THE FINE STRUCTURE OF THE DIGESTIVE TUBULES OF THE MARINE BIVALVE *CARDIUM EDULE*

By G. OWEN
Department of Zoology, Queen's University, Belfast

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[Plates 39 to 50]

CONTENTS

The epithelium lining the digestive tubules of *Cardium edule* consists of three cell types, namely mature digestive cells, mature secretory cells and immature flagellated cells. Both the secretory and flagellated cells exhibit a pronounced basiphilia and occur in well-defined crypts. The secretory cells are pyramidal in shape and characterized by the possession of a well-developed granular endoplasmic reticulum and Golgi apparatus. Golgi vesicles derived from the latter migrate to the apical region of the cell where they release their contents into the lumen of the tubules. It is possible that the secretion contains enzymes and although it is likely that such enzymes would function primarily in the lumen of the tubules they may also be the source of the weak proteolytic activity which has been recorded in the gastric fluid of many bivalves. The immature flagellated cells are columnar in shape and possess a poorly developed endoplasmic reticulum and numerous free ribosomes. Although no evidence for this was obtained it is suggested that they may serve to replace either or both of the mature cell types. The digestive cells vary from cuboidal to columnar, possess distinctive Golgi elements with characteristic intracisternal membranous elements, and are capable of ingesting exogenous material from the lumen of the tubule. The process of ingestion was examined following feeding experiments with (*a*) a mixture of iron oxide and colloidal graphite (Aquadag), (*b*) whole blood from pigeon and (*c*) ferritin. Individual particles of graphite were enclosed in phagosomes by a process of phagocytosis, while the proteins haemoglobin and ferritin were ingested by a process of pinocytosis; the membrane enclosing the pinocytic vesicles possesses a characteristic outer granular coat. The contents of both the phagocytic and pinocytic vesicles were transferred to larger bodies considered to be primarily phagosomes in the sub-apical regions of the cell. These possess an interconnecting system of membrane-bound channels which ramifies through the apical cytoplasm. Phagolysosomes deeper in the cytoplasm of the cell were identified by the presence of exogenous material and a positive reaction to tests for acid phosphatase activity. They showed changes in appearance which could be put into a series suggestive of the progressive intracellular digestion of the ingested material.

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Introduction

While most workers are agreed that the digestive diverticula of the majority of bivalves are concerned primarily with intracellular digestion, there have been a number of reports that in at least some species these organs may also serve a secretory function (Mansour 1946; Mansour & Zaki 1946; Owen 1956; Morton 1956; Reid 1965). There is some evidence that the solution to this problem may be linked with a second problem, namely the number of cell types present in the epithelium lining the blind ending tubules of the diverticula. Following the earlier work of Yonge (1926) most workers believe that the epithelium lining the tubules of the majority of bivalves consists of only one cell type—a digestive cell—which is continuously replaced by darkly staining, undifferentiated, pyramid-shaped cells present in the crypts of the tubules. Some doubt was cast on this view when it was reported that the darkly staining cells of many, if not all, bivalves are flagellated and appear to undergo a secretory cycle (Owen 1955, 1956). Certainly, similar cells present in the diverticula of many gastropods have long been thought to have a secretory (excretory) function.

Recently, Sumner (1966*a*), following a histochemical investigation of the digestive diverticula of a number of species of freshwater bivalves, concluded that the digestive cells and the darkly staining cells, which he aptly described as basiphil cells, were distinct cell types and that the latter were probably secretory in function. In what appears to be the only published account of an electron-microscope study of the digestive tubules of bivalves, Sumner (1966*b*) later confirmed his conclusions on the existence of at least two cell types. The animal studied was the freshwater bivalve *Anodonta anatina*. This present study extends Sumner's observations on the fine structure of the digestive tubules of bivalves with an electron-microscope study of these organs in the marine bivalve *Cardium edule*. A preliminary investigation of the ingestion of material by the digestive cells and of the distribution of acid phosphatase within these cells has also been undertaken. The results are discussed in the light of recent observations on the uptake of material by cells and the phago-lysosome concept of intra-cellular digestion.

Materials and methods

Specimens of *Cardium edule* were collected from Strangford Lough and kept in aerated sea water in the laboratory. For routine studies on the structure of the digestive tubules portions of the digestive diverticula were removed and fixed within 24 h of the animals being taken from the beach.

In preparation for light microscopy, portions of the digestive diverticula were fixed either in Bouin's fluid or in a modified Bouin–Dubosq fixative (Atkins 1937). Following dehydration, they were embedded in ester wax and sections 5 to 8 μm thick were routinely stained with Mayer's haemalum, Alcian blue and orange G in clove oil.

For electron-microscopic examination, portions of the digestive diverticula were fixed for 2 h in 2 % glutaraldehyde made up in 3 % NaCl and buffered at pH 7.2. The fixed material was washed in 0.2 M sucrose (pH 7.2) and post-fixed in 1 % osmic acid (pH 7.2) for 1 h. Following dehydration the tissue was embedded in Araldite resin and sections cut on an LKB Ultrotome were stained in alcoholic uranyl acetate (5 min) and lead citrate (5 min). The sections were examined in either an Akashi TRS 50 or an AEI EM 6B microscope.

Feeding experiments

Feeding experiments were carried out to enable preliminary observations to be made on the mode of ingestion of exogenous material by the cells lining the digestive tubules. Animals were provided with finely particulate material which they would filter from the sea water and which could be readily identified in electron micrographs. No attempt was made to estimate the suspensions quantitatively nor was it possible under the conditions of the experiments to determine accurately the time and duration of feeding. Materials used were (*a*) a mixture of colloidal graphite (Aquadag) and iron oxide, (*b*) a suspension of whole pigeon's blood and (*c*) Cd^{2+}-free ferritin. The animals were kept in aerated sea water for 24 to 48 h after removal from the beach. Suspensions of the above materials were then added to the sea water and left for 2 h, at the end of which time the animals were transferred to clean sea water. Individual animals were removed and portions of the digestive diverticula excised and fixed at regular intervals during the course of the experiments.

Acid phosphatase

Frozen sections 50 μm thick were cut from portions of digestive diverticula previously fixed in glutaraldehyde and thoroughly washed in 0.2 M sucrose as previously outlined. The sections were incubated for 20 min at 20 °C in a Gomori medium and rinsed thoroughly in the sucrose solution before being post-fixed for 1 h in 1 % osmic acid, dehydrated and embedded in Araldite resin. Controls were run by omitting the substrate from, or by adding 10^{-2} M NaF to, the incubation medium.

Observations

As in other bivalves, the digestive diverticula of *Cardium edule* consist of numerous blind-ending tubules which communicate with the stomach by way of partially ciliated main ducts and non-ciliated secondary ducts (Owen 1955). The tubules are more or less circular in cross-section, surrounded by a sheath of collagen fibres, and external to this a system of smooth-muscle fibres forming a meshwork rather like a string bag. A similar arrangement of muscle fibres round the tubules has been found in all the species of bivalves examined. Those associated with the tubules of *C. edule* are shown in figure 7, plate 41, the circularly arranged fibres (*cf*) lying within the longitudinally orientated fibres (*lf*) and both running external to the skeletal collagenous sheath (*cs*). On occasions the basal regions of the cells lining the tubules are markedly indented by the underlying muscle fibres, suggesting that the latter are contracted.

Figure 1 is a semi-diagrammatic representation of a T.S. through a digestive tubule of *C. edule* as seen under the light microscope. The larger digestive cells (*dc*) lining the tubules are characterized by the presence distally of irregularly shaped and apparently empty vesicles while the basal regions are occupied by smaller spherical inclusions which colour faintly with Alcian blue. Basiphil cells (*bc*) occur in three or four well-defined groups occupying crypts which extend the length of the tubule. Apart from flagella (*f*), which undoubtedly arise from cells situated in the crypts, little detail of the structure of these cells can be made out under the light microscope.

The basiphil cells

In view of Sumner's (1966*a*) optical microscopic observations on the digestive diverticula of *Sphaerium*, an important question is whether the crypts contain more than one type of basiphil cell. Examination of large numbers of electron micrographs suggests that the crypts of the

tubules of *C. edule* contain two types of basiphil cell only one of which is flagellated. Figure 2, plate 39, is a low-power survey electron micrograph of the crypt of a tubule of *C. edule*. Most of the cells (*MBC*) possess structural features similar to those which characterize the now well known acinar cell of the mammalian exocrine pancreas. They are more or less pyramidal in shape (figure 5*A*) with the broad base resting on a basement membrane and the tapering apical region, which bears microvilli (*m*) some 20 μm long and 1.5 μm in diameter, bordering the lumen of the tubules. The basal plasma membrane shows little infolding and this is also true of the lateral plasma membranes which distally are linked with those of adjoining cells by septate desmosomes (*sd*, figure 4 (plate 40), figure 5). Adjacent plasma membranes are on average 12.5 nm apart while the septa of the desmosomes are repeated at intervals of 12.5 nm; well-developed terminal bars (*tb*) occur distal to the septate desmosomes.

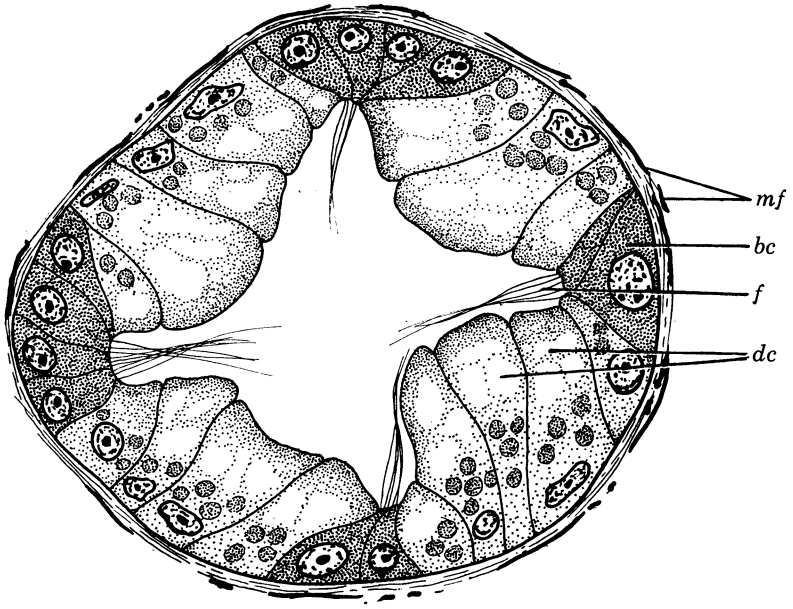
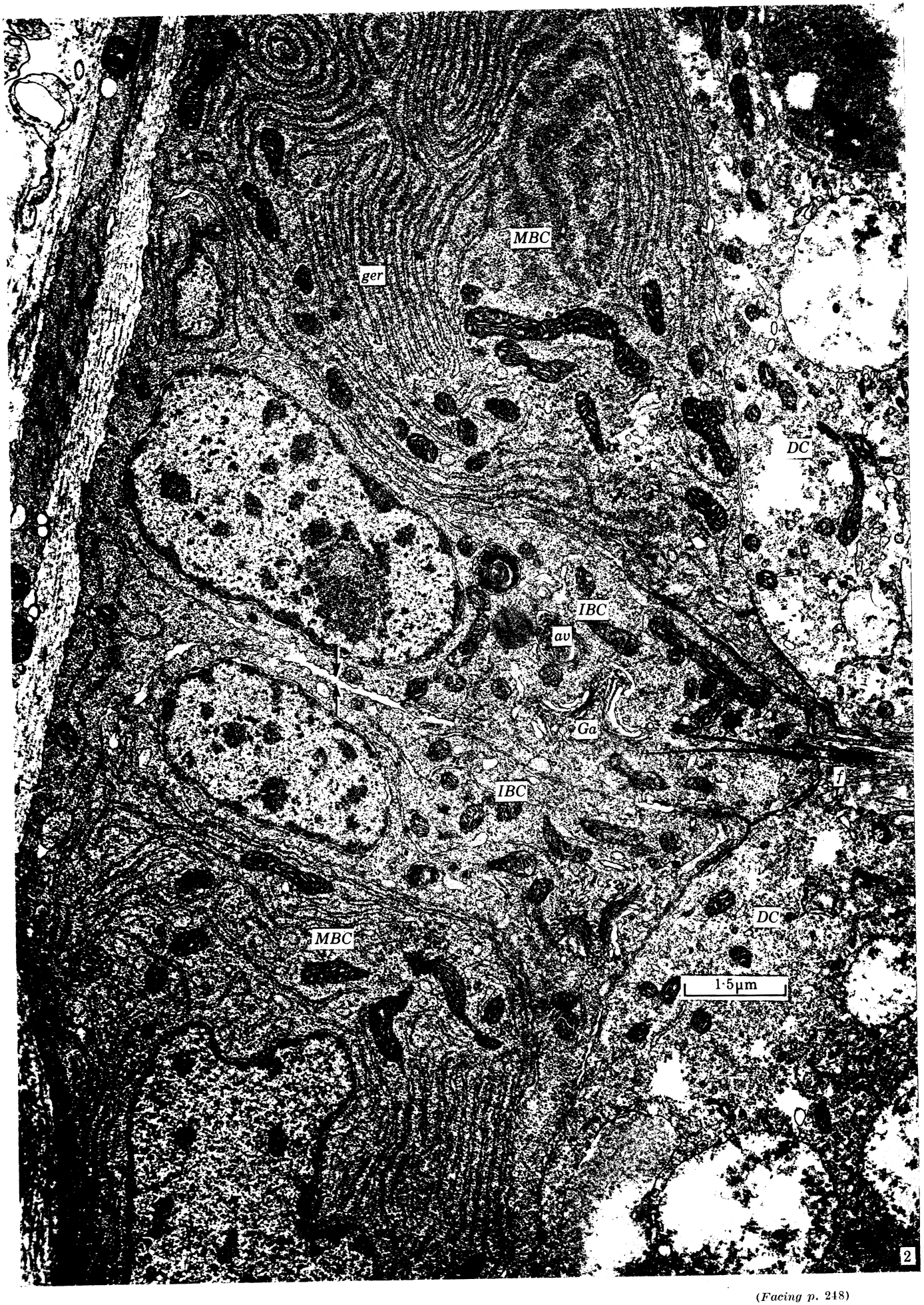


FIGURE 1. Semi-diagrammatic drawing of a transverse section through a digestive tubule of *C. edule* as seen under the light microscope. Basiphil cells (*bc*), apparently all of the same type, are clustered in well-defined crypts with the larger digestive cells (*dc*) between.

