution of pigment

Figure 16. *Arbacia lixula*. Portion of a section of the axial organ showing the characteristic concentrations in the terminal caps (*tc*) of brown reducing pigment, later shown to be melanin (p. 222). Fixed Rossmann. Masson-Fontana argentaffine reaction, counterstained Mayer's carmalum. Scale 72 μm.

Figure 17. *Diadema antillarum*. A portion of a section of the axial organ showing the extensive deposition of melanin (*mn*) in the peripheral lacunae (p. 220) revealed by Lillie's method (see p. 221). Fixed Carnoy. Stained Lillie's (1956) Nile blue method. Scale 80 μm.

3. *Lipid*

Material from all three species was tested directly for phospholipids by means of Sudan black B and for neutral fats by Sudan III and Oil red O. These dyes, dissolved in 60% triethyl phosphate as recommended by Gomori (1952, p. 96), were applied to frozen sections of both fixed (10% formalin) and fresh material.

In every case staining was diffuse and non-selective, except that in *Diadema* some of the pigment stained with Sudan black B, (see p. 222). There was therefore no indication of any significant storage of lipid.

The presence of lipid-bound reducing groups was revealed in *Arbacia* only by the tetrazolium reaction, the use of which has already been reported in connexion with the demonstration of protein-bound reducing groups (p. 214).

4. *Pigment*

Pigment in the axial organs of various echinoids has been described by earlier workers and indeed inspired the name 'brown gland'. Thus Hamann (1887) noted a yellow-brown pigment in the axial organ of *Arbacia pustulosa* (= *lixula*), mostly in the periphery, and thought that it was produced by amoebocytes. Prouho (1887) noted a similar distribution of pigment in *Dorocidaris papillata* and considered it identical with that in the perivisceral fluid and haemal system. Our observations confirm the existence of pigment in the axial organs of all three species, but the amounts vary considerably. The pigments are of two main types, brown or black and orange to red. These will be considered separately below. The brownish pigments have commonly been supposed to be excretory.

(*a*) *Reddish pigment*

The occurrence of a reddish pigment resembling echinochrome A in the axial organ of *Diadema* has already been reported. The absorption spectrum of crude extracts in acidified di-ethyl ether resembled that of the naphthaquinone pigment in the same solvent obtained from other parts, including spines, of this urchin (Millott 1957). A more recent re-examination of the widely distributed reddish pigment of *D. antillarum* has shown that it is indeed echinochrome A (Thompson, private communication 1965).

Echinochrome has long been known to exist in *Arbacia* (McMunn 1885), but more recent work has shown that in the species *lixula* it exists in several forms (see Thomson 1962) in addition to echinochrome A. The presence of echinochrome A in the amoebocytes of the coelomic fluid however, suggests that some, at least, of the pigment in the axial organ is likely to be in this form.

In fresh axial organs of the species examined, this reddish pigment is conspicuous only in *Arbacia*. The amount varies greatly but it is sometimes sufficient to colour the organs deep red. In *Diadema* and *Paracentrotus*, and sometimes in *Arbacia*, the organ is coloured orange by the pigment which changes to red on fixation in acid solutions. The change may be striking, particularly in *Arbacia* and *Diadema*, and is not only an overt expression of a change in colour with pH but also of a change in pigment distribution. This can be seen directly, red pigment being discharged from the surface of the organ into the fixative. Thus, when fixed in Carnoy, the internal tissues of the organ may turn bright red. Such colour

spreads and eventually disperses into the fixative, although in all three species sufficient may persist to be obvious in unstained wax sections of the organ. It will now be evident that such sections cannot be used to determine the original distribution of this pigment. The situation is complicated by the presence of amoebocytes in the organ, for some of these cells are known to contain naphthaquinone (Ball & Cooper 1949; Millott 1957). It is, therefore, essential to discover whether the red colour is entirely due to the contents of these cells. Accordingly freshly prepared frozen sections of unfixed material were examined.

In *Diadema* and *Paracentrotus* reddish pigment is present in localized areas throughout the lacunae and less often in the canaliculi. It is almost invariably associated with intact or degenerating amoebocytes and their remains. The pigment is similarly distributed in *Arbacia* but there is much more of it: moreover, in addition to that associated with amoebocytes, conspicuous amounts occur in the cytoplasm of irregular lacunar cells resembling those already described as producing secretion. The amount of pigment discharged was sometimes sufficient to colour the coagulum in the lacunae.

In all three species pigment in the red form is characteristic of the ubiquitous actively moving amoebocytes, whereas the orange is often associated with their aggregated and disorganized remains. The cell fragments, including their contained spheroids, appear in all shades from red to golden-yellow.

Taken together these facts suggest that the golden-yellow pigment is derived from the red in the course of amoebocyte degeneration. This fits, to some extent, with the known instability of the pigment when freed from the amoebocytes of *Diadema*, for disintegration of these cells on a microscope slide can be watched and the liberated pigment can be seen to change from red to orange and finally to brown or black (Millott 1957).

The notion is further substantiated by more exact evidence from microspectrophotometric examination of freshly cut frozen sections. In such sections of *Paracentrotus*, amoebocytes that appear histologically normal are red, the cytoplasm showing a broad absorption in the visible range between 500 and 600 nm with a peak at 550 to 580 nm. In amoebocytes which show signs of degeneration the region of maximal absorption in the visible spectrum shifts progressively towards the blue while absorption at longer wavelengths declines. The change appears to culminate in the production of an orange pigment, which absorbs maximally in the visible range at about 470 nm. The pigment in the lacunae surrounding the amoebocytes shows parallel changes which appear to culminate in the production of a pigment also absorbing maximally at about 470 nm.

The reddish pigment in different regions of the axial organ of *Arbacia* and *Diadema* shows a similar range of variation, the region of maximum absorption varying between 550 and 470 nm. Similar variation occurs in the reddish pigment of amoebocytes present in the organs.

Though by no means complete, the foregoing data suggest that the red pigment in the amoebocytes and in the lacunae of the axial organs of all three species undergoes similar changes. It is not yet possible to explain these changes in chemical terms. Although echinochrome is known to change from red to yellow with change in pH (Ball 1936), additional factors are likely to be involved in the living axial organ. Thus, redox changes, instability of the pigment and its possible association with protein, are factors that must be envisaged. These observations, when combined with the histological evidence already

presented, confirm the suggestion that the pigment in the lacunae is derived from the breakdown of amoebocytes. In the case of *Paracentrotus* and *Diadema* all the pigment seems to arise in this way, but in *Arbacia* some of it may be derived from the irregular secretory cells of the lacunae.

(*b*) *Black and brown pigment*

In all three species this pigment is widely distributed. Although extending deeply into the centre of the axial organ it tends to be more concentrated at the periphery, where it is found in the lacunar system. It is notably absent from the canaliculi, except where these spaces are invaded by cells from the surrounding lacunae. The distribution of the pigment in *Arbacia* and *Diadema* is characteristic. In the former, most of it is aggregated around the blind endings of the embayments of the central lumen (p. 203), forming conspicuous terminal caps to these regions (figure 16, plate 12). In *Diadema*, most of the pigment is concentrated in the extreme periphery, where it accumulates in superficial pockets of the lacunar system, sometimes in such impressive quantities as to form nodules on the surface of the organ (figure 17, plate 12). When viewed externally these pockets of pigment impart to the organ its characteristic pardine appearance.

Unlike the reddish pigment the brown or black pigments did not change or diffuse on fixation. This is confirmed by the similar appearance and distribution of these dark pigments in unstained frozen and in paraffin sections.

The pigment is frequently associated with amoebocytes either enclosed within them or free in the vicinity of their disorganized remains. In *Arbacia* as in *Diadema* there may be considerable quantities, but in *Paracentrotus* there is relatively little, and most of it is contained within amoebocytes.

In all three species pigment may be finely granular, compacted into irregular masses or formed into characteristic spheroids. In addition, some of the pigment of *Paracentrotus* is in highly distinctive bodies with rhomboid facies.

These dark pigments could be of several chemical types, including ommochrome, lipofuscin and melanin. Their identification is difficult and our findings are necessarily somewhat tentative.

Ommochrome is distinguished from the other two types of pigment by solubility, reaction to concentrated sulphuric acid and oxidation-reduction properties (Fox & Vevers 1960).

The solubility of the pigment in the axial organ was tested by treating paraffin sections for 5 to 10 min with 10 M formic acid or 5 % HCl in methanol or for 16 h with 6 M NaOH. The appearance of the sections was compared before and after treatment. In addition the treated sections were compared with untreated controls taken from the same series. In *Arbacia* and *Paracentrotus* the pigment in the treated sections appeared unaffected and thus may be regarded as insoluble. In *Diadema*, however, a proportion of the pigment sometimes dissolved in the alkali when this was more dilute.

The characteristic violet colour given by ommochromes on treatment with concentrated sulphuric acid was not shown by any of this pigment.

The oxidation-reduction properties of the pigment were tested by treating sections with potassium borohydride. None of the red colour characteristic of reduced ommochromes was produced.

There is therefore no evidence that the brownish pigments are ommochromes.

Melanin and lipofuscin are more difficult to identify, but a number of useful tests have been devised.

Both behave in a characteristic way towards oxidizing agents, though in the case of lipofuscin such behaviour may change with age. Accordingly, sections of the axial organs of the three species were first subjected to the Masson–Fontana argentaffine reaction, which showed that much, though not all, of the brown pigment had reducing properties.

The reducing properties of the pigment were also revealed by Schmorl's method (as given by Pearse 1960), which depends on the reduction of ferricyanide to ferrocyanide in the presence of ferric salts, with the production of Prussian blue or Prussian green. Owing to shortage of material it was possible to perform this test only on *Diadema*. The general distribution of reducing pigment was the same as that shown by the Masson–Fontana reaction, but in our slides the pigment appeared green as well as blue or in intermediate hues. It would appear that all these shades indicate reducing properties even though the precise mechanism whereby they are produced is a matter of opinion (Pearse 1960).

This variation in behaviour is matched by a difference in the response of the brown pigment of *Diadema* to other oxidizing agents. Thus, bromine bleaches all the brown pigment of *Arbacia* and *Paracentrotus* but not all of that in *Diadema*. Here such variation suggests that the pigment in *Diadema* is a mixture.

Attempts were made to differentiate the pigments by the Nile blue method (Hueck 1912) which is stated to stain lipofuscin blue but to leave melanin, which is bleached by the test, colourless. Two variants were employed. In one, bleaching in hydrogen peroxide was performed before staining (Gurr 1958). In the other the order was reversed (Pearse 1960).

In *Diadema* a positive reaction with both variants indicated the presence of lipofuscin. We must add, however, that pigment staining the diagnostic blue colour was mixed with that which appeared in hues varying from blue to green. This may be due to blending the blue colour produced in the test with the natural yellowish-brown colour of the pigment, some of which perhaps resists bleaching by the hydrogen peroxide used in the test. This notion was substantiated by examining slides which had been subjected to the action of hydrogen peroxide alone, when it was seen that much of the pigment resisted bleaching and remained pale brown in colour.

Further attempts were made to differentiate the pigments by using Lillie's methods (1956), When sections were treated with Nile blue at a pH below 1·0 and mounted in an aqueous medium the pigment in the axial organ stained in all three species. The coloration was not altogether satisfactory. Some of the pigment remained unstained. Some of the pigment appeared dark green, indicating melanin, some especially in *Paracentrotus*, was yellowish-green. In *Diadema* it varied almost continuously between the dark blue or greenish blue prescribed for lipofuscin and the dark green for melanin. Again some of the pigment remained unstained.