As in the acinar cell of the mammalian pancreas, much of the cytoplasm of the cell is filled with flattened cisternae of granular endoplasmic reticulum (figure 2, plate 39; figure 5; *ger*) which are usually arranged in parallel arrays in the basal, lateral and circumnuclear regions of the cell. Elsewhere, the granular reticulum, at least in section, presents a more fragmented appearance and the enclosed cisternae confer a vacuolated appearance on these regions of cytoplasm. The nuclei (figure 5*A*, *n*) tend to be somewhat elongated along the axis parallel

DESCRIPTION OF PLATE 39

FIGURE 2. Low-power survey electron micrograph of a section through a crypt of a digestive tubule showing the two types of basiphil cells, namely non-flagellated, granular endoplasmic reticulum cells (*MBC*), and immature flagellated cells (*IBC*). On either side of the crypt can be seen portions of digestive cells (*DC*). The arrows indicate the wider intercellular gap which frequently occurs between the lateral plasma membrane of the flagellated cells and adjoining cells.



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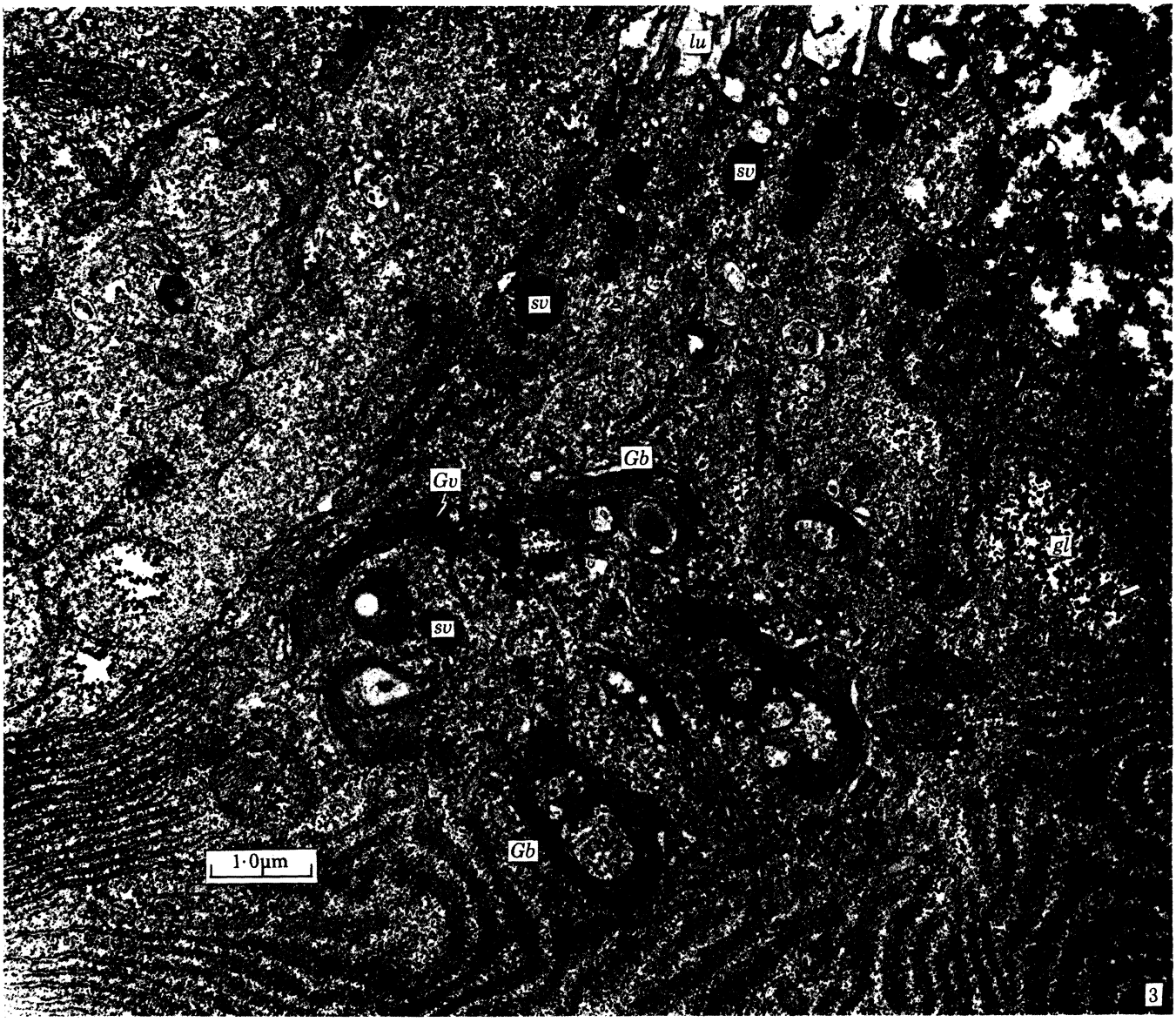
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3


0·4μm
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4

with the base of the cell and the continuity of the granular reticulum with the outer membrane of the nuclear envelope, which is similarly studded with ribosomes, is frequently evident; numerous pores penetrate the nuclear envelope.

The Golgi apparatus consists of a number of Golgi bodies situated in the supranuclear region of the cell and arranged more or less concentrically to enclose an extensive cup-shaped Golgi zone (figure 5*A*, *Ga*). Each Golgi body (figure 3, plate 40; *Gb*) consists typically of a stack of curved, saucer-shaped saccules, the inner members of which tend to show an increasing distension of their peripheral margins to form Golgi vesicles (*Gv*). The contents of both the saccules and the peripheral vesicles are of moderate electron density. Adjacent to the Golgi bodies are invariably two other types of membrane-bound vesicles, namely microvesicles of

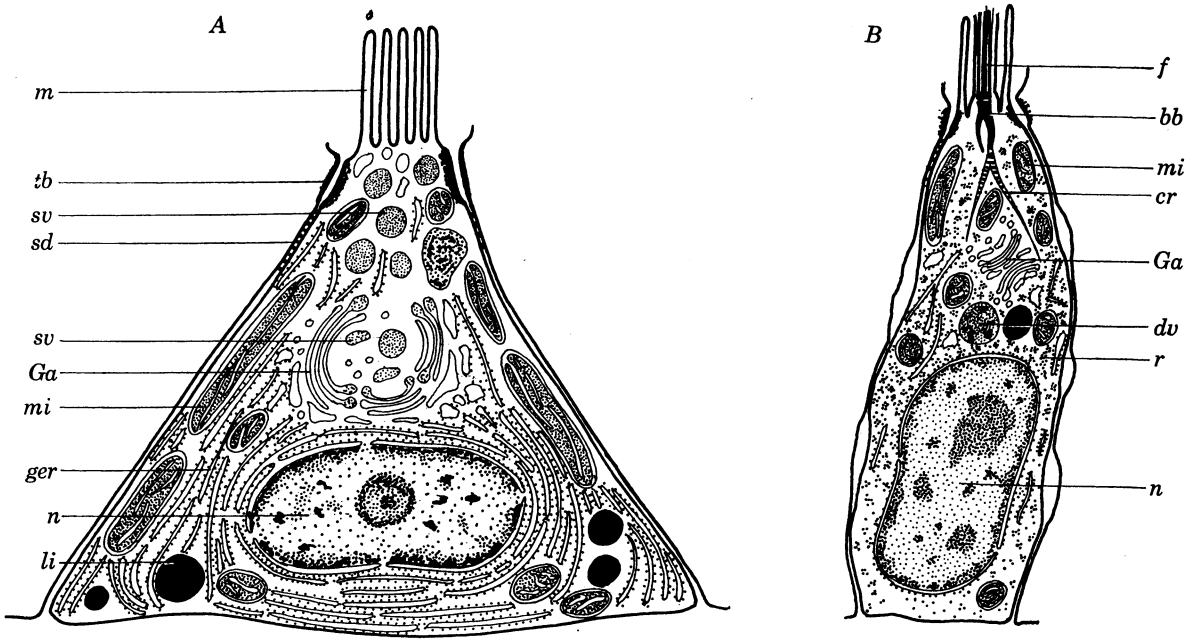


FIGURE 5. Stylized drawings of median sections through the two types of basiphil cells found in the crypts of the tubules: *A*, non-flagellated *ger* cell, and *B*, immature flagellated cell.

more or less constant diameter and without any coating of ribosomes, and larger, less numerous, and occasionally irregularly shaped, secretory vesicles (figure 3, plate 40; figure 5*A*; *sv*), the contents of which are of similar electron density to those of the Golgi vesicles. Similar secretory vesicles are also frequently, although not invariably, present in the distal regions of the cell (figures 3 and 4, plate 40; figure 5; *sv*) where their contents tend to be slightly more electron-dense than those in the vicinity of the Golgi apparatus. Also present in the apical regions of the cell are apparently empty membrane-bound vesicles of varying sizes (figures 3 and 4, plate 40).

DESCRIPTION OF PLATE 40

FIGURE 3. Electron micrograph of the supra-nuclear region of a *ger* cell showing the well-developed Golgi apparatus, consisting of a number of Golgi bodies with associated Golgi vesicles. Secretory vesicles occur both in the region of the Golgi apparatus and in the apical region of the cell adjoining the lumen.

FIGURE 4. Electron micrograph of the apical regions of two *ger* cells, showing secretory vesicles (*sv*), terminal bars (*tb*) and septate desmosomes (*sd*).

Mitochondria (figure 5*A*, *mi*) occur throughout the cell but tend to be more numerous in the peripheral regions. Some are considerably elongated and the cristae tend to run in all directions. Finally, there may be present, generally in the basal regions of the cell, an occasional lipid sphere (*li*) and in some specimens conspicuous clusters of glycogen granules (figure 3, plate 40, *gl*).

The differences between the flagellated cells and what might be termed the granular endoplasmic reticulum cells, i.e. *ger* cells, are most clearly shown in figure 2 (cf. *MBC* and *IBC*), one of the few electron micrographs obtained showing the full extent of both cell types from basement membrane to apical surface; the differences are also represented diagrammatically in figures 5*A*, *B*. The lateral margins of the apical regions of the flagellated cell taper markedly toward the lumen of the tubule but the general form of the cell is columnar rather than pyramidal. Correlated with this columnar form the nucleus tends to be elongated along the long axis of the cell. Perhaps the most striking difference, apart from the flagellum (figures 2 and 6, plates 39 and 41; *f*) which projects from the apical region of the cell into the lumen of the tubule is the general scarcity of endoplasmic reticulum, although free ribosomes (figure 5*B*, *r*) are numerous and scattered throughout the cytoplasm. The Golgi apparatus (figure 2; plate 39, figure 5*B*, *Ga*) is not as prominent as that of a *ger* cell and consists of only one or two stacks of saccules with their convex surfaces disposed back to back. Secretory vesicles of the type present in the *ger* cell do not occur either in the vicinity of the Golgi apparatus or in the apical regions of the cell (cf. *MBC* and *IBC* in figure 6). The mitochondria are essentially similar to those of the *ger* cell but tend to be evenly distributed throughout the supra-nuclear cytoplasm. Also present in this region, as in the *ger* cells, are one or more membrane-bound vesicles of varying dimensions and whose contents show considerable variation (figure 2, *av*). Positive results obtained with tests for acid phosphatase suggest they are autophagic vacuoles. Finally, there can frequently be observed over limited regions a marked increase in the intercellular distance between the lateral plasma membranes of the flagellated cells and those of adjoining cells (see arrows in figure 2).

The digestive cell

The digestive cells form a simple columnar epithelium lining the blind ending tubules and extend between the crypts containing the basiphil cells (figure 1). The basal and lateral plasma membranes show little folding and interdigitation and, as for the basiphil cells, well-developed terminal bars and septate desmosomes serve to link adjoining cells. The apical surface bears microvilli (figure 8; figure 9, plate 42; *m*) which project into the lumen (*lu*) of the tubule but their number and degree of development shows considerable variation depending apparently on the phase of activity present in the cell (cf. figures 9 and 17, plates 42 and 46). Scattered throughout the cytoplasm (figure 8; figure 9, plate 42) are mitochondria (*mi*), Golgi elements (*Ga*) showing a number of peculiar features, free ribosomes, elements of both smooth and granular endoplasmic reticulum (*ger*) and in many cells clusters of lipid spheres (*li*) usually located in the basal regions of the cell. The most characteristic feature of the digestive cell, however, is the presence of numerous membrane-bound vesicles (*p* 1–4) which fill the cell to such an extent that the basally situated nucleus (*n*) is frequently irregular in section due to the presence of adjacent vesicles. For descriptive purposes it is convenient to consider the vesicles in two main groups, the first comprising relatively large macrovesicles which are readily visible under the light microscope and the second smaller microvesicles scattered throughout

the cell. The macrovesicles range from 20 to 40 μm in diameter and are all bounded by a triple-layered membrane of similar appearance and dimensions to that of the apical plasma membrane. They exhibit considerable variation, and although it is probable, as discussed later, that the differences represent phases in a continuous sequence it is convenient to distinguish four main types.

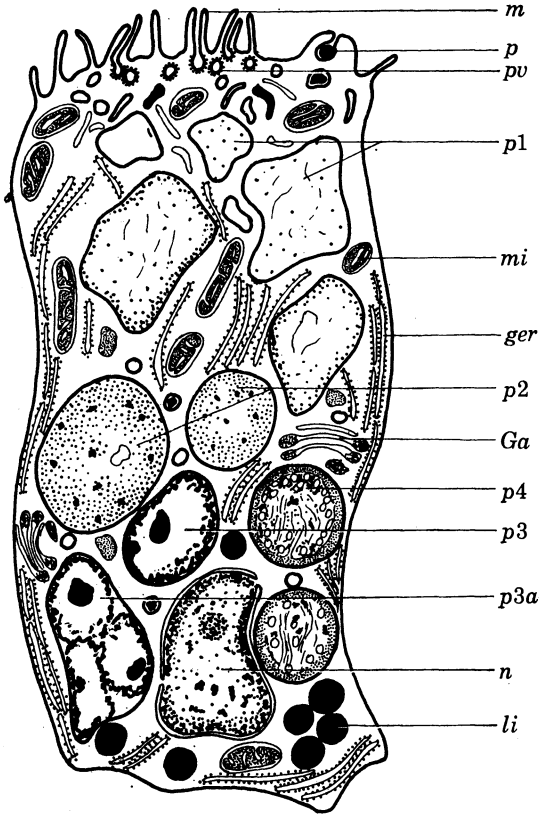


FIGURE 8. Stylized drawing of a median section through a digestive cell showing particularly the various types of macrovesicle (*p*1 to *p*4) typically present.