A refinement suggested by Lillie (1956), employing the same stain followed by acetone extraction and mounting in resinous media, was more satisfactory. Here, lipofuscins stained at pH 1·0 lose the stain on acetone extraction, whereas melanins retain it. In *Arbacia* and *Paracentrotus* all the pigment retained the stain and thus appears to be melanin. In *Diadema*, only a proportion of the pigment remained stained. The remainder of the

pigment which lost its stain thus appears to be lipofuscin. These results confirm those of the previous tests, namely that so far as reducing pigments are concerned *Arbacia* and *Paracentrotus* contain melanin, whereas *Diadema* has both melanin and lipofuscin.

Tests were made to determine whether the lipofuscin was of fatty acid or phospholipid origin.

Evidence of phospholipid was sought by using Sudan staining. Sudan black B is known to stain both neutral fat and phospholipid. The reaction of the axial organ to such dyes as Oil red O and Sudan III which stain neutral fats has already been discussed on p. 218, where it was shown that tests for such fat were negative. Therefore, reaction of the pigment with Sudan black B should indicate the presence of phospholipid. The method used was that of McManus (1946) as given by Pearse (1960) and involved the initial bleaching of the melanin which is said not to affect the subsequent staining of lipofuscin. Two alternative bleaching methods were used, namely by hydrogen peroxide or by a saturated aqueous solution of bromine. Each was allowed to act for 48 h at room temperature. The bromine proved more effective.

In *Arbacia* and *Paracentrotus* none of the pigment of the axial organ stained with Sudan black B, which agrees with the foregoing indication that all of it is melanin. In *Diadema* much of the unbleached pigment stained, though we found that the staining was affected by the method of bleaching. Thus, after bleaching with hydrogen peroxide none of the pigment stained. It was after bromine bleaching that the positive reaction was obtained, particularly in the peripheral portions of the lacunar system, where the reacting pigment appeared densely black among reddish or brownish bodies, representing the remaining unstained, unbleached or partly bleached pigment. Some of the lipofuscin pigment therefore appears to be derived from phospholipid.

To determine whether any of the lipofuscin arises from fatty acid, sections similarly bleached were stained for 2 to 5 min in a saturated solution of Sudan IV dissolved in equal volumes of acetone and 70% ethanol. None of the pigment of the axial organ stained, indicating that none is so derived.

The previous indication that some of the brownish pigment is melanin was confirmed by a test more recently devised by Lillie (1957). This involves the uptake of ferrous ions by melanin, and very high specificity has been claimed for it (Pearse 1960). Accordingly paraffin sections were subjected to this test, combined in some cases with van Gieson as a counterstain. Pigment staining the diagnostic dark green was widely distributed throughout the axial organs of all three species. This method therefore confirms the presence of melanin.

It was noted, however, that not all of the pigment stained in the way prescribed by Lillie (1957), some appearing blue.

The foregoing tests, indicating the presence of both melanin and lipofuscin, do not account for the pigment of nuclear origin (p. 224). This pigment fits neither category, for although it is bleached by bromine, it is not bleached by hydrogen peroxide, it is unaffected by Sudan dyes and does not reduce ammoniacal silver nitrate. Bearing in mind that some of the axial organ pigment stained blue with Lillie's ferrous ion uptake test, it was suspected that the overall reactions were being complicated by the existence of an iron-containing pigment.

These suspicions were confirmed by treating sections from the axial organ of *Arbacia*, *Diadema* and *Paracentrotus* with an acidified solution of potassium ferrocyanide, when the pigment of nuclear origin stained selectively deep blue. The intensity of staining was such as often to obscure the brown colour which suggests that Prussian blue had been formed as a result of interaction with ferric iron. The reaction is due to the pigment itself, for occasionally in *Paracentrotus* the umistakable facies of the empty envelopes corresponding to pigment bodies were observed, but they did not stain.

It is therefore reasonable to suspect that the bluish hues produced in Lillie's ferrous ion test which employs potassium ferricyanide may be linked in some way with the iron-containing brown pigment of nuclear origin.

It must remain uncertain whether the foregoing represent all the types of brown or black pigment to be found. In *Diadema*, at least, the changes observed in amoebocytes which disintegrate *in vitro* suggest that some of this pigment could arise from echinochrome which under certain conditions becomes unstable yielding darkly coloured compounds, possibly polymers. The problem is not easy to solve for not only are the properties of such naphthaquinone derivatives unknown but both melanin and echinochrome are associated with the spheroids of amoebocytes so that the two types of dark pigment would be difficult to distinguish histologically. In any event the processes leading to blackening in both cases involve oxidation and indeed may even be linked (Jacobson & Millott 1953; Millott 1957).

Some mention should now be made of the association of all the types of black and brown pigment into complexes.

The association of such pigment with acid mucopolysaccharide in *Arbacia* and *Diadema* has already been mentioned (p. 212). It recalls the similar association described by Zimmermann & Eastham (1959) in the mammalian retina, and in wider context the frequently reported association of melanin with protein.

Such associations may be due to the carriage of pigment in formed bodies both within and outside cells. This appears to be so at least with the iron-containing pigment and with the melanin, for in the former instance the distinctive rhomboid bodies are sometimes seen without their pigment and in the latter, stainable spheroids may remain evident after their associated melanin has been bleached.

Origin of pigment

All the available evidence suggests that much of the pigment in the axial organ arises from amoebocytes. In echinoids generally, pigment is ubiquitous and so are amoebocytes as well as signs of their disintegration (Cuénot 1891 *a*, *b*). The widespread degeneration of amoebocytes in *Arbacia* is described by Donnellon (1938) and in *Diadema* by Millott (1957). In *Arbacia* the red pigment of the amoebocytes is chemically closely similar to that found in other situations (McClendon 1912; Ball & Cooper 1949). Moreover, in *Diadema*, the red pigment extracted from the axial organ has the same spectral absorption as that extracted from amoebocytes (Millott 1957).

Again, the red echinochrome liberated by disintegration into the various cavities of the axial organ often becomes orange. In *Diadema* such changes are matched by those shown by amoebocytes *in vitro*. Nevertheless, it should be borne in mind that in *Arbacia* at least

there are indications that the red pigment may also arise from the transformation of other cells in the lacunae (p. 219).

There is comparable evidence concerning the origin of the black and brown pigments. In *Diadema*, Jacobson & Millott (1953) showed that some of the melanin arises in association with the spheroids of certain amoebocytes, and that these cells contain phenolases. The story has now been carried farther by the demonstration of tyrosine in some of the amoebocytes containing spheroids, so that both enzyme and substrate, as well as melanin,

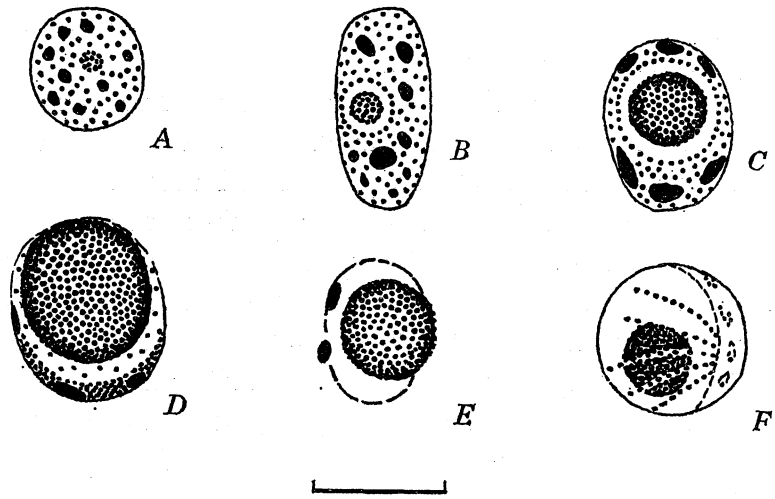

FIGURE 18. *Diadema antillarum*. Selected stages arranged in sequence to show the accumulation within nulcei of iron-containing pigment (p. 224). *A* to *E* show the accumulation in the form of a single brown body which eventually comes to occupy most of the nucleus, while the remaining nuclear contents degenerate and form a rim or halo around the pigment spheroid (*D* and *E*). In some cases (*F*) the nuclear contents disintegrate leaving only an ill-defined envelope around the accumulated pigment. Fixed Susa. Stained Delafield's haematoxylin and eosin. Scale 5 μm.

are now known to occur in these cells. In view of this and of the ubiquitous signs of amoebocyte degeneration in the axial organ, it is but reasonable to suspect that much of the melanin found in the axial organ also arises in amoebocytes and is perhaps transported to the organ by these cells and liberated into the lacunar system when they degenerate.

The iron-containing pigment can also be traced to amoebocytes, but in this instance to their nuclei. In the axial organ of *Diadema*, nuclei giving rise to the characteristic iron-containing bodies can be seen in the lacunae, in the wall of the canaliculi and in the investing peritoneum. In the same echinoid, however, nuclei giving rise to these pigment bodies have been seen in other situations, for example, in the epidermis and in the connective tissue generally.

Although a substantial proportion of the nuclei are affected in *Arbacia* and *Paracentrotus* as well as in *Diadema*, the pigment accumulated within them often remains in the form of inconspicuous spheroids. The process is more conspicuous however in the numerous, apparently isolated nuclei among the extensive pigment deposits of the axial organ such as those forming the terminal caps in *Arbacia* or those in the lacunar system of *Diadema*. The changes are especially striking in the nuclei of amoebocytes, particularly those of *Paracentrotus*. In these situations, the pigment accumulates in such quantity as to form prominent

spheroids or rhomboids (p. 220) which may be extruded from the nucleus into the cytoplasm or remain *in situ* to fill and distend the nucleus, reducing its original substance to a mere rim or halo around the enclosed pigment (figure 18).

The process contributes to the extensive and widespread cell degeneration or transformation already mentioned and its frequent association with disintegrating amoebocytes may account for the wide distribution of isolated, and apparently effete nuclei, many of which contain pigment bodies. The quantity of pigment arising in this way may therefore be substantial.

The site of origin of the lipofuscin found in *Diadema* remains unknown.

Discussion

Viewed in retrospect the foregoing results confirm the glandular nature of the axial organ previously emphasized (Millott & Vevers 1964), but as a result of the present investigation we would re-emphasize that it is misleading to categorize the organ in a simple fashion. This is particularly pertinent, bearing in mind the misapprehension that has arisen in the past following attempts to fix a more specific label to it (see Millott & Vevers 1964). Despite the recent strictures of Boolootian & Campbell (1966) we continue to regard the old term 'axial organ' as adequate to denote a structure of complex function, much of which is imperfectly understood.

The foregoing investigation has clarified some of the fundamental structural features of the axial organ but much has yet to be learned, particularly concerning the development of the various types of cavity enclosed within it. These cavities have therefore been distinguished by simple descriptive names that imply nothing concerning their developmental origin.

In our preceding communication (Millott & Vevers 1964) we drew attention to the strategic position of the organ, in that it lies at the confluence of the perivisceral coelom, the water vascular system and the so-called 'haemal' system. Some features of this intercommunication and their potential significance in *Strongylocentrotus purpuratus* have also been recognized by Boolootian & Campbell (1964, 1966). As an outcome of the present study the precise relations of these important systems become clearer, for the free communication between the lacunae and the contractile vessel has been demonstrated. Furthermore, free communication of the lacunar and canalicular systems has been shown in areas where the canalicular wall breaks down. This means that communication exists between the perivisceral coelom, the lacunar system and the contractile vessel via the canalicular system and its openings on the surface of the organ. It also means that cells as well as fluid and secretion can pass between them. The direction in which they pass requires further study, but histological appearances associated with the production of cysts at times of injury or infection (Millott 1966 and below) and with the formation of the peninsulas of cells already described (p. 208), show clearly that cells enter the canaliculi from the lacunae. Histological evidence also suggests, despite the contentions of Boolootian & Campbell (1966), that at least some of the substances which appear to arise within the organ leave it to enter the pervisceral coelom (p. 207). To interpret such appearances as indicating passage in the reverse direction would require much contriving! At the same

time on the basis of available evidence we not do wish to deny that other cells or other substances may pass in either direction.