Type 1 (figure 8; figure 9, plate 42; *p*1)

This type of macrovesicle is found exclusively in the sub-apical regions of the cell and exhibits considerable variation both in shape and size. In contrast to the remaining types of macrovesicle, which are more or less spherical in form, it is frequently irregular in section and generally contains only small amounts of membrane-like and/or granular material and occasionally moderately electron-dense material distributed round the periphery. It is almost certainly this type of macrovesicle which is readily visible in light microscope preparations as irregularly shaped, apparently empty, vesicles in the distal regions of the cell (figure 1).

Type 2 (figure 8; figures 9 and 10, plates 42 and 43; *p*2)

This type of vesicle occurs mainly in the mid-regions of the cell and is characterized by a granular content of moderate electron density which is more or less uniformly distributed throughout the vesicle; embedded in this material are small highly electron-dense granules. The investing membrane rarely appears entire, a number of 'gaps' or 'breaks' being visible in section (figure 15, plate 45). Moreover, there is frequently present within this outer membrane,

and separated from it by some 20 to 40 nm, short lengths of apparently isolated membrane giving the vesicle wall in these regions a double or triple structure.

Type 3 (figure 8; figures 9 and 10, plates 42 and 43; *p*3)

The characteristic features of this type of macrovesicle are its apparently empty appearance compared with type 2 and fairly regular spherical form compared with type 1. The empty appearance results from the apparent concentration of the contents in more or less discrete electron-dense clumps distributed round the periphery. The bounding membrane again shows a number of 'gaps' in section and is separated from the peripherally distributed contents by a distinct electron light zone some 10 nm in width (figure 15, plate 45, *x*). A modified form of this vesicle is sometimes present in the basal regions of the cell (figure 17, plate 46, *p*3*a*). It comprises what appear to be a number of type 3 macrovesicles enclosed within a common investing membrane.

Type 4 (figure 8; figure 11, plate 43; *p*4)

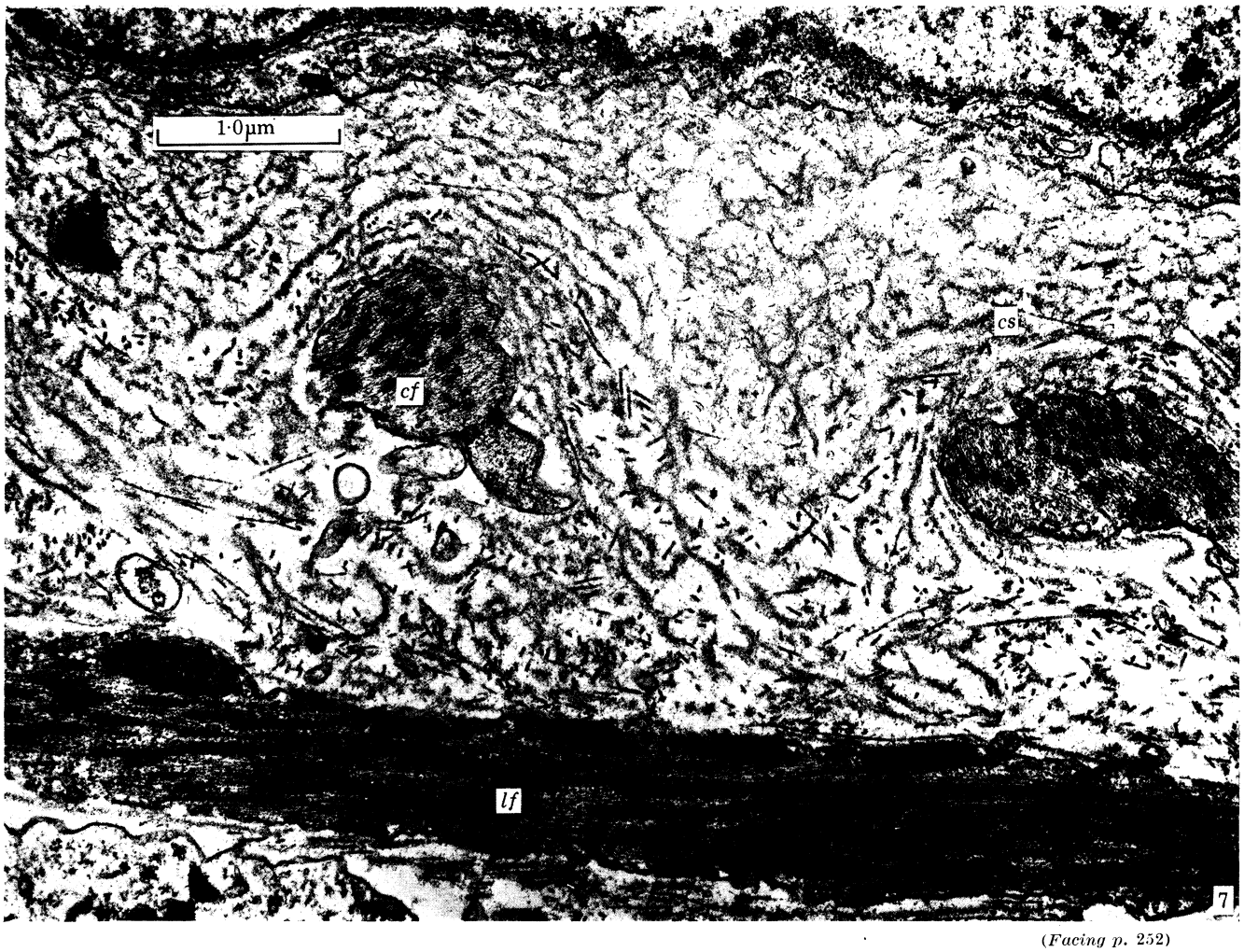
This type of vesicle is usually, although not invariably, confined to the basal regions of the cell. The contents range from fine fibrous and granular material to scattered portions of membrane and membrane-bound microvesicles (figure 11, plate 43). In some cases the latter are scattered throughout the vesicle while in others they tend to be distributed round the periphery of the vesicle. The characteristic feature of the type 4 macrovesicle, however, is the possession of a coat or wall, some 0.1 μm thick, composed of fine, moderately electron-dense material (figure 12, plate 44; *s*). This coat is invariably bounded on its outer surface by a triple-layered membrane which in section follows a sinuous course and gives an impression of blebbing. In what could be interpreted as a mature type 4 macrovesicle (figure 11, plate 43), a similar membrane is also present against the inner surface of the coat, but in this case it is frequently not continuous and may also show deep infoldings extending into the body of the vesicle.

The second group of membrane-bound vesicles—the microvesicles—is varied in appearance but can be subdivided on the basis of the dimensions of the limiting membrane. The first group comprises microvesicles which, like the macrovesicles, are bounded by a triple-layered membrane of similar dimensions to that of the apical plasma membrane, namely 8 nm. Such microvesicles are numerous in the apical regions of the cell (figures 18 and 21, plate 47). Their profiles range from circular to elongate in section and they are occasionally branched. There is also variation in the contents. Some are apparently empty while others contain fine, granular, electron dense material. Microvesicles which are similarly bounded by an 8 nm membrane are scattered throughout the cytoplasm between the macrovesicles (figures 11 and 15, plates 43 and 45; *mv*). They are always circular in profile, some 0.15 μm in diameter, and contain a central mass of highly electron-dense material. The second type of microvesicle which can be readily distinguished is bounded by a triple-layered membrane of similar dimensions to that of the Golgi saccules, namely 6 nm in width (figure 15; *mvl*). They are slightly irregular in outline and

Description of plate 41

Figure 6. Electron micrograph of the apical regions of an immature cell (*IBC*), bearing a single flagellum (*f*), and an adjoining secretory cell (*MBC*) with secretory vesicles (*sv*).

Figure 7. Electron micrograph of the connective tissue sheath surrounding a digestive tubule and showing circular (*cf*) and longitudinal (*lf*) muscle fibres.



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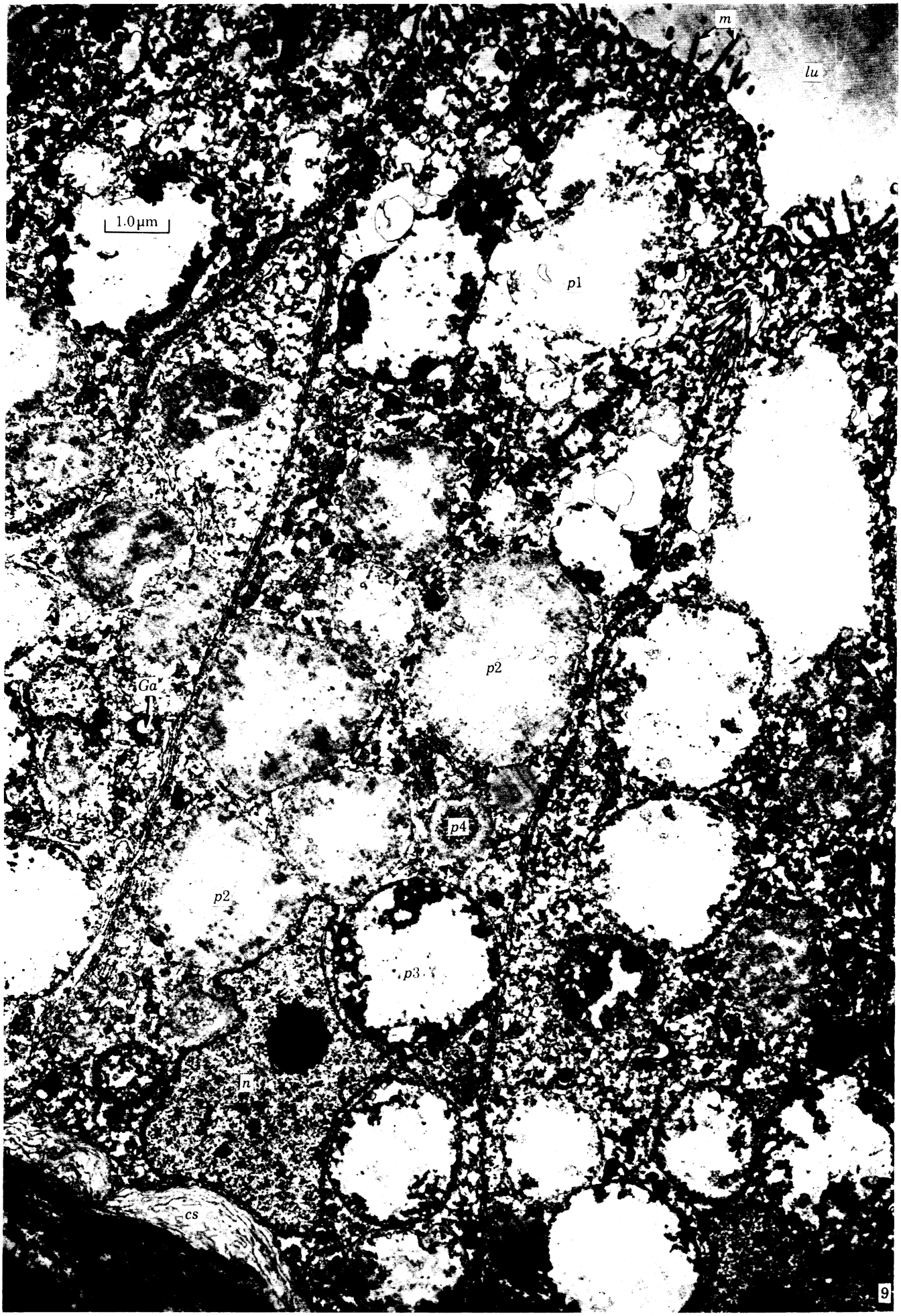
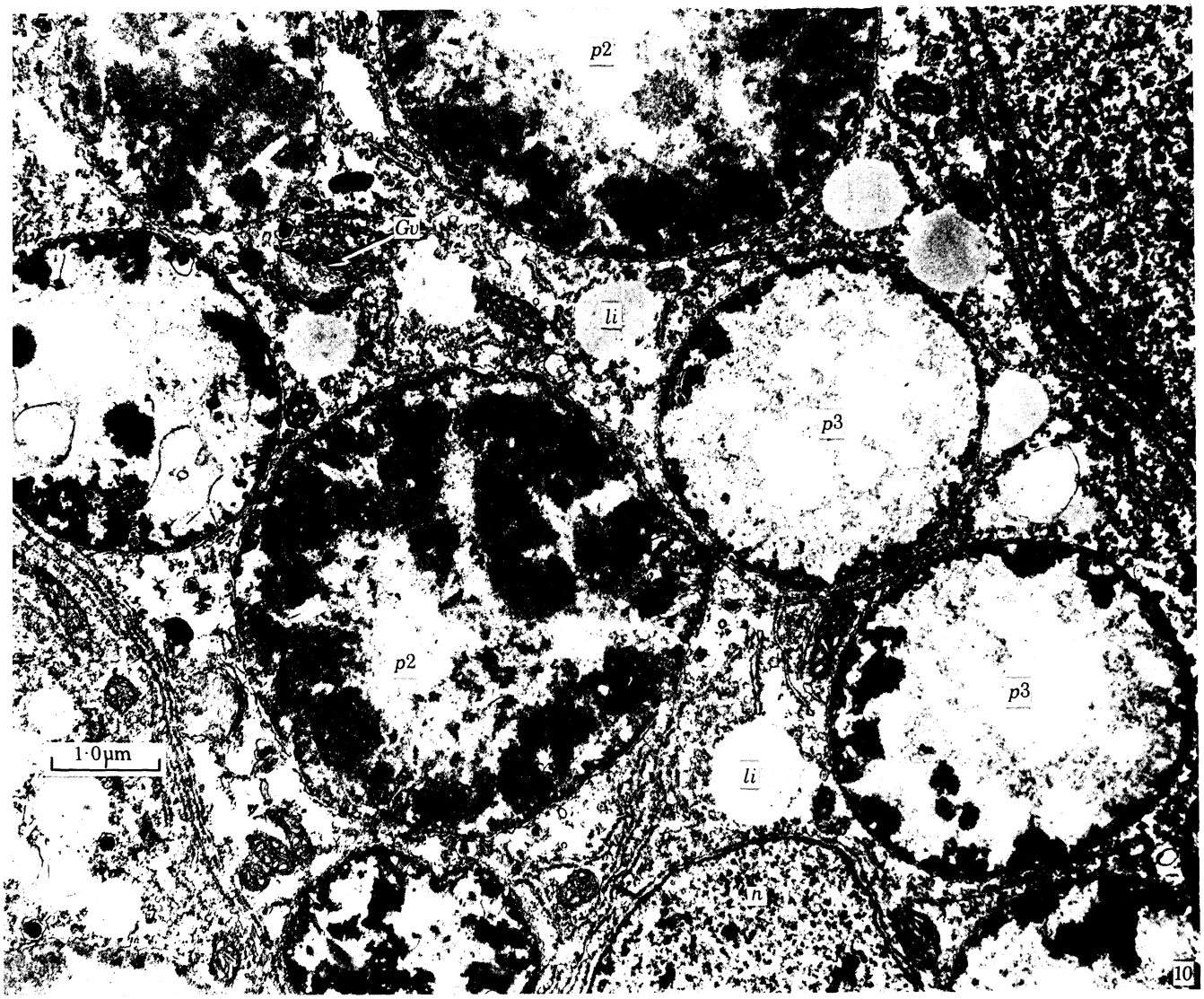


FIGURE 9. Electron micrograph of a digestive cell showing the general columnar form of the cell and the four types of macrovesicle (*p*1 to *p*4) typically present.