As concerns function, histological evidence of the discharge of considerable quantities of secretion into spaces within the organ or into the perivisceral coelom from the free surface (p. 207) hints at an important secretory activity that matches the strategic position. Again the frequent appearance of degenerating amoebocytes also agrees with the time-honoured notion that the organ plays some part in excretion.

The clearest demonstration of a function has been reported by Millott (1966) in connexion with defence against invading organisms. Thus in *Strongylocentrotus* and *Arbacia punctulata*, when foreign organisms are introduced into the perivisceral coelom, or when the perivisceral coelomic fluid is induced to form a clot (as might occur in nature when the fluid is exposed by damage to the test), the axial organ may become distended and the canaliculi may protrude from the surface as blisters. At the same time a striking concert of activity may become evident in the organ whereby the foreign organisms and effete amoebocytes are compounded into a cyst (see below).

In addition, however, Boolootian & Campbell (1964) have clearly shown the existence of a contractile component in the axial organ of *Strongylocentrotus purpuratus*. Although the extent of the contractile vessel in the three species examined here is not easy to trace by the simple study of sections, Boolootian & Campbell's studies have shown that it can be extensive. Moreover, it is stated (Boolootian, private communication) to move large quantities of fluid. Slow rhythmic contractions in the main organ have also been seen by one of us (N.M.) in *Arbacia punctulata*, and though the contractions were observed only once they were unmistakable. The existence of pervasive muscle fibres in the organ must surely be related to this activity.

Though contractions clearly occur their function is unknown. Millott & Vevers (1964) have pointed out the organ cannot be regarded simply as a heart and there are difficulties in speaking of a circulation in echinoids. It has been suggested that the rhythmic contractions may be related to the decontaminating function of the axial organ (Millott 1966). If the contractions are important in this connexion the significance of the canaliculi and their openings into the perivisceral coelom, may be in part explicable as providing a route by which contaminants may enter the organ from the perivisceral coelom or by which phagocytic cells may leave it.

Despite the glandular character of the organ, the function of its secretion is still incompletely known, though demonstration of the chemical nature of some of the substances produced has important implications. The presence of acid mucopolysaccharide may well be related to the production of cysts by a somewhat elaborate mechanism comparable to that revealed in *Strongylocentrotus droebachiensis* and *Arbacia punctulata* (Millott 1966). Contaminating cells or organisms artificially introduced into the perivisceral coelom, were shown to be wrapped with phagocytes, in layers of coagulum.

This mucin may have other protective functions. Thus, it could be discharged from the organ under conditions of stress, e.g. injury to the test, and so help in the formation of a protective clot over the injury. Such clots are well known to form when perivisceral coelomic fluid comes into contact with the environment. Here the binding or precipitative action of mucus may also be relevant in trapping organisms that might otherwise invade

the perivisceral coelom at the point of injury. In this connexion it will be recalled that considerable quantities of acid mucopolysaccharide appear to be produced, moreover, some of it appears in the openings of the canaliculi and passes off from the peritoneum investing the organ into the perivisceral coelom.

The precise means whereby the secretion is carried from the axial organ is not clear. The powerful ciliation of the surface has already been reported in *Arbacia punctulata* (Millott 1966) and such currents might sweep secretion from the surface of the organ into the coelomic fluid. On the other hand, no inward or outward currents were detected at the openings of the canalicular system in *A. punctulata*. Rhythmic contractions of the contractile vessel might play some part in moving secretion. In the present state of our knowledge only a simple pumping action is envisaged. Such action might not only assist expulsion of secretion from the canaliculi into the perivisceral coelom but it might also assist the movement of secretion from the lacunar system into the contractile vessel.

The function of the other identified carbohydrate, namely glycogen, is not entirely clear. Although some is associated with muscle fibres the remainder is stored in the lining of the canaliculi from which it may be discharged into the canalicular lumen. From here it may pass into the perivisceral coelom, or in regions where the canalicular wall is incomplete, it could enter the lacunar system and so reach the contractile vessel, but its fate, like its function, must remain for the moment a matter for conjecture (see Burton 1964).

Readily detectable quantities of protein or amino acids appear in the secretion in the lacunar and canalicular systems, but there is no clear evidence of enzymic activity. However, the tests that could be used were severely restricted by the limited material available. Some of this substance clearly contains 3-indolyl derivatives, which could well be tryotophan. This might be significant in two particular connexions. First, since tryptophan is a fairly common constituent of proteins, some of which function as hormones, the possibility of hormone production in the axial organ should be borne in mind. Secondly, such indole derivatives might be related to pigment production. Although the precise chemistry of melanogenesis is as yet uncertain, following the initial work of Bloch (1917) and Raper (1926) there is now substantial indication that 5:6-dihydroxyindole may be involved (Thomson 1962; Piatelli, Fattorusso, Magno & Nicolaus 1963). Since in *Diadema*, some of the amoebocytes have been shown to produce a black pigment with the properties of melanin, by a mechanism involving tyrosinase (Jacobson & Millott 1953), it is reasonable to suspect that the clear indication of indole derivatives in these cells might presage the presence of melanin intermediates. Thus although melanin itself does not stain by benzylidene condensation (Lea 1949; Glenner & Lillie 1957), its precursors react positively to Ehrlich's test (Lea 1949).

The situation is far from clear, however, for not only is the general subject of melanogenesis fraught with considerable difficulty (Thomson 1962; Nicolaus 1962), but the steps by which melanin is formed in *Diadema* are unknown. The positive reaction to the *DMAB*-nitrite test has so far been recorded only for compounds with the 3-indolyl configuration, such as tryptophan (Adams 1958), whereas the indole derivatives forming the intermediate stages postulated in the classical Raper scheme of melanogenesis and those shown by Piatelli *et al.* (1963) to be part of the polymer of *Sepia* melanin are not of this type.

Therefore, lacking evidence of ommochromes, for which tryptophan (or its derivatives such as 3-hydroxykynurenine) have been cited as chromogens, the possible relation of tryptophan to pigment production remains uncertain.

The occurrence of pigment in considerable quantities is a striking feature of the axial organ of *Arbacia* and *Diadema*, though the dominant pigments in each case are different, that of the former being echinochrome and those of the latter being melanin, lipofuscin and a pigment of nuclear origin. Relatively little is known concerning the function of these pigments (Vevers 1963), especially in relation to the axial organ. As regards the naphthaquinone, the experiments reported by Vevers (1963) suggest that such pigment has the property of inhibiting the growth of blue-green algae. Lesions are common in regular echinoids and contamination by calcicolous algae could be a serious factor in survival. In view of the production within the axial organ of cysts incorporating contaminating organisms it is possible that the naphthaquinone present in the organ may form another important aspect of its decontaminating function.

On the other hand, the older view that the axial organ served as a site of pigment excretion could still be correct. Indeed the two views are not incompatible, in the sense that at least the red pigment unloaded in the organ might be put to good use.

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References

Adams, C. W. M. 1957 A *p*-dimethylaminobenzaldehyde-nitrite method for the histochemical demonstration of tryptophane and related compounds. *J. Clin. Path.* **10**, 56–62.

Adams, C. W. M. 1958 The application of histochemical methods for protein identification in the study of certain pathological problems. *Proc. Roy. Soc. Med.* **51**, 339–343.

Ball, E. G. 1936 Echinochrome: its isolation and composition. *J. Biol. Chem.* **114**, vi.

Ball, E. G. & Cooper, O. 1949 Echinochrome: its absorption spectra, pK-volume, and concentration in eggs, amoebocytes and test of *Arbacia*. *Biol. Bull. mar. biol. Lab., Woods Hole* **97**, 231–232.

Becker, S. W., Praver, L. L. & Thatcher, H. 1935 An improved 'paraffin section' method for the dopa reaction. *Archs. Derm. Syph.* **31**, 190–195.

Bensley, R. R. & Gersh, I. 1933 Studies on cell structure by the freeze-drying method. 2. The nature of the mitochondria in the hepatic cell of *Amblystoma*. *Anat. Rec.* **57**, 217–237.

Bloch, B. 1917 Das Problem der Pigmentbildung in der Haut. *Arch. Derm. Syph.* **124**, 129–208.

Boolootian, R. A. & Campbell, J. L. 1964 A primitive heart in the echinoid *Strongylocentrotus purpuratus*. *Science* **145**, 173–175.

Boolootian, R. A. & Campbell, J. L. 1966 The axial gland complex. *Nature, Lond.* **212**, 946–957.

Bulmer, D. 1959 Dimedone as an aldehyde blocking reagent to facilitate the histochemical demonstration of glycogen. *Stain Tech.* **34**, 95–98.

Burton, M. P. M. 1964 Haemal system of regular echinoids. *Nature, Lond.* **204**, 1218.

Casselman, W. G. B. 1959 *Histochemical technique*. London: Methuen.

Cuénot, L. 1891*a* Etudes sur le sang et les glandes lymphatiques dans la serie animale. 2. Invertébrés. *Arch. Zool.* (*Ser.* 2) **9**, 365–670.

Cuénot, L. 1891*b* Etudes morphologiques sur les échinodermes. *Arch. Biol.* **11**, 313–680.

Danielli J. F. 1946 A critical study of techniques for determining the cytological position of alkaline phosphatase. *J. Exp. Biol.* **22**, 110–117.

Donnellon, J. A. 1938 An experimental study of clot formation in the perivisceral fluid of *Arbacia*. *Physiol. Zool.* **11**, 389–397.

Fedotov, D. M. 1924 Zur Morphologie des axialen Organkomplexes der Echinodermen. *Z. wiss. Zool.* **123**, 209–304.

Fox, H. M. & Vevers, G. 1960 *The nature of animal colours*. London: Sidgwick and Jackson.

Glenner, G. G. & Lillie, R. D. 1957 The histochemical demonstration of indole derivatives by the post-coupled *p*-dimethylaminobenzylidene reaction. *J. Histochem. Cytochem.* **5**, 279–296.

Glenner, G. G. & Lillie, R. D. 1959 Observations on the diazotization-coupling reaction for the histochemical demonstration of tyrosine: metal chelation and formazan variants. *J. Histochem. Cytochem.* **7**, 416–422.

Gomori, G. 1952 *Microscopic histochemistry*. Chicago: University Press.

Griffiths, D. E., Morrison, J. F. & Ennor, A. H. 1957 The distribution of guanidines, phosphagens and *n*-imidino phosphokinases in echinoids. *Biochem. J.* **65**, 612–617.

Gurr, E. 1958 *Methods of analytical histology and histochemistry*. London: Leonard Hill (Books).

Hamann, O. 1887 Beiträge zur Histologie der Echinodermen. *Jena. Z. Naturwiss.* **21**, 87–262.

Hofmann, C. K. 1871 Zur Anatomie der Echinen und Spatangen. *Niederl. Arch. Zool.* **1**, 11–112.

Hueck, W. 1912 Pigmentstudien. *Beitr. path. Anat. allg. Path.* **54**, 68–232.

Jacobson, F. W. & Millott, N. 1953 Phenolases and melanogenesis in the coelomic fluid of the echinoid *Diadema antillarum* Philippi. *Proc. Roy. Soc.* B **141**, 231–247.

Koehler, R. 1883 Recherches sur les échinides des cotes de Provence. *Ann. Mus. Hist. nat. Marseille* **1**, (*Mem.* 3), 1–167.

Kugler, J. H. & Wilkinson, W. J. C. 1961 The basis for the histochemical detection of glycogen. *J. Histochem. Cytochem.* **9**, 498–503.

Lea, A. J. 1949 Significance of Ehrlich's reaction in cases of melanuria. *Arch. Path.* **47**, 211–214.