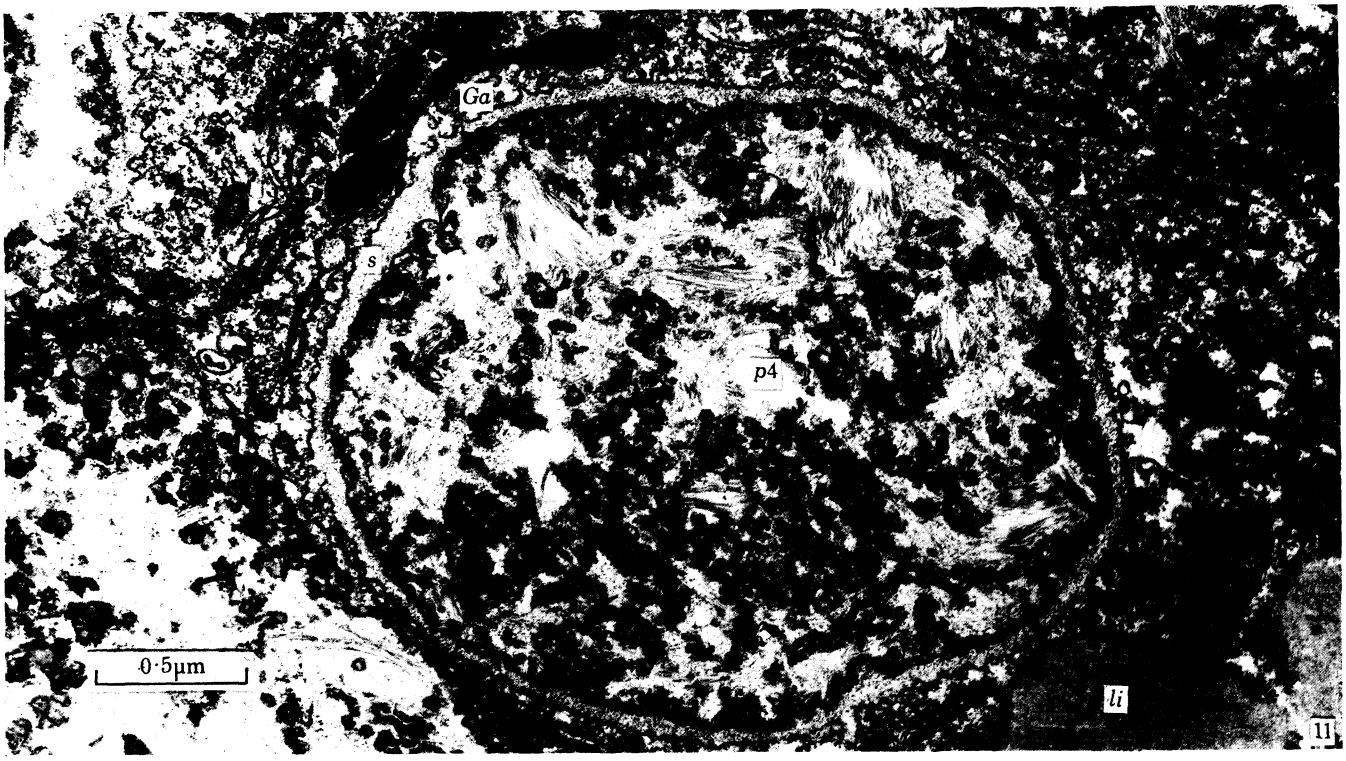
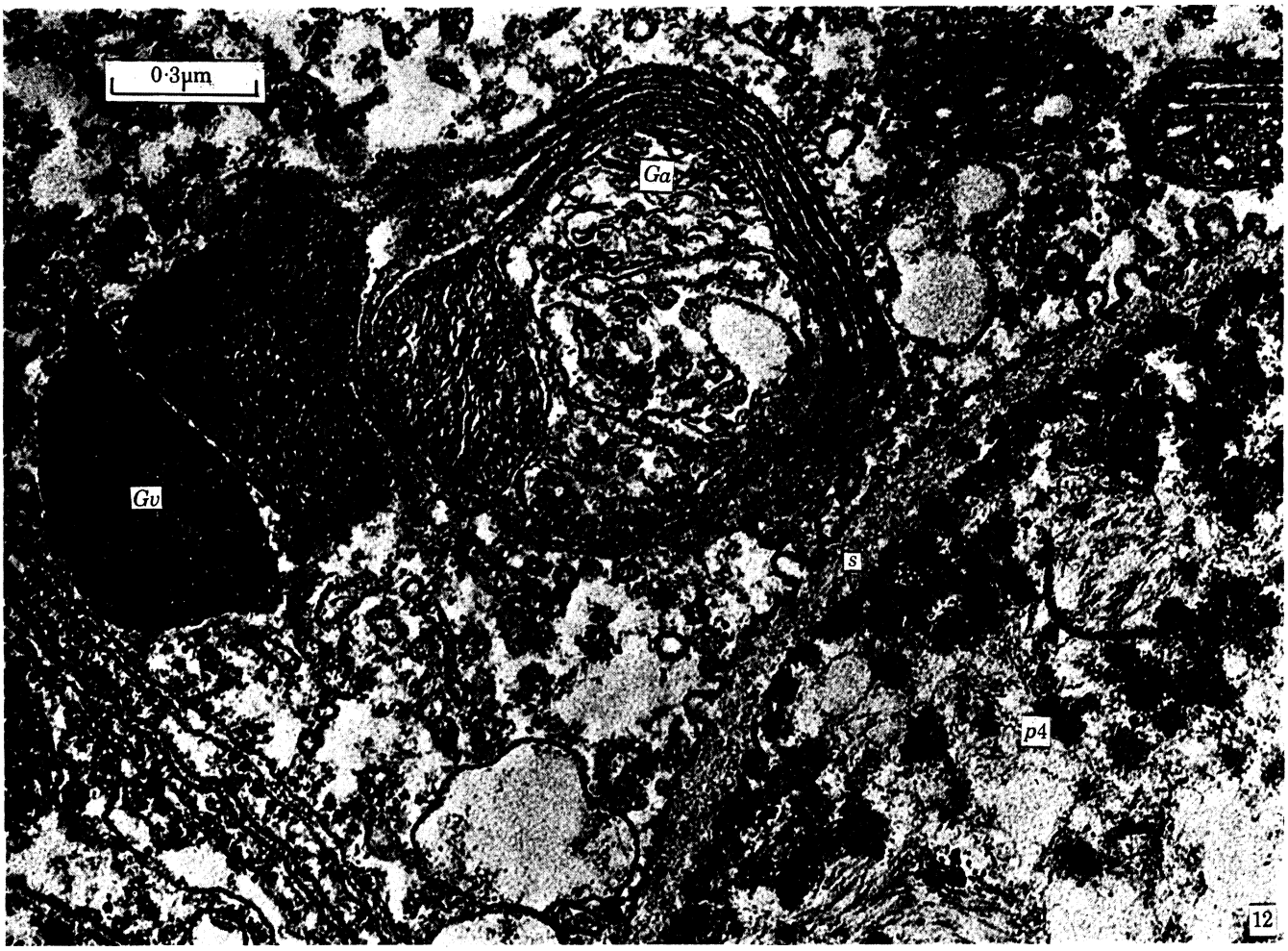


FIGURE 10. Electron micrograph of the mid-region of a digestive cell showing details of two types of macrovesicles (*p2*, *p3*) which may represent phagolysosomes and residual bodies respectively.

FIGURE 11. Electron micrograph of a type 4 macrovesicle, showing the characteristic coat which surrounds this type of vesicle; note the adjacent Golgi body (*Ga*).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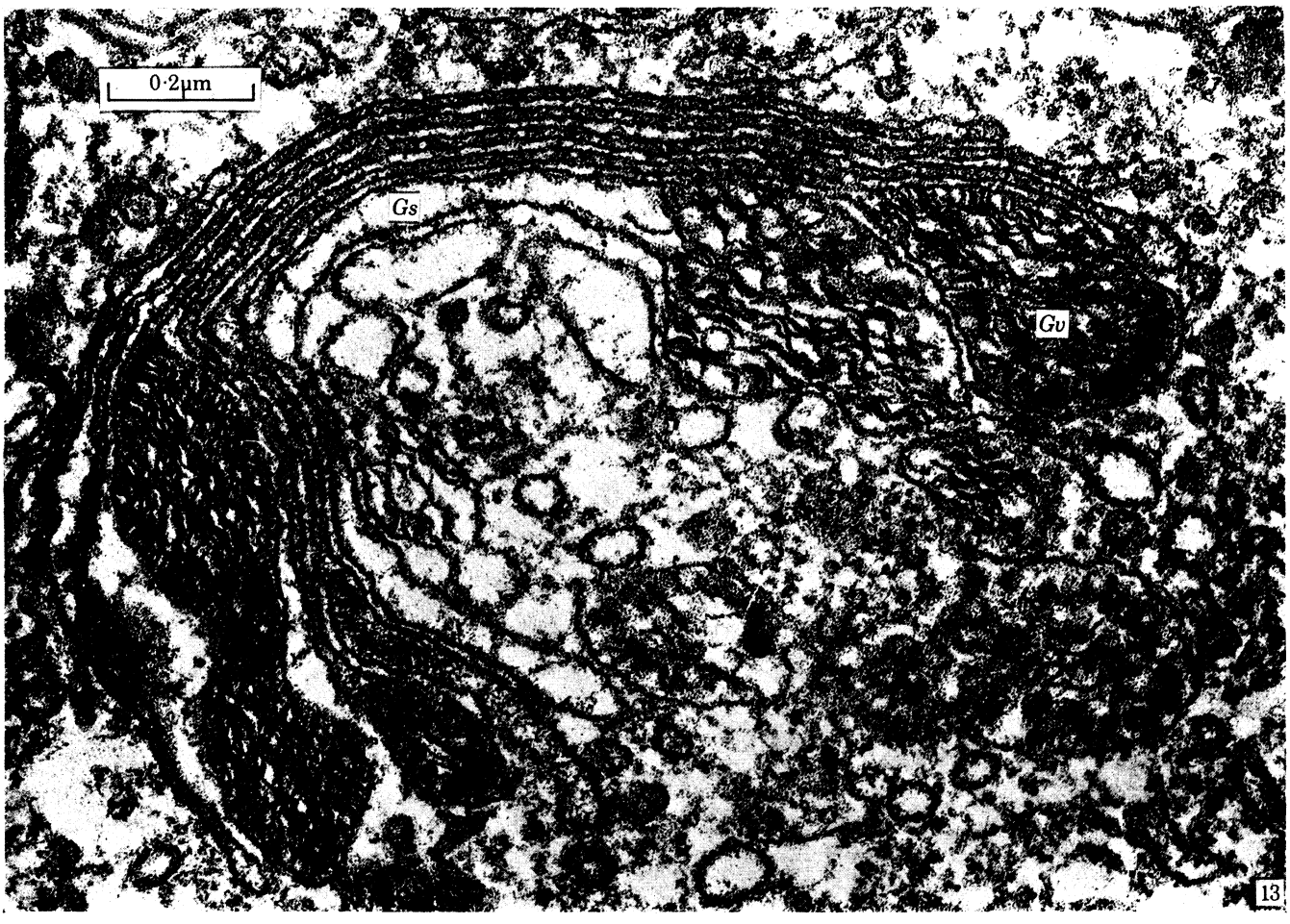
p4
12


0·2μm
Gs
Gv
13

Description of plate 44

Figure 12. Electron micrograph, showing details of the characteristic Golgi bodies present in the digestive cells and also details of the coat which surrounds the type 4 macrovesicles.

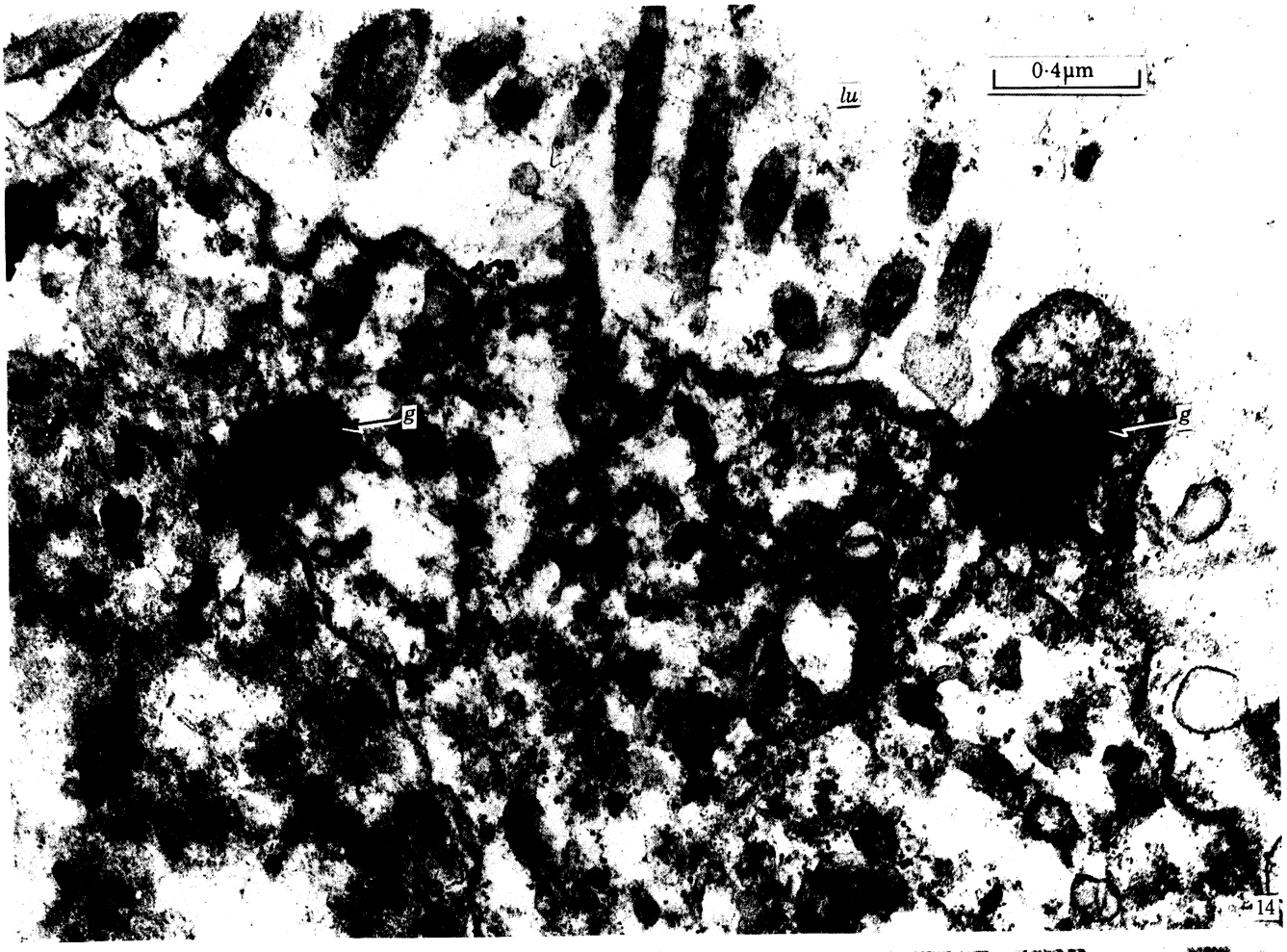
Figure 13. High-power electron micrograph showing further details of the Golgi bodies of the digestive cell and in particular the characteristic membranous elements present within the peripheral dilations of the Golgi saccules.

Description of plate 45

Figure 14. Electron micrograph of the apical region of a digestive cell from an animal fed a mixture of iron oxide and 'Aquadag' and showing phagocytosis of single particles of graphite from the lumen of the tubule.

Figure 15. Electron micrograph showing details of the membranes surrounding type 2 and type 3 macrovesicles. Note the characteristic clear zone (*x*) between the membrane and the electron-dense contents of the type 3 macrovesicle. Also shown are two types of microvesicle.

Figure 16. Electron micrograph of the apical region of a digestive cell from an animal fed 6 h with a mixture of iron oxide and 'Aquadag'. Both substances are present within type 1 macrovesicles, the iron oxide as fine, electron-dense particles and the graphite (*g*) as larger irregularly shaped particles.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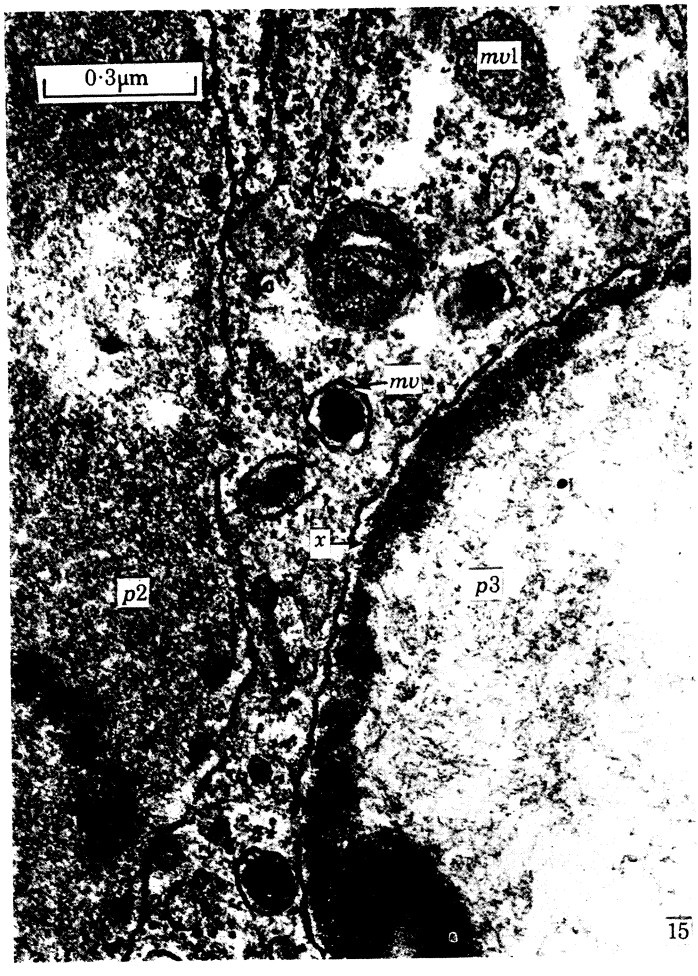
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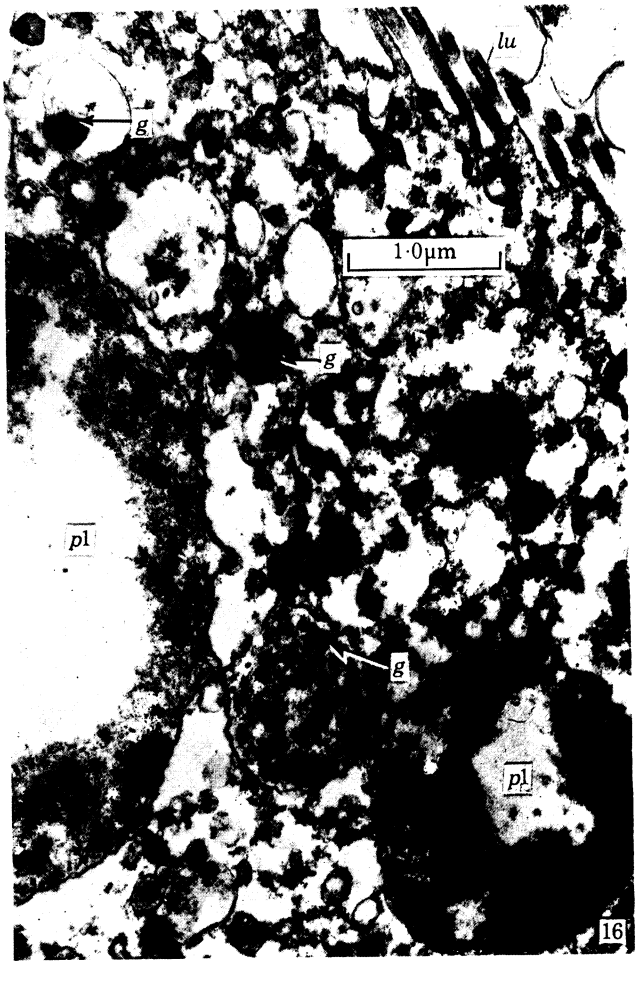
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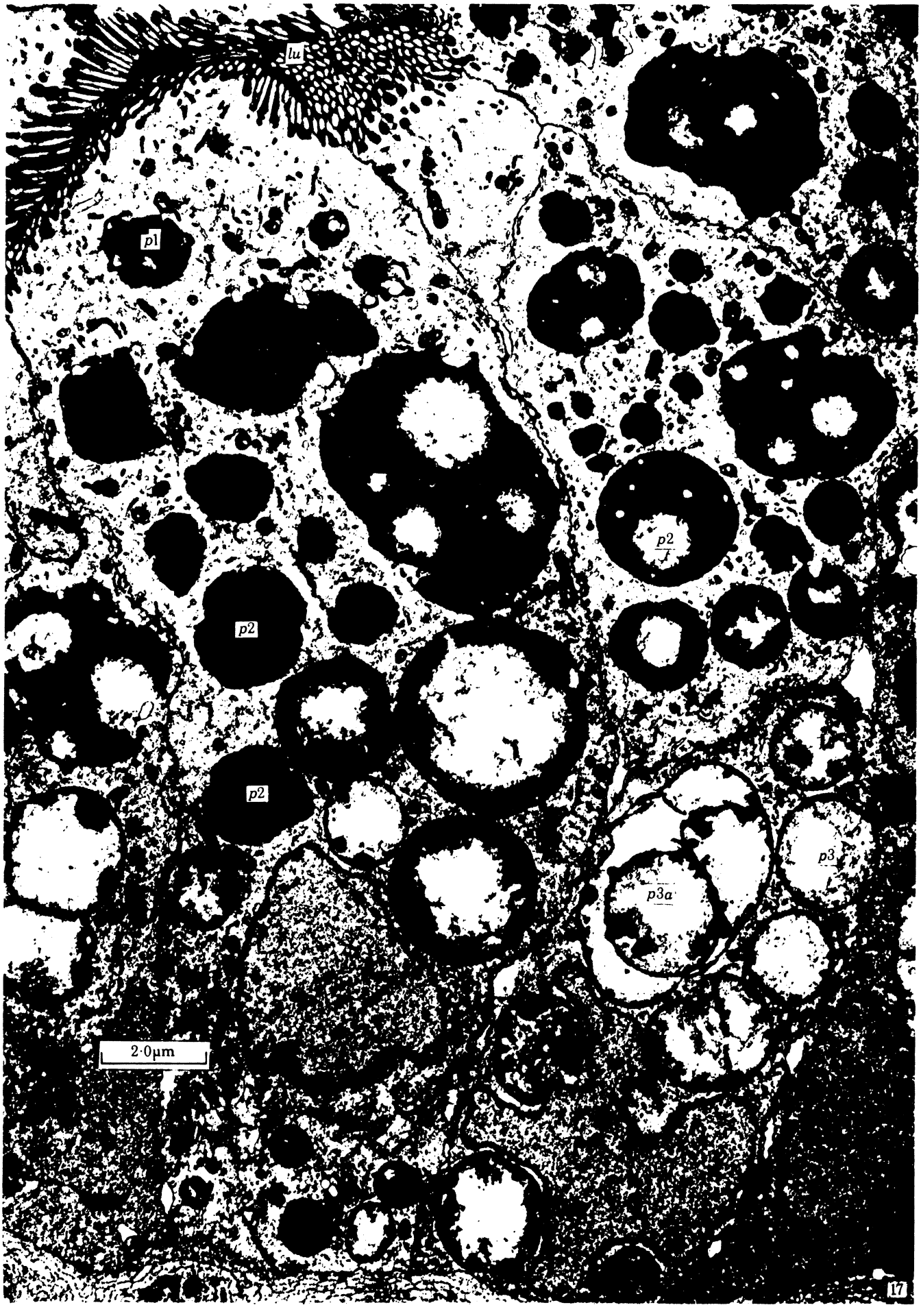
16


lu
p1
p2
p2
p2
p3
p3a
2·0μm

Description of plate 46

Figure 17. Electron micrograph of a digestive cell from an animal fed pigeon blood for 8 h (cf. figure 9, plate 42). Note the increase in the number of microvilli projecting into the lumen and the presence of haemoglobin within the type 1 and type 2 macrovesicles. There is also a marked increase in the electron density of the peripherally distributed contents of the type 3 and 3*a* macrovesicles.

Description of plate 47

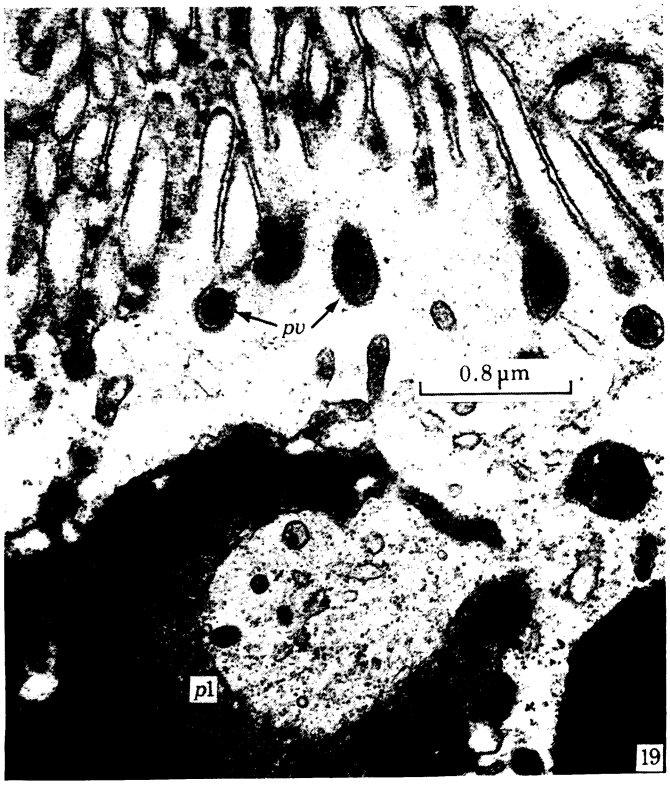
Figure 18. Electron micrograph of the apical region of a digestive cell from an animal fed pigeon blood for 3 h and showing numerous coated pinocytic vesicles containing electron-dense material and small amounts of similar material within a type 1 macrovesicle.