Leipoldt, F. 1893 Das angebliche Exkretionsorgan der Seeigel, untersucht an *Sphaerechinus granularis* und *Dorocidaris papillata*. *Z. wiss. Zool.* **55**, 585–625.

Lillie, R. D. 1956 A Nile blue staining technic for the differentiation of melanin and lipofuscins. *Stain. Tech.* **31**, 151–153.

Lillie, R. D. 1957 Ferrous ion uptake. A specific reaction of the melanins. *A.M.A. Arch. Path.* **64**, 100–103.

Lison, L. 1953 *Histochimie et cytochimie animales*. Paris: Gauthier-Villars.

McClendon, J. F. 1912 Echinochrome, a red substance in sea urchins. *J. Biol. Chem.* **11**, 434–441.

McManus, J. F. A. 1946 The demonstration of certain fatty substances in paraffin sections. *J. Path. Bact.* **58**, 93–95.

McManus, J. F. A. & Mowry, R. W. 1958 Effects of fixation on carbohydrate histochemistry. *J. Histochem. Cytochem.* **6**, 309–316.

McMunn, C. A. 1885 On the chromatology of the blood of some invertebrates. *Q. Jl microsc. Sci.* **25**, 469–490.

Mason, H. S. 1956 Structures and functions of the phenolase complex. *Nature, Lond.* **177**, 79–81.

Messina, L. 1957 Prime osservazioni sulla natura chimica del liquido periviscerale di *Arbacia lixula*. *Pubbl. Staz. Zool. Napoli* **30**, 127–131.

Millott, N. 1957 Naphthaquinone pigment in the tropical sea urchin *Diadema antillarum* Philippi. *Proc. Zool. Soc. Lond.* **129**, 263–72.

Millott, N. 1966 A possible function for the axial organ of echinoids. *Nature, Lond.* **209**, 549–596.

Millott, N. & Vevers, H. G. 1964 Axial organ and fluid circulation in echinoids. *Nature, Lond.* **204**, 1216–1217.

Mowry, R. W. 1958 Improved procedure for the staining of acidic polysaccharides by Muller's colloidal (hydrous) ferric oxide and its combination with the Feulgen and the periodic acid-Schiff reactions. *Lab. Invest.* **7**, 566–576.

Mowry, R. W. 1963 The special value of methods that color both acidic and vicinal hydroxyl groups in the histochemical study of mucins with revised directions for the colloidal iron stain, the use of alcian blue G8X and their combination with the periodic acid-Schiff reaction. *Ann. N.Y. Acad. Sci.* **106**, 402–423.

Nicolaus, R. A. 1962 Biogenesis of melanins. *Rassegna di medicina sperimentale.* Anno IX, Suppl. N. 1. 1–32.

Pearse, A. G. E. 1960 *Histochemistry: applied and theoretical.* London: Churchill.

Perrier, E. 1875 Recherches sur l'appareil circulatoire des oursins. *Arch. Zool. exp.* (1) **4**, 605–643.

Piatelli, M., Fattorusso, E., Magno, S., & Nicolaus, R. A. 1963 The structure of melanins and melanogenesis. III. The structure of sepiomelanin. *Tetrahedron* **19**, 2061–2072.

Prouho, H. 1887 Recherches sur le *Dorocidaris papillata. Arch. Zool. exp. gen.* (2) **5**, 213–380.

Raper, H. S. 1926 The tyrosinase-tyrosine reaction. V. Production of 1-3,4-dihydroxyphenylalanine from tyrosine. *Biochem. J.* **20**, 735–742.

Rappay, G. 1963 Blocking the ninhydrin-Schiff reaction with copper ions. *Nature, Lond.* **200**, 274.

Schinke, H. 1950 Bildung und Ersatz der Zellelement der Leibeshohlenflussigkeit von *Psammechinus miliaris* (Echinoidea). *Z. Zellforsch. mikr. Anat.* **35**, 311–331.

Seiji, M. & Fitzpatrick, T. B. 1958 cited from Fitzpatrick, T. B. & Kukita, A. 1959 Tyrosinase activity in vertebrate melanocytes. *Pigment cell biology*, ed. M. Gorden. New York: Academic Press.

Seiji, M., Fitzpatrick, T. B. & Birbeck, M. S. C. 1961 The melanosome: a distinctive subcellular particle of mammalian melanocytes and the site of melanogenesis. *J. Invest. Derm*, **36**, 243–252.

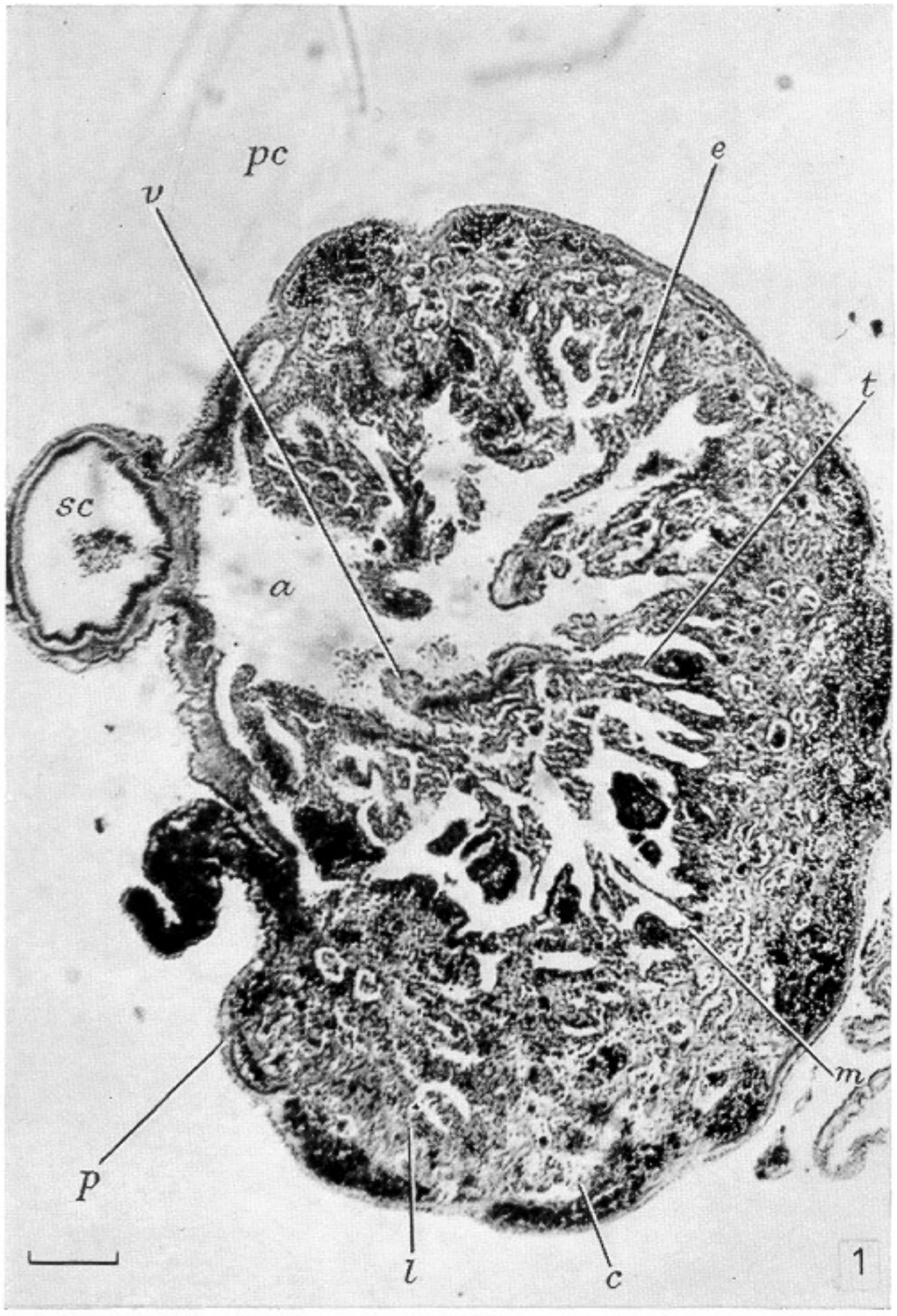
Thomson, R. H. 1962 *Comparative biochemistry* **3** (Ed. Florkin, M. and Mason, H. S.). New York and London: Academic Press.

Vevers, G. 1963 Pigmentation in the echinoderms. *Proc. XVI Int. Congr. Zool. Washington D.C.* **3**, 120–123.

Weiss, L. P., Tsou, K.-C. & Seligman, A. N. 1954 Histochemical demonstration of protein-bound amino groups. *J. Histochem. Cytochem.* **2**, 29–49.

Yasuma, A. & Ichikawa, T. 1953 Ninhydrin-Schiff and alloxan-Schiff staining: a new histochemical staining method for protein. *J. lab. clin. Med.* **41**, 296–299.

Zimmerman, L. E. & Eastham, A. B. 1959 Acid mucopolysaccharide in the retinal pigment epithelium and visual cell layer of the developing mouse eye. *Amer. J. Ophthalm.* **47**, 488–499.



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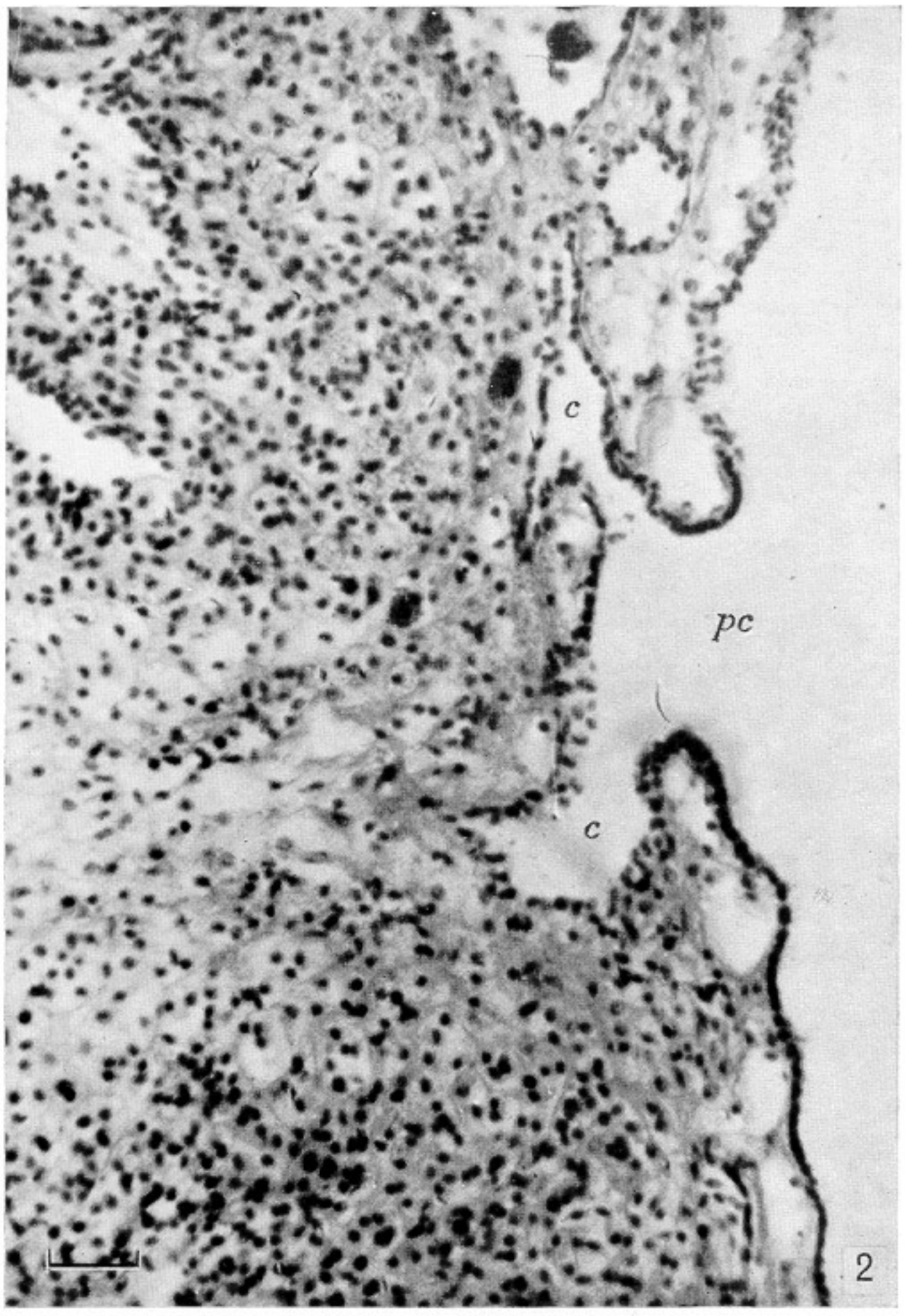

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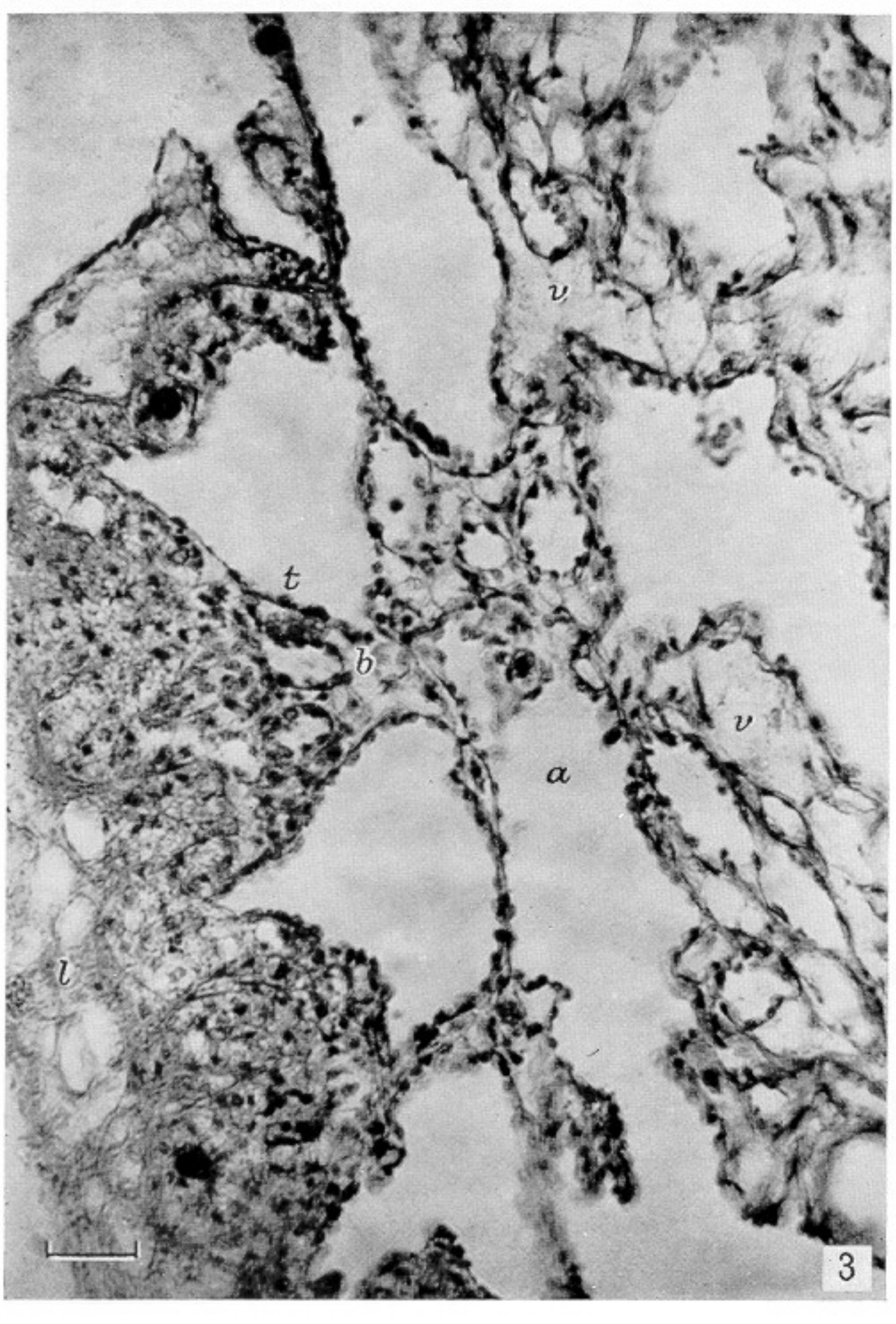

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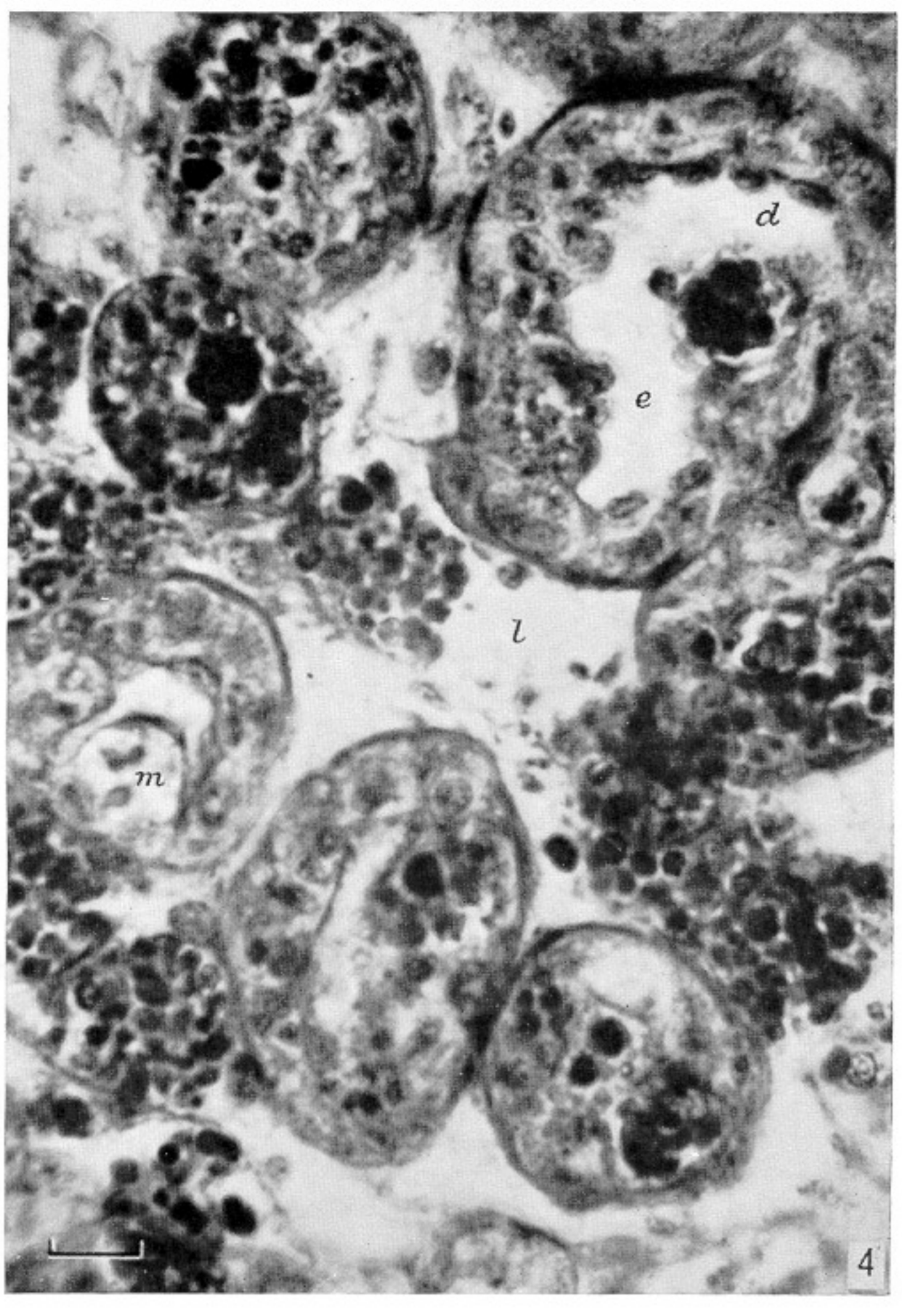

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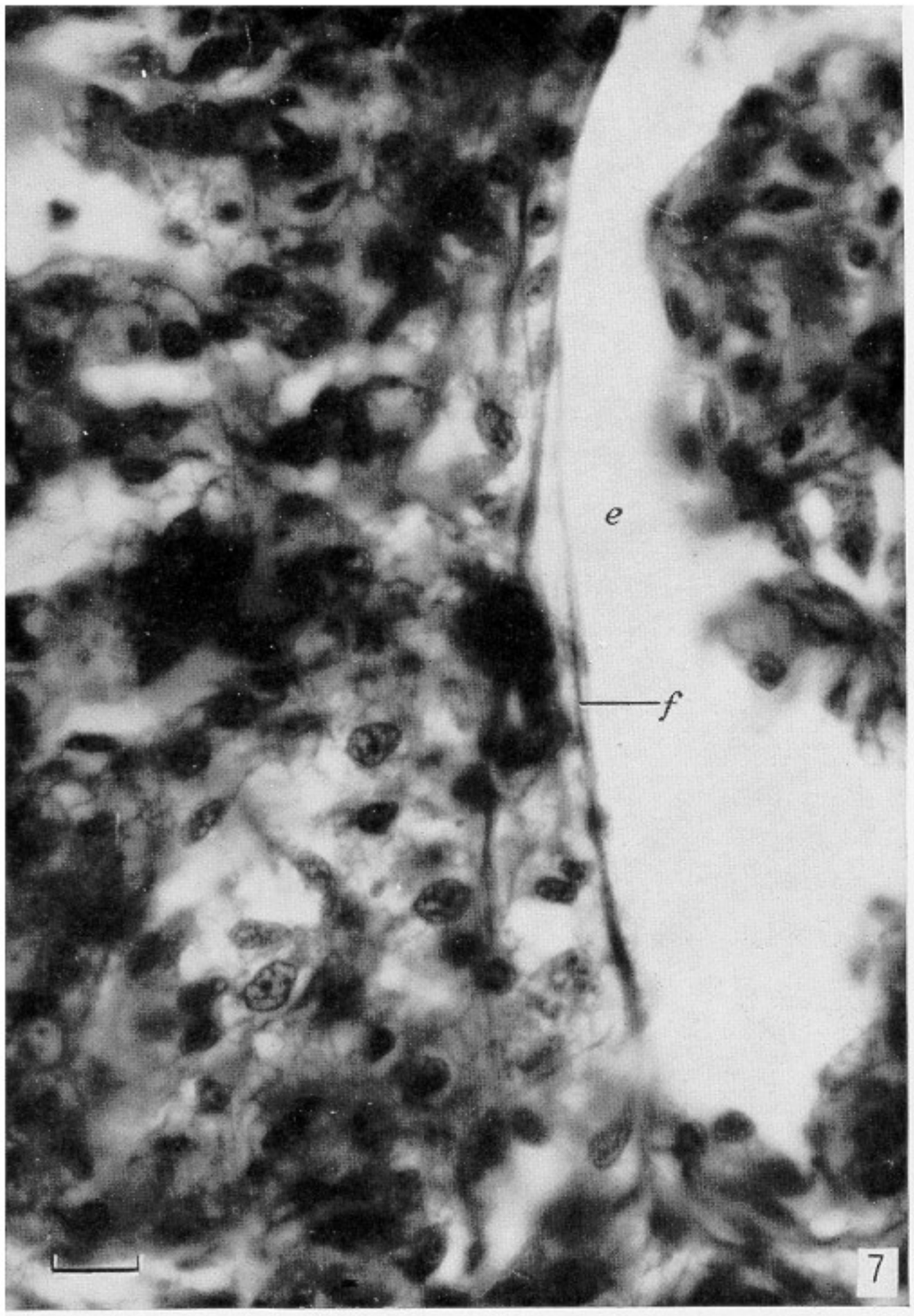