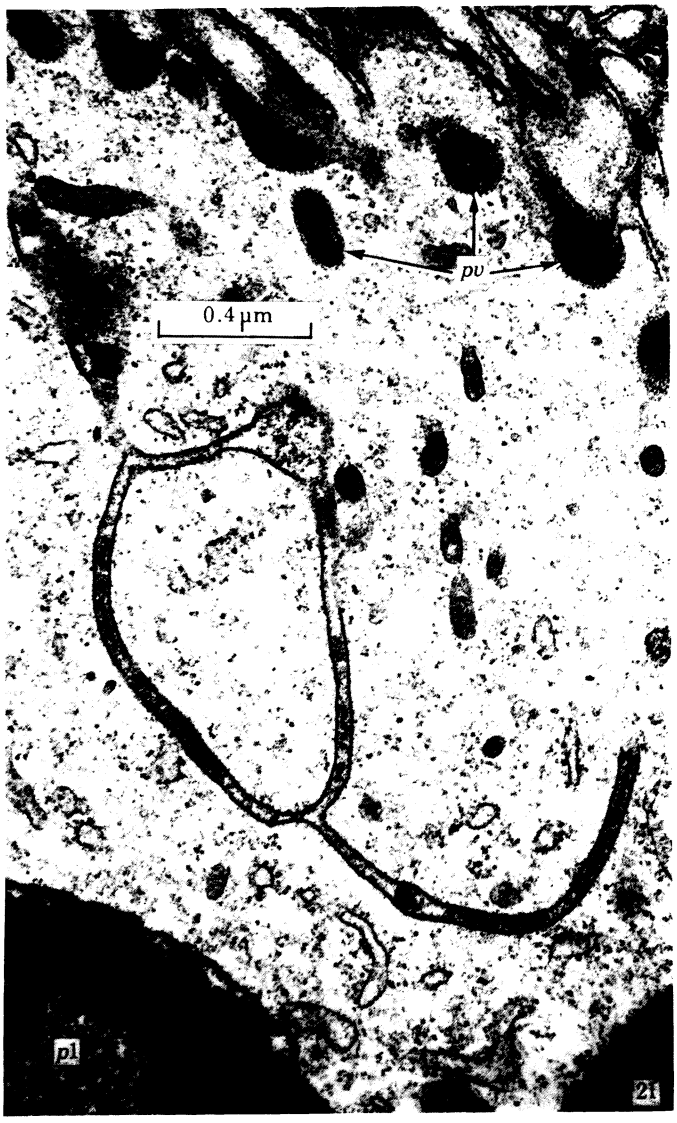
Figure 19. As for figure 18 but from an animal fed pigeon blood for 8 h. Note the increased amount of electron-dense material within the type 1 macrovesicle.

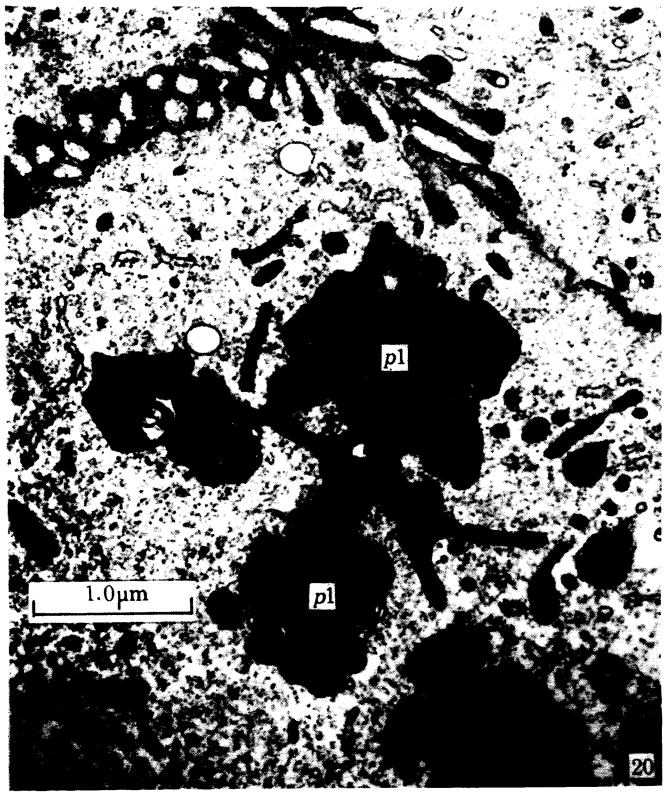
Figure 20. Electron micrograph of the apical region of a digestive cell from an animal fed pigeon blood showing the connexions between adjacent type 1 macrovesicles.

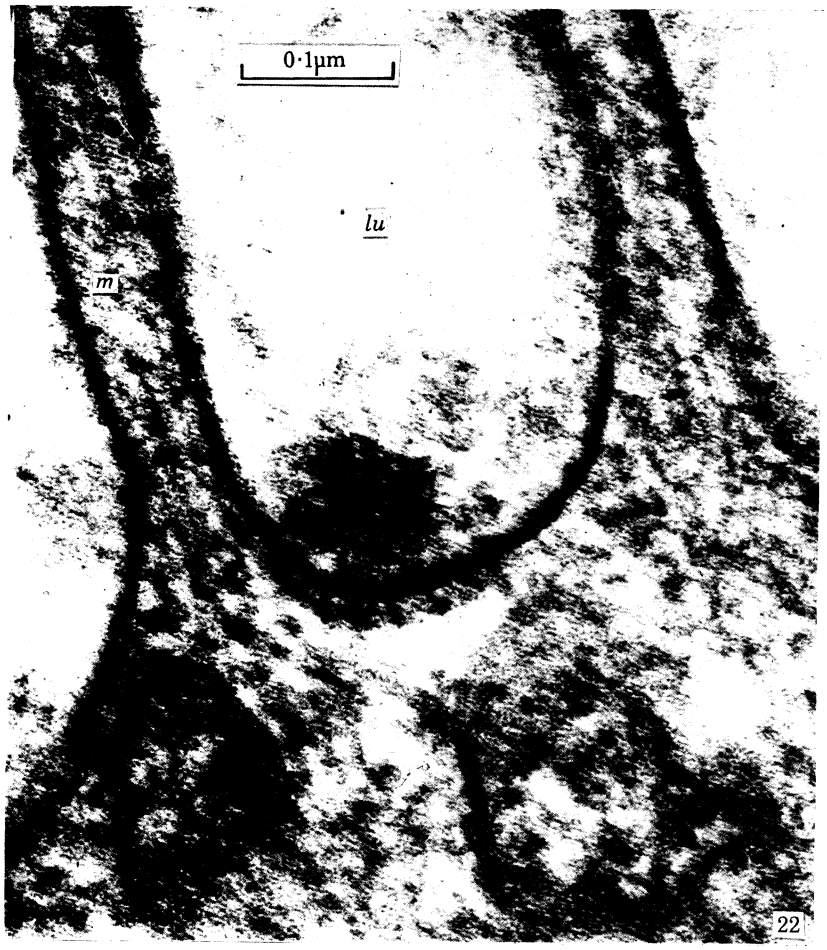
Figure 21. Electron micrograph of the apical region of a digestive cell from a blood fed animal showing a portion of the branched endocytic tubule system in the apical cytoplasm.

Phil. Trans. Roy. Soc. Lond. B, volume 258, plate 47





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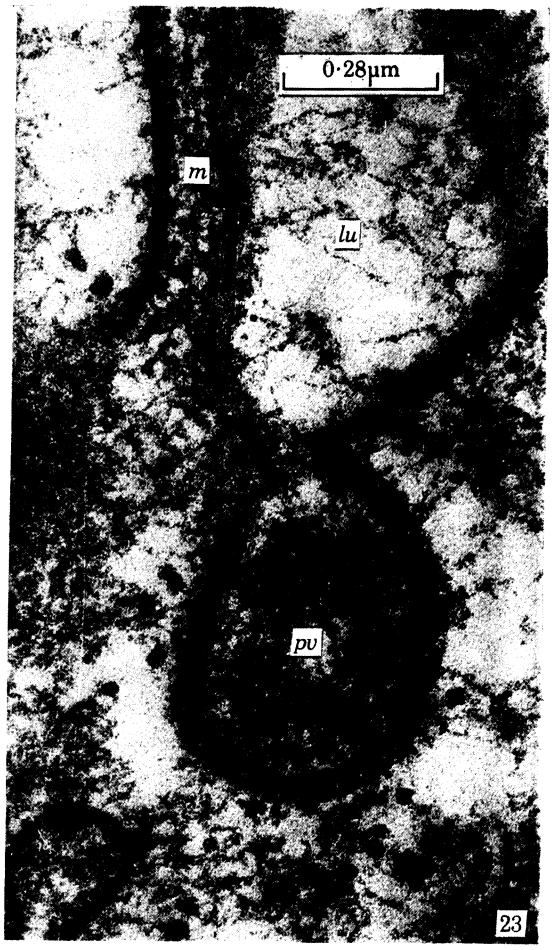
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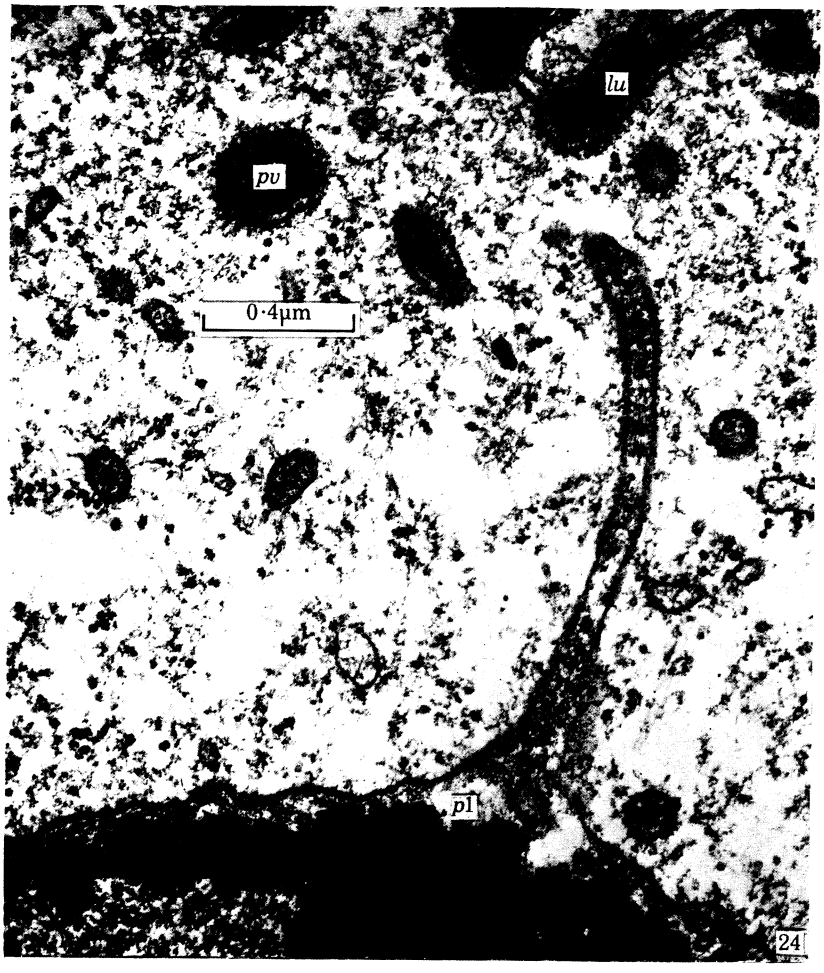
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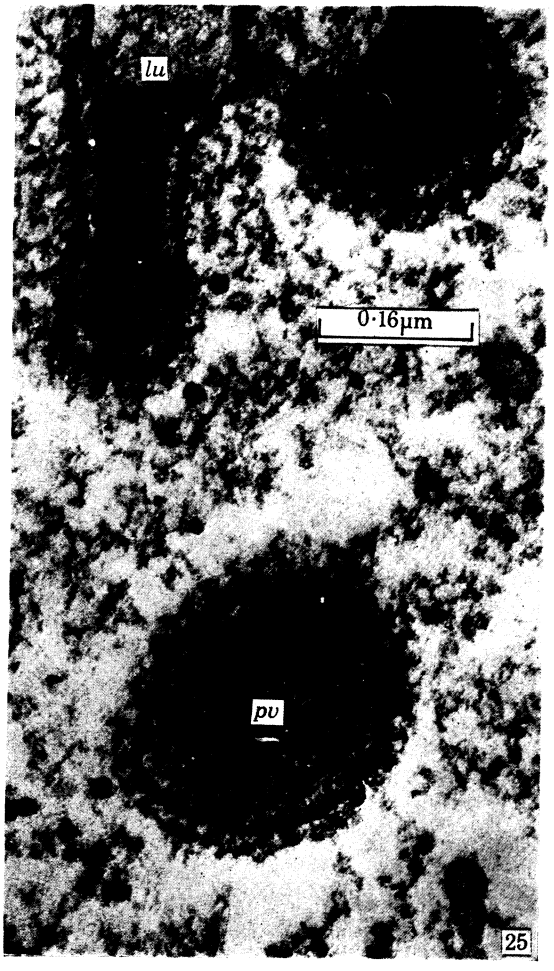
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average some 0.3 μm in diameter. They occur mainly between the macrovesicles and are uniformly filled with a fine granular material of moderate electron density.

Each cell contains a number of Golgi complexes distributed in the mid and lower regions of the cell (figure 8; figure 9, plate 42; *Ga*). The disposition of the elements comprising each Golgi complex is fairly typical, namely a stack of flattened membrane-bound saccules, markedly curved, and with their peripheral portions, showing increasing distension toward the concave side of the stack (figures 12 and 13, plate 44). In the adjacent cytoplasm are numerous membrane-bound microvesicles and a smaller number of larger vesicles (*Gv*) apparently derived from the peripheral regions of the saccules (*Gs*). The interesting and characteristic feature of the Golgi complex of the digestive cell is the presence of membranous elements within the distended regions of the saccules and also within the vesicles derived from these. The exact disposition of these membranous elements is difficult to determine but they appear to enclose sinuous flattened sacs which extend across the Golgi vesicles rather like the cristae of a mitochondrion. Whether these sacs are in the form of closed compartments distinct from the bounding membrane of the Golgi complex or whether they are formed by invaginations of the bounding membrane has not been determined. What does seem clear is that the contents of these flattened compartments are electron-light while the surrounding material within the Golgi vesicle is electron-dense. In those vesicles which have separated from the saccules the contents become increasingly electron-dense and the membranous elements obscured (figure 12, plate 44, *Gv*).

The remaining cell organelles are all fairly typical. Mitochondria are distributed throughout the cell but with possibly slightly greater numbers in the upper half (figure 8, *mi*). Elements of granular endoplasmic reticulum (*ger*) occur throughout the lower two-thirds of the cell but are most conspicuous near the lateral plasma membrane, while profiles of smooth endoplasmic reticulum are less obvious and appear to be mainly restricted to the mid and upper regions of the cell.

Ingestion of materials by the digestive cell

The materials used in feeding experiments consisted of inorganic substances in the form of a mixture of iron oxide and colloidal graphite (Aquadag) and organic materials fed separately in the form of Cd^{2+}-free ferritin and whole blood of pigeon. With the exception of the last, all the materials found their way into the blind-ending tubules and into at least some of the digestive cells. In the case of whole pigeon blood, intact erythrocytes were found in the main ducts of the digestive diverticula but were never observed in the lumen of the tubules. Haemoglobin released from erythrocytes, however, filled the lumen of the tubules and was readily ingested by the digestive cells. It is not the intention in this paper to follow in detail the fate of the ingested material but rather to indicate certain general features of the ingestion of substances by the digestive cells.

Figure 16, plate 45, shows the apical region of a digestive cell from a cockle which had been

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Description of plate 48

Figures 22, 23, 25. Electron micrographs showing stages in the formation of the coated pinocytic vesicles at the apical surface of digestive cells from blood fed animals.

Figure 24. Electron micrograph of the apical region of a digestive cell from a blood fed animal, showing electron-dense material, almost certainly haemoglobin, in the lumen of the tubule, coated pinocytic vesicles, and a type 1 macrovesicle. Note the tube-like extension from the macrovesicle which is probably part of the endocytic system shown in figure 21, plate 47.

presented 3 h earlier with a mixed suspension of iron oxide and colloidal graphite. Numerous particles of both substances have accumulated within type 1 macrovesicles (*p*1). The mode of ingestion of the larger graphite particles is indicated in figure 14, plate 45, which shows a single particle of graphite (*g*) enclosed in a membrane-bound vesicle at the apical surface of the cell. The surface of the cell at this point bulges into the lumen (*lu*) of the tubule and is devoid of microvilli. It is reasonable to assume that the particle has been recently phagocytosed. Single particles of graphite were frequently observed in microvesicles in the apical cytoplasm between the plasma membrane and the type 1 macrovesicles. It would appear that following phagocytosis of single particles of graphite and their enclosure within a microvesicle they are transferred to the larger type 1 macrovesicles deeper within the cell where they accumulate. The mode of ingestion of the smaller particles of iron oxide is not clear since they were never identified with certainty within apical microvesicles. It is possible that they are ingested by a process of pinocytosis, since the ability of the digestive cells to ingest material by pinocytosis in addition to phagocytosis is clearly demonstrated when the exogenous material is haemoglobin.

Figure 17, plate 46, is a low-power electron micrograph of a digestive cell from an animal which had been presented 8 h earlier with whole pigeon blood. Comparison of this micrograph with that of figure 9, plate 42, clearly illustrates the more obvious changes which have occurred. There has been a tremendous increase in the number of microvilli at the free surface of the cell while many of the macrovesicles, and particularly those belonging to types 1 and 2 (*p*1, *p*2) are filled with an amorphous, electron-dense material which it is reasonable to assume is haemoglobin released from erythrocytes. Figures 18 and 19, plate 47, show two stages in this process of ingestion of haemoglobin. The former shows the apical region of a digestive cell from an animal which had been presented with whole pigeon blood 3 h earlier while the latter shows a similar region of a cell from an animal which had been fed 8 h earlier. The increased accumulation of haemoglobin in the type 1 macrovesicles (*p*1) is evident while in both electron micrographs there is abundant evidence of intense pinocytic activity (*pv*) in the depths of the crypts between the microvilli.

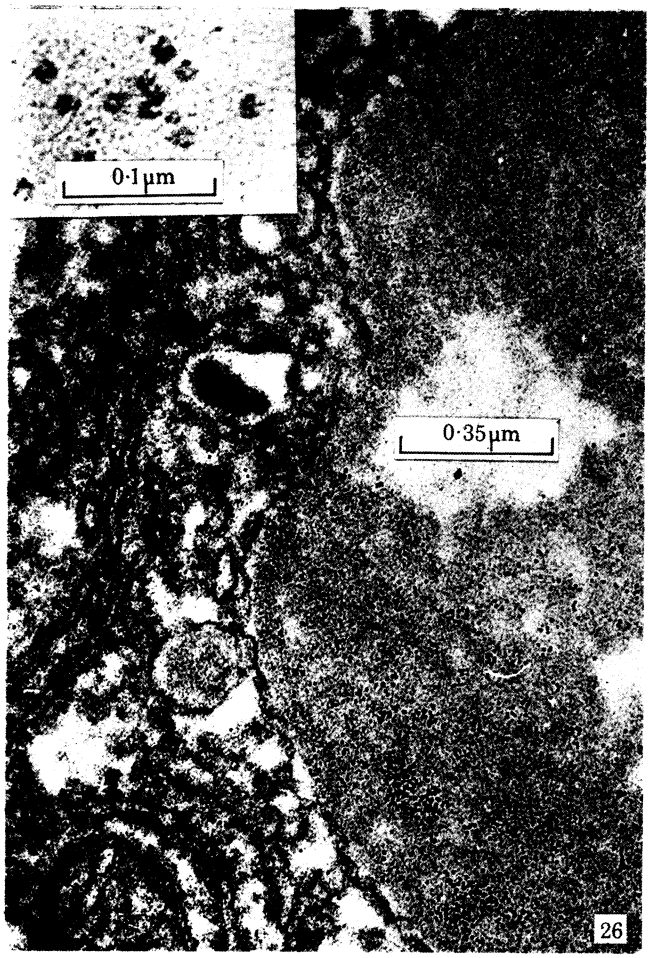
It is not difficult to follow the formation of the pinocytic vesicles since the plasma membrane in the regions where they are forming, and the bounding membrane of the vesicles after they are formed, is characteristically coated on the cytoplasmic surface by a single row of granules or spheres, some 8 nm in diameter and spaced 25 nm apart (figures 22, 23 and 25, plate 48). The first indication of the formation of a vesicle is a shallow depression of the plasma membrane which is coated on its cytoplasmic surface by such a row of granules (figure 22, plate 48). Small amounts of haemoglobin appear to be adsorbed on to the lumenal surface of the membrane. The depression deepens (figure 23, *pv*) and is finally pinched off to form a spherical, membrane-bound vesicle, some 25 to 100 nm in diameter, enclosing exogenous material in the form of electron-dense haemoglobin and coated on its cytoplasmic surface by the characteristic granules

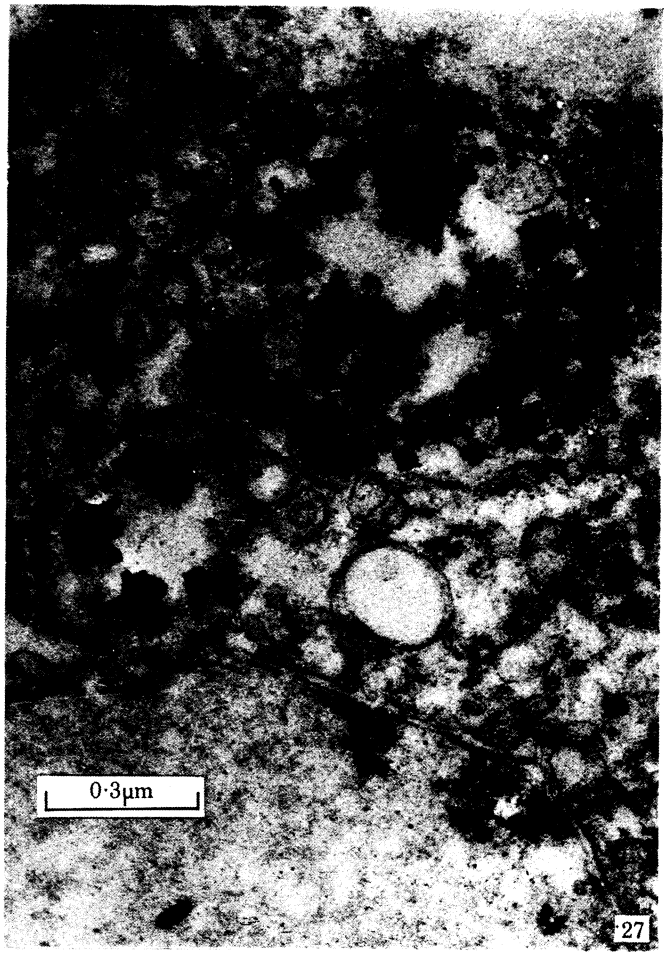
Description of plate 49

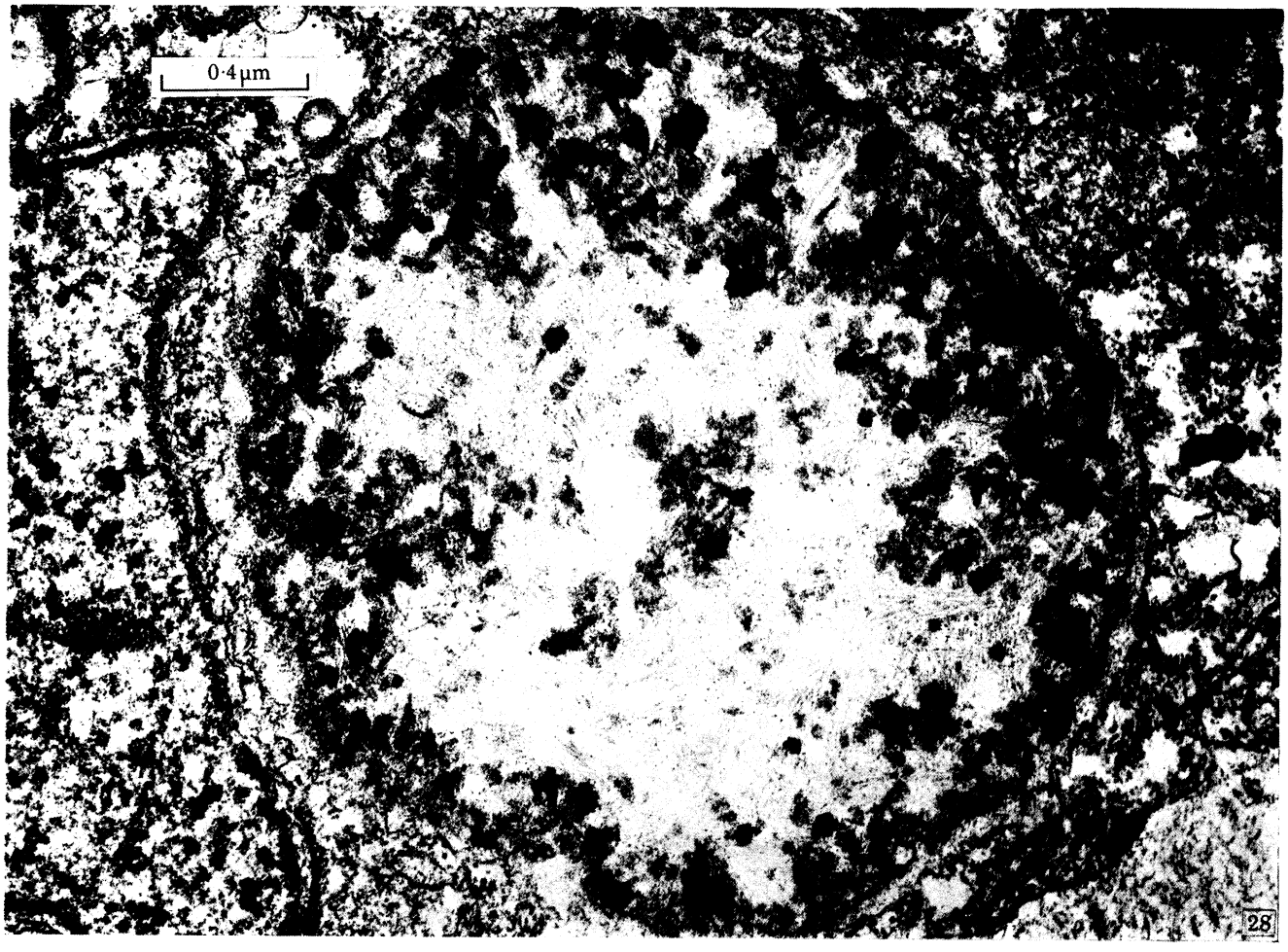
Figure 26. Electron micrograph of a portion of a type 2 macrovesicle from a ferritin fed animal showing the accumulation of ferritin readily identified by its characteristic molecular configuration (inset).

Figure 27. Electron micrograph of a type 1 macrovesicle after treatment for the demonstration of acid phosphatase activity.

Figure 28. Electron micrograph of a type 4 macrovesicle after treatment for the demonstration of acid phosphatase activity.







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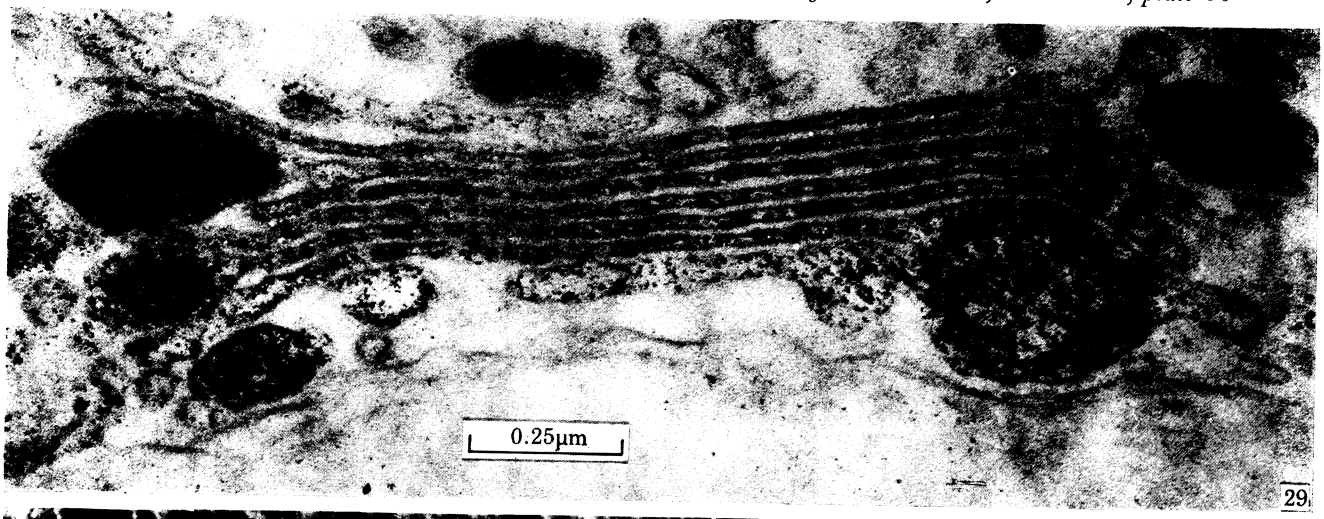