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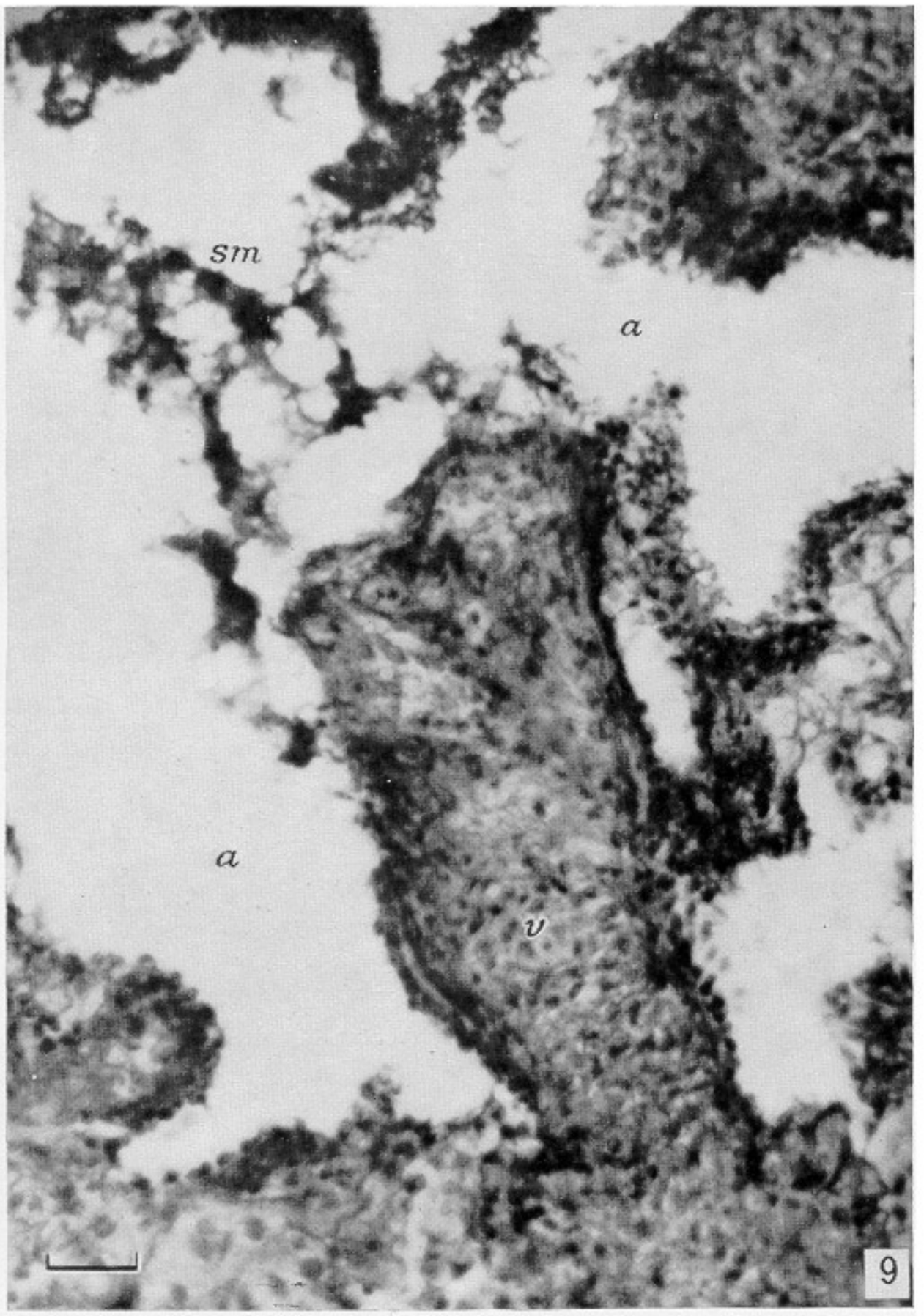

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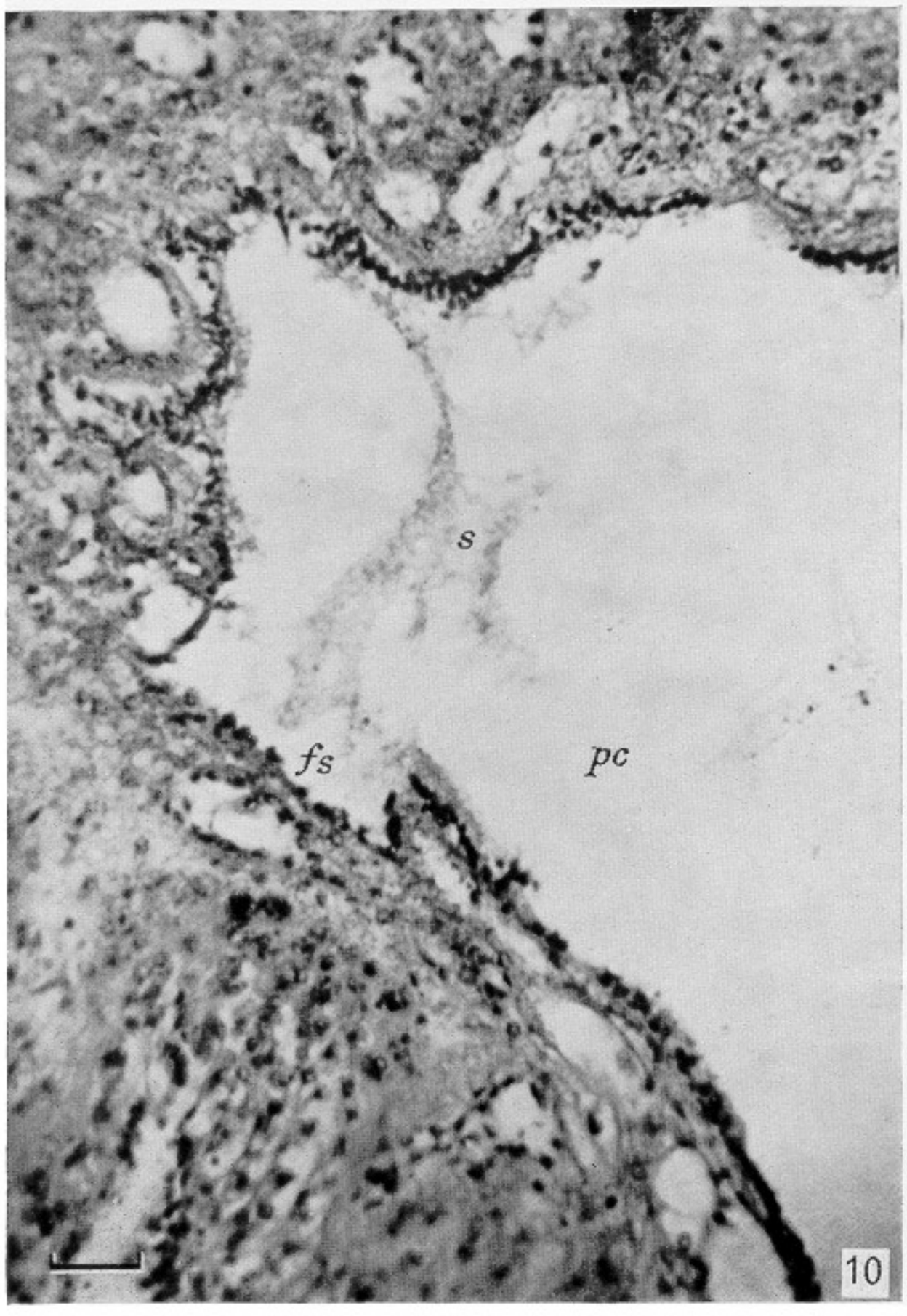

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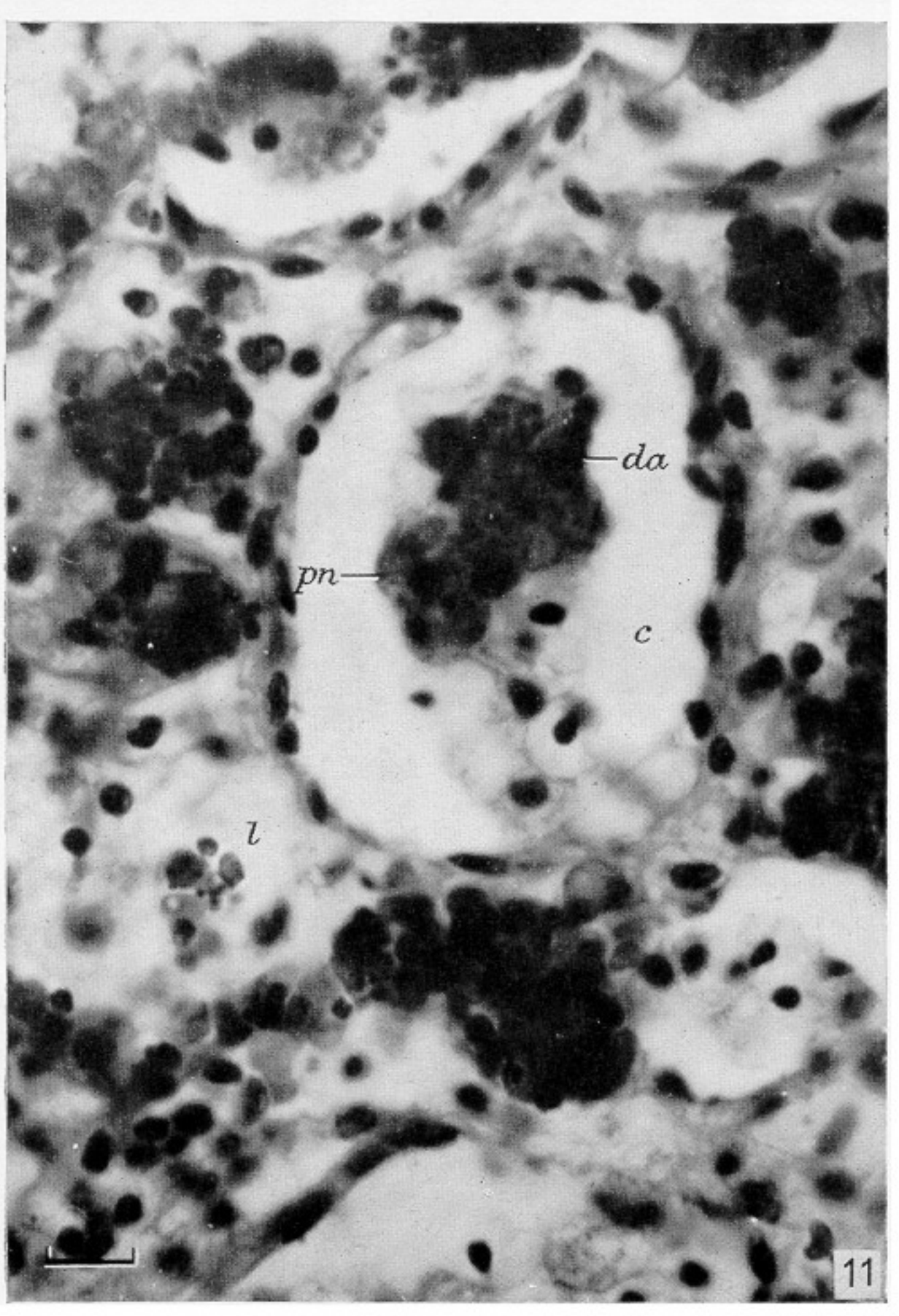

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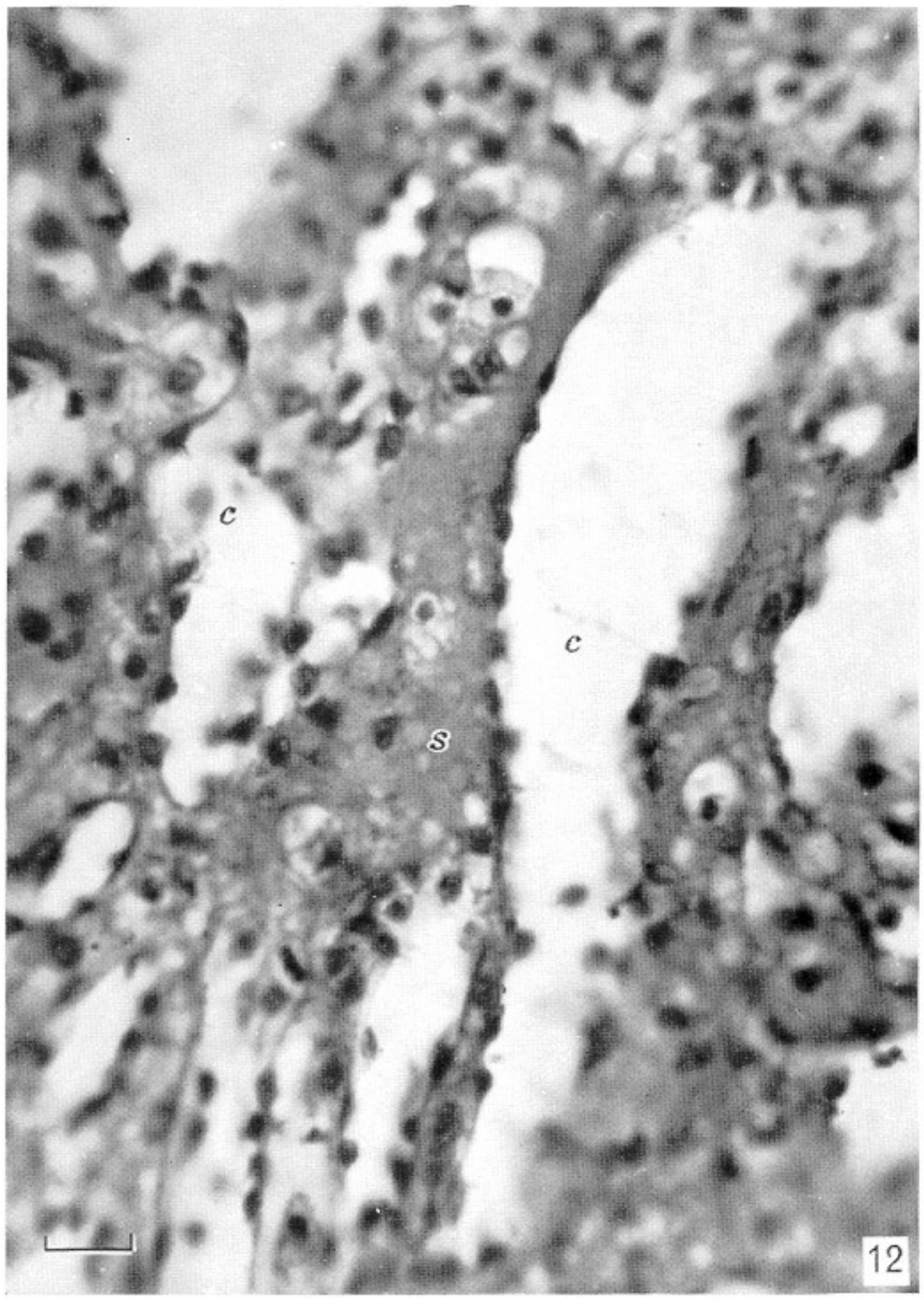

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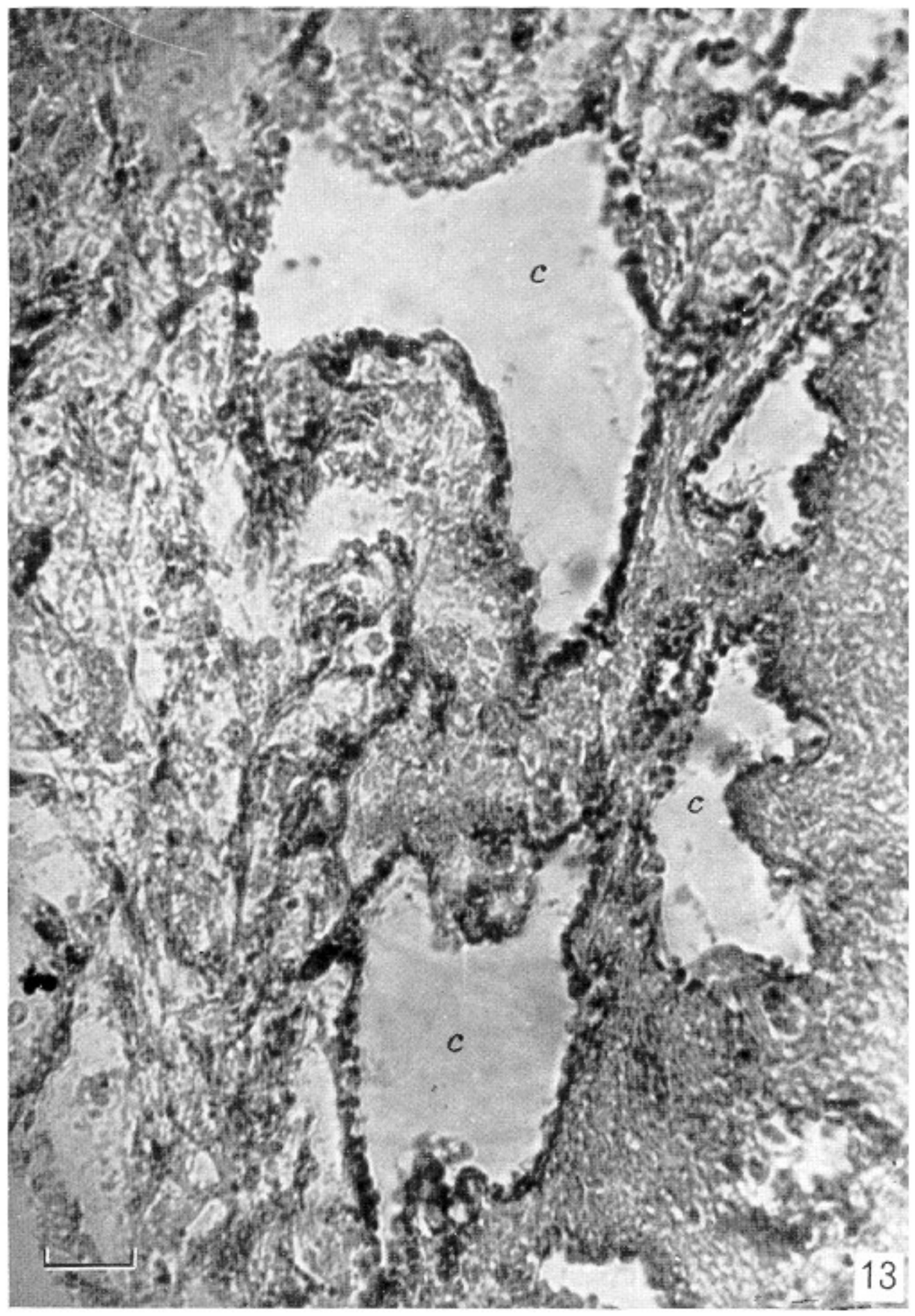

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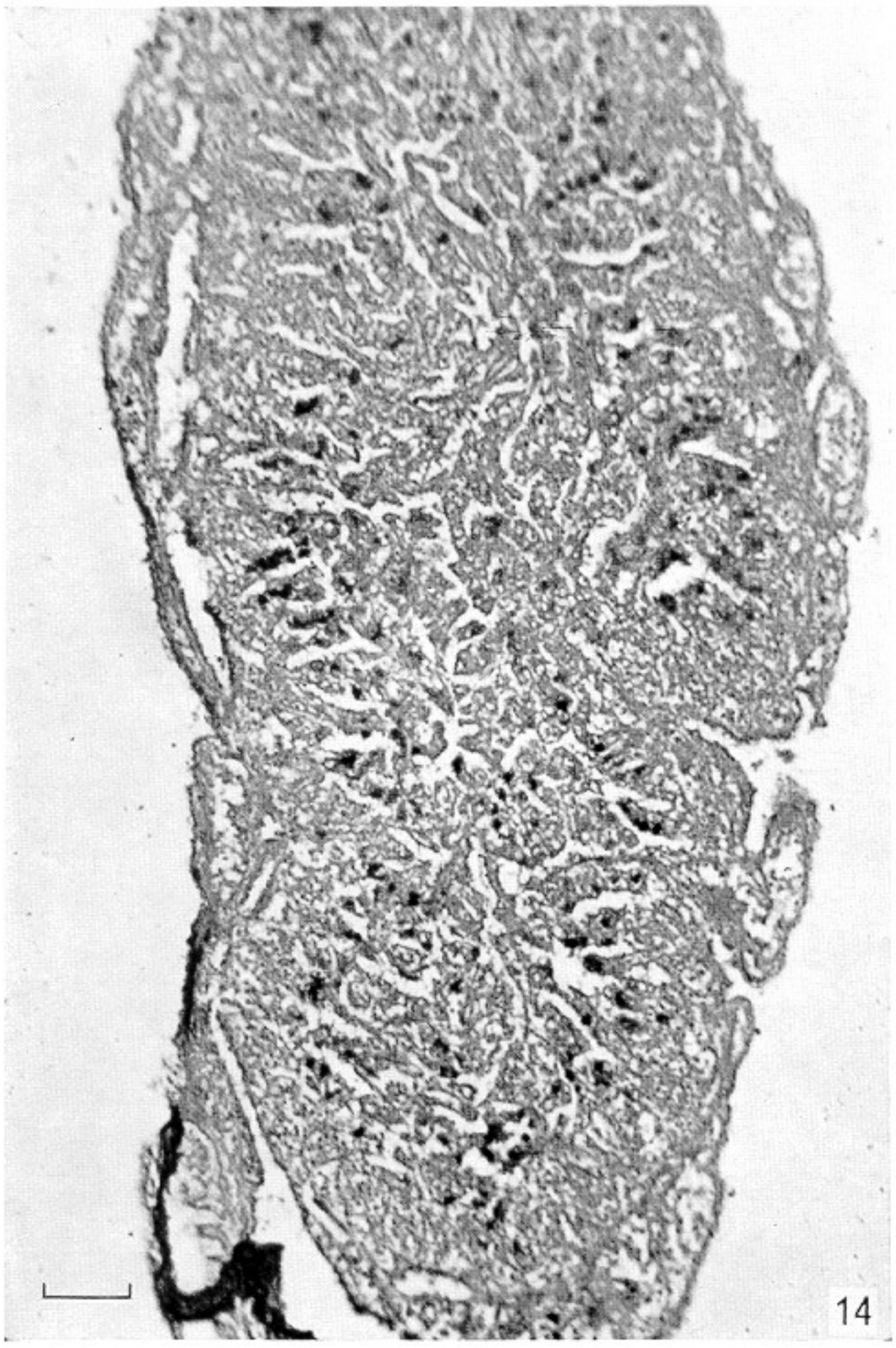