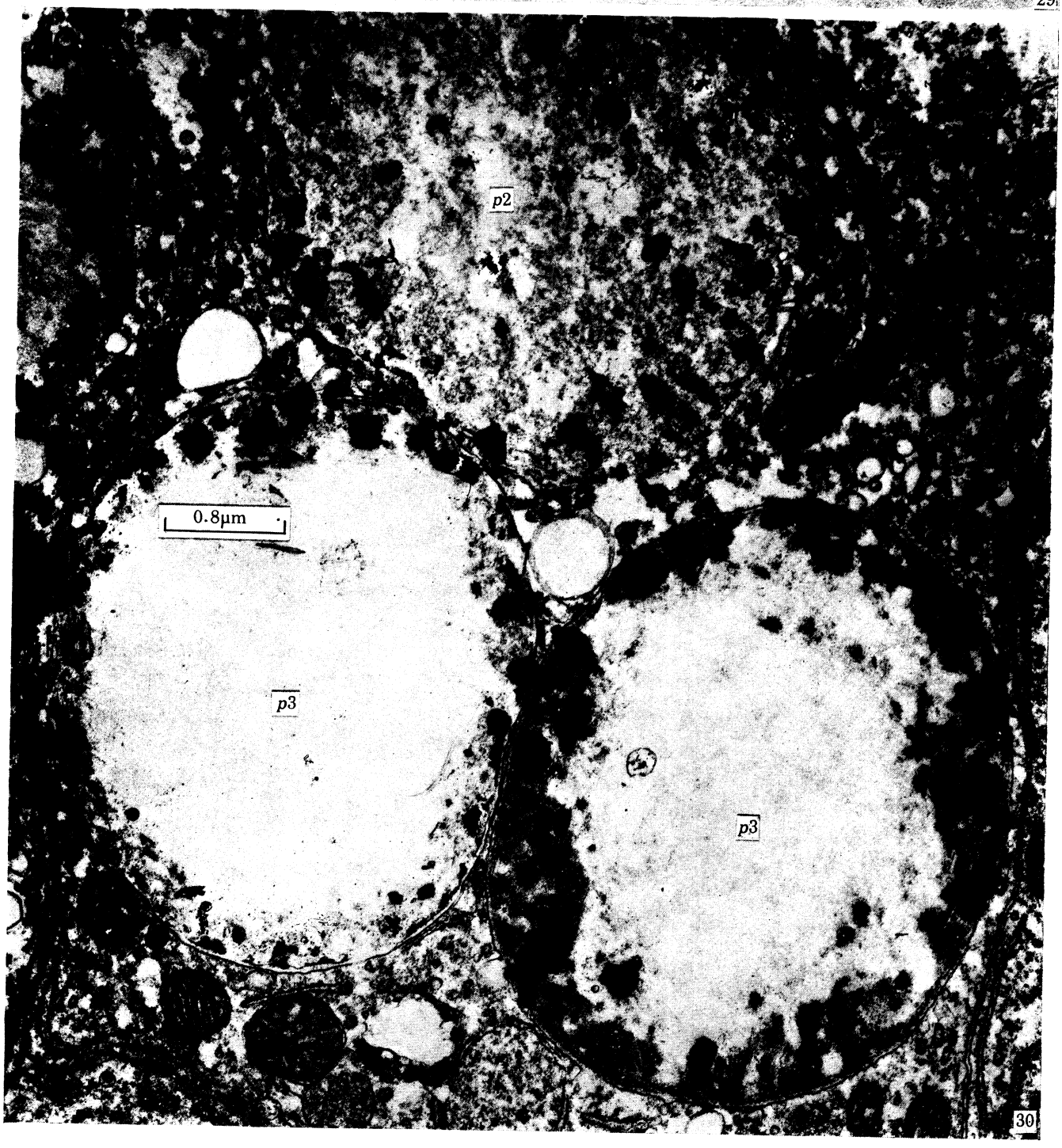

(figure 25, plate 48, *pv*). Such coated depressions, pits and vesicles are common at the surface of the digestive cell within 1 h of feeding with whole pigeon blood, but even after 8 h or more the vesicles present deeper within the cytoplasm lack the granular coat and moreover are frequently elongated in outline and suggestive of sections through tubular structures. Indeed, a study of figures 20, 21 and 24, plates 47 and 48, suggests that there is present, at least in digestive cells actively ingesting haemoglobin, an apical endocytic network of membrane-bound tubules approximately 50 nm in diameter and which open into the type 1 macrovesicles and also serve to interconnect these vesicles (figures 20 and 24, plates 47 and 48). This intracellular system of tubules, however, was never observed to be connected to the surface of the cell, although in some cases individual tubules did extend close to the surface plasma membrane.

The subsequent fate of the haemoglobin which accumulates in the type 1 macrovesicles is difficult to determine. The contents of type 2 macrovesicles certainly exhibit an increased electron-density suggesting that haemoglobin, or the products of haemoglobin digestion, are present (figure 17, plate 46, *p*2). A similar change appears to take place in the contents of type 3 (*p*3, *p*3*a*) macrovesicles but in this case it is more difficult to determine since the contents of these vesicles exhibit considerable electron density in unfed animals. Apart from a possible decrease in their number there appeared to be no obvious change in the type 4 macrovesicles.

The distribution of ingested material within the digestive cells was more readily followed in ferritin-fed animals. As in blood fed animals, coated pits and vesicles were common at the apical surface of the digestive cells of ferritin fed animals although ferritin particles were never clearly identified in association with these pits and vesicles. Moreover, there was never the same dramatic increase in the number of microvilli at the apical surface of the cells. There was, however, no doubt about the accumulation of ferritin particles within type 1 and type 2 macrovesicles (figure 26, plate 49) which were frequently filled with ferritin particles readily identified by their molecular configuration. Ferritin particles were also present within type 3 macrovesicles although never in the same high concentrations. They tended to occur in small clumps associated with the electron-dense material which is characteristically present round the periphery of these vesicles. The presence of ferritin particles within type 4 macrovesicles was difficult to determine with certainty. Electron-dense particles of the same dimensions as ferritin particles were frequently present but rarely did they show clearly the molecular configuration of ferritin.

Localization of acid phosphatase activity

In frozen sections of glutaraldehyde-fixed material prepared for optical microscopy the digestive cells lining the tubules showed intense acid phosphatase activity while the basiphilic cells in the crypts and the epithelia lining the main and secondary ducts were clear. Electron micrographs showed that the bulk of the activity associated with the digestive cells was confined to the macrovesicles, where the reaction product appeared as clumps of coarse, electron-opaque precipitate (figures 27, 28 and 30, plates 49 and 50). Small isolated masses of reaction product were also observed in the cytoplasm between the macrovesicles, and although they appeared on

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Description of plate 50

Figure 29. Electron micrograph of the Golgi body of a digestive cell after treatment for the demonstration of acid phosphatase activity.

Figure 30. Electron micrograph showing type 2 and type 3 macrovesicles after treatment for the demonstration of acid phosphatase activity.

occasions to be enclosed within membrane-bound microvesicles it was not possible to correlate these with structures seen in routinely prepared material. This was due to the fact that the reaction product obscured all detail and more important the various types of microvesicle identified in routinely prepared sections could usually be identified within the same cell of phosphate-treated material clear of reaction product. It must be added, however, that the occurrence of reaction products, even within the macrovesicles, was spasmodic. Thus on occasion, while all four types of macrovesicle within the same cell contained reaction product, those of adjacent cells were either clear or the product was present in only some of the macrovesicles. Fine deposits of lead were also occasionally observed in association with the Golgi complexes both within the cisternae of the flattened saccules and of the distended peripheral vesicles (figure 29, plate 50). Finally, and although they were not visible in the optical microscope, small amounts of reaction product were invariably present within the autophagic vesicles of the basiphilic cells.

Discussion

The main aim of this study was to provide a general picture of the fine structure of the digestive tubule which could serve as a basis for further work on other species and also provide preliminary information on the functions of the cells present in the tubule. Although optical microscopic examination of the digestive diverticula of bivalves has long demonstrated a differentiation of the cells lining the tubules into dark-staining cells and more numerous vacuolated, digestive cells, it has been widely accepted that these represent but one cell type. Following the earlier work of Yonge (1926), the former are regarded as nests of immature cells which divide to replace the mature digestive cells (Morton 1956). The results of the present study, however, confirm the conclusions of Sumner (1966*b*), drawn from studies of *Anodonta*, that there are at least two distinct cell types present in the tubule epithelium. As the descriptions given earlier show, the *ger* cells present in the crypts have a fine structure typical of protein-secreting cells, such as the acinar cells of the mammalian pancreas.

The concept that the crypts of the tubules contain young undifferentiated cells is, however, not necessarily mistaken. Sumner (1966*a*) has suggested that the groups of basiphil cells present in the crypts of the tubules of *Anodonta* consist of both mature and immature cells. This also appears to be the situation in *Cardium*. The structure of the flagellated, columnar cells is consistent with that of young immature cells, particularly in the possession of numerous free ribosomes and the absence of a well-organized endoplasmic reticulum. Also significant is the pronounced intercellular 'gap' which frequently separates these cells from adjoining cells. It is reasonable to suggest, therefore, that they may serve to replace either the pyramidal *ger* cells, or the digestive cells, or both. The question, however, is far from settled for the bivalves as a whole. Unpublished observations on the fine structure of the digestive tubules of the protobranch *Nucula* show that in this species the groups of basiphil cells are composed solely of secretory *ger* cells, each of which bears a single flagellum.

Results obtained here amply confirm the long-held view that the digestive cells are specialized for the uptake and intracellular digestion of exogenous material directed to the tubules from the stomach. Exogenous material derived from the lumen of the tubules was identified in all four types of macrovesicle, although notably less frequently in type 4 than in the other types. Similarly, acid phosphatase activity was demonstrated in all four types, although less frequently in type 1 than in the other types. Thus, while types 2 and 3 macrovesicles undoubtedly qualify as

phagolysosomes (Straus 1967; Gahan 1967), the classification of types 1 and 4 is less certain. The former, despite the occasional demonstration of acid phosphatase activity, could be primarily phagosomes within which the ingested material initially accumulates, while the latter could represent primary lysosomes rather than phagolysosomes. In either event it is reasonable to assume that all four types of macrovesicle comprise elements of the digestive system of the cell, and the main points for discussion are the uptake of exogenous material into the system, the source of enzymes, or at least of acid phosphatase, present within the system, and finally the sequence of events within the system.

There is considerable speculation concerning the origin of the hydrolytic enzymes which can be demonstrated within phagolysosomes. The general view is that primary lysosomes (de Duve & Wattiaux 1966) are held in reserve by the cell to be released into phagosomes to form phagolysosomes where the hydrolytic enzymes act upon the exogenous material. The available evidence suggests that the primary lysosomes may have different origins in different cell types, in some cases being formed directly from the endoplasmic reticulum and in others from specialized Golgi vesicles (Novikoff, Essner & Quintana 1964). In this study particular attention was paid to the characteristic Golgi bodies of the digestive cell. The occurrence of membranous elements within the Golgi vesicles appears to be a constant and characteristic feature of this organelle in the digestive cell of bivalves since it has been noted in all species studied (unpublished observations). It was at first thought that these peculiar Golgi vesicles gave rise to primary lysosomes but the evidence obtained from studies of acid phosphatase activity is confusing. In one batch of tissue fine lead deposits were observed associated with the Golgi saccules and vesicles, the strength of the reaction appearing to increase with the formation of the vesicles. Similar, although less dense, deposits of lead were also associated with the endoplasmic reticulum and the nuclear envelope and it was concluded that much if not all of this activity represented a false positive reaction. Certainly in subsequent batches of tissue processed to demonstrate acid phosphatase activity the Golgi saccules and vesicles associated with them were clear of lead deposits. It is possible, and indeed likely, that differences in the distribution of acid phosphatase activity may be correlated with different phases of the digestive cell. R. W. McQuiston (personal communication), who is carrying out a parallel study on the small inter-tidal bivalve *Lasaea rubra*, has found that the Golgi vesicles in this species do exhibit a true reaction to tests for acid phosphatase activity. In neither species, however, was fusion of the Golgi vesicles with any of the macrovesicles observed. An alternative possibility is that the Golgi vesicles undergo a rapid metamorphosis into the much larger type 4 macrovesicles and that the latter represent primary lysosomes. An objection to this interpretation is that the type 4 macrovesicles are enclosed within an 8 nm membrane while the Golgi vesicles are limited by a 6 nm membrane.

The ability of the digestive cells to ingest exogenous material from the lumen of the tubules has been amply demonstrated and it is clear that the ingested material is accumulated initially within the large type 1 macrovesicles. No evidence was obtained that these are ever formed directly at the cell surface and it is reasonable to assume that the various pits and microvesicles present in the apical cytoplasm are involved in the transfer of exogenous material from the lumen to the type 1 vesicles. Indeed it is clear that at least in blood-fed animals many of the smooth-coated microvesicles present in the apical cytoplasm form part of a branched intracellular tubule system which interconnects the type 1 phagosomes and extends throughout the apical cytoplasm of the cell. Despite a careful search, however, direct connexions between this apical system of tubules and the lumen were never observed.

The 'coated' pits and vesicles, on the other hand, are formed at the cell surface and because of their characteristic granular coat the part they play in the uptake process is not difficult to follow, at least in blood-fed material. Similar coated pits and vesicles occur at the surface of the oocyte of the mosquito *Aedes aegypti*, and Roth & Porter (1964) concluded that they were responsible for the uptake of yolk proteins. In this case the coat consisted of a border of fine bristles, about 20 nm long, rather than granules, and it was tentatively suggested that the characteristics of the coat may be associated with the nature of the material adsorbed on to the membrane. This does not seem likely in the case of the digestive cell of *Cardium*, since the characteristics of the coat appeared similar in blood-fed, ferritin-fed, and freshly collected animals.

Although it is clear that the subsequent fate of the coated pinocytic vesicles must be linked with the apical tubule system and type 1 macrovesicles, the exact nature of the relationship between them is not clear from the present study. One possibility, frequently expressed in association with pinocytic uptake of exogenous material, is that the pinocytic vesicles lose their coat as they move deeper into the apical cytoplasm and progressively fuse with one another to finally form the larger type 1 macrovesicles or phagosomes. Well-developed type 1 macrovesicles, however, were present in the digestive cells of all the animals examined. Moreover, while clear-cut evidence of a well-developed intracellular tubule system was obtained only from blood-fed animals, the apical regions of the digestive cells of freshly collected animals did show a confused vesicular appearance which could be interpreted as representing profiles of a closely packed, interconnecting system of tubules. It is possible that the apical tubule system and the type 1 macrovesicles are permanent structures within the cell and the function of the coated pinocytic vesicles is to transfer exogenous material from the lumen to the endocytic system of tubules, which in turn opens into the larger type 1 macrovesicles. In other words, the pinocytic vesicles may be regarded as temporary mouths which regulate the entry of material into the cell while the tubule system and type 1 macrovesicles serve as the first part of an alimentary system, namely the crop, the prime function of which is to accumulate and store material prior to further processing.

The digestive cells are also capable of ingesting material by phagocytosis. Korn