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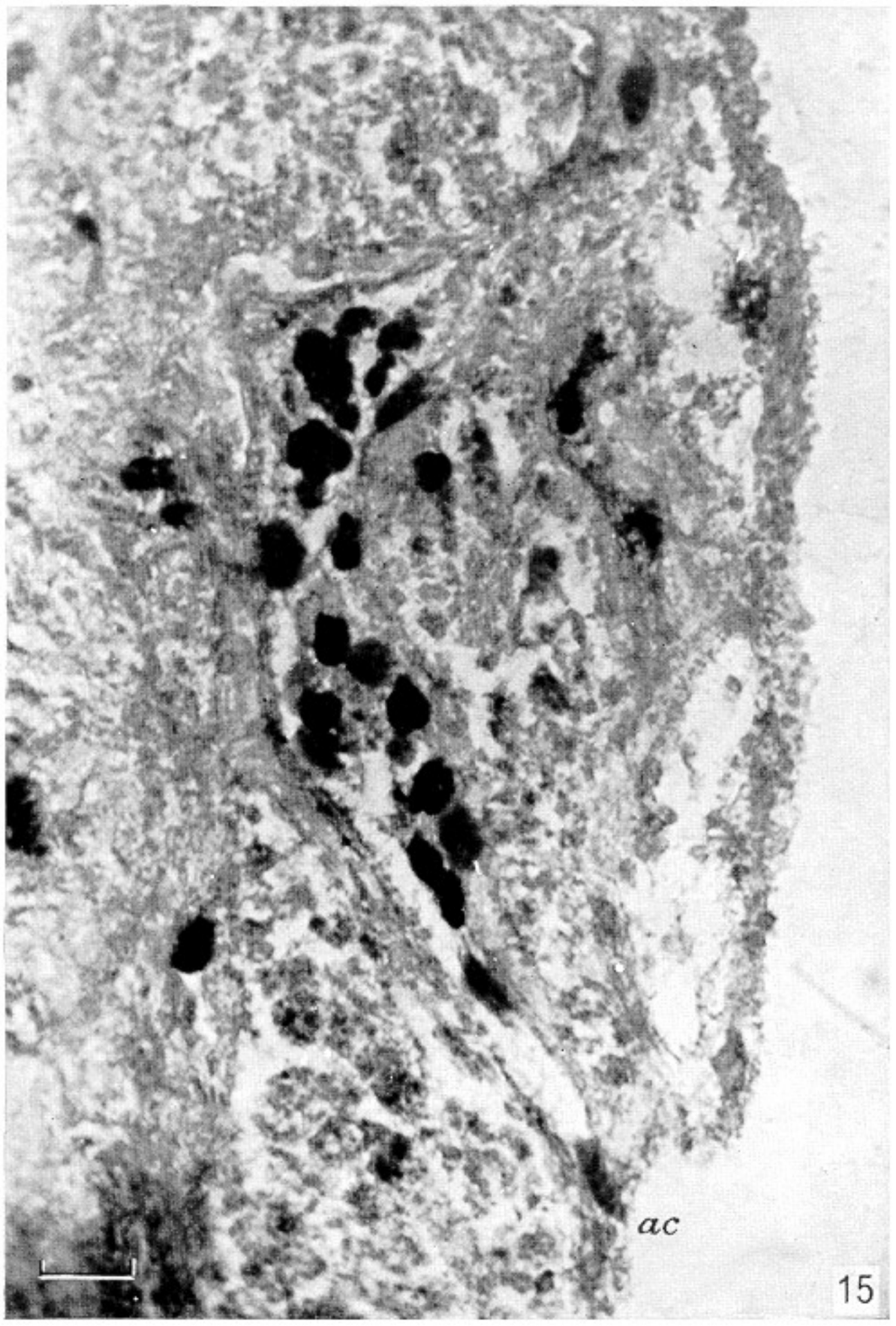

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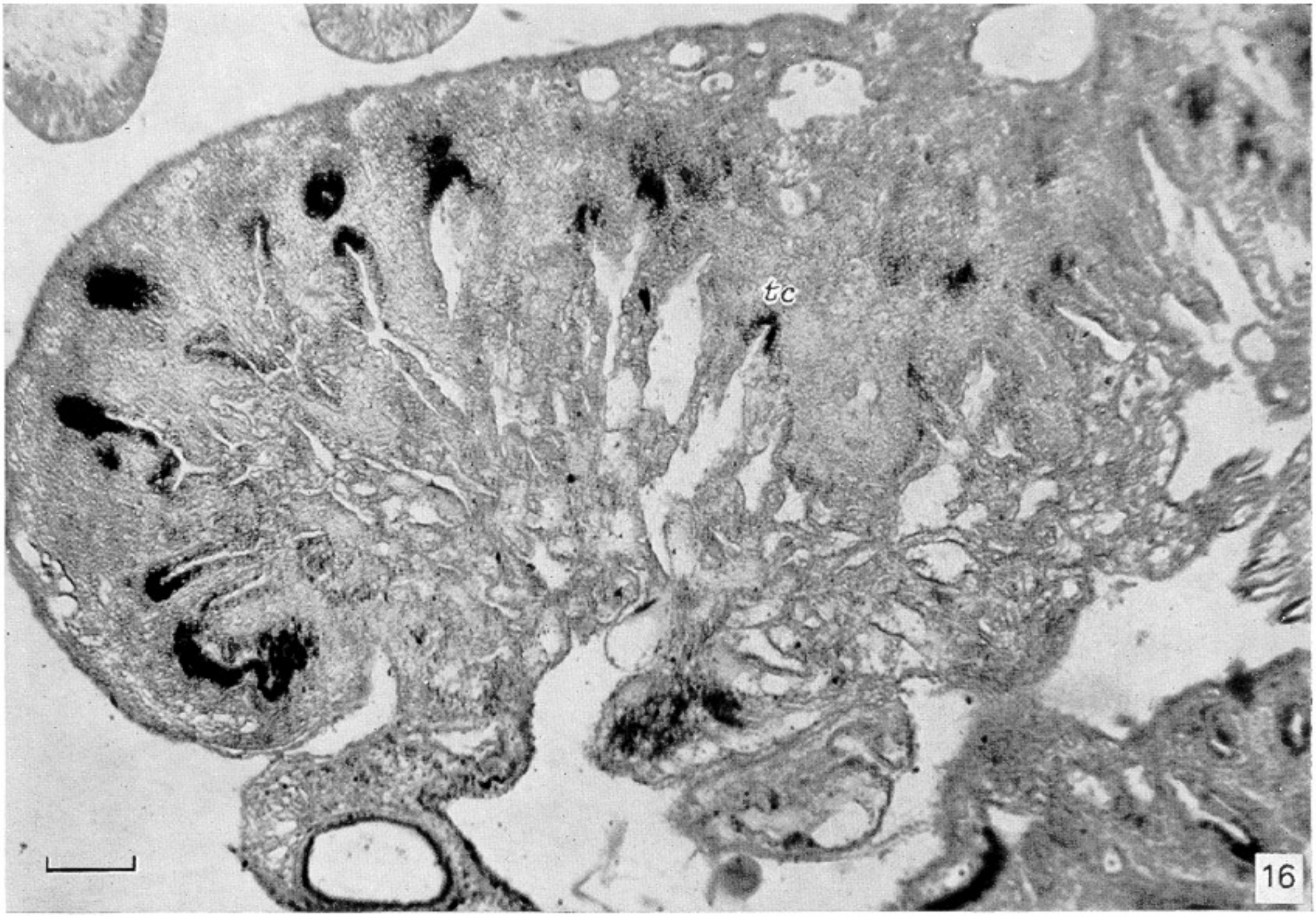

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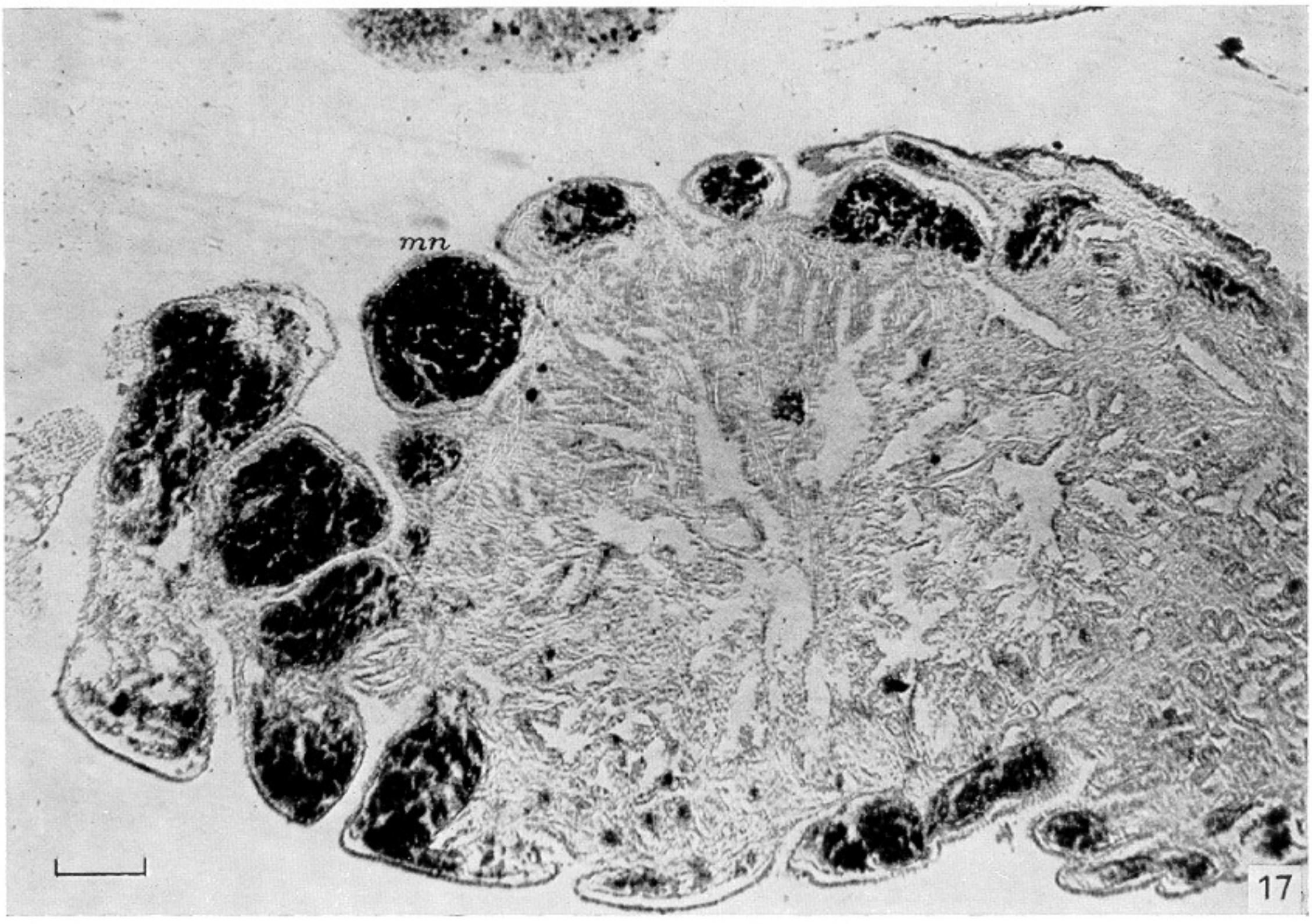

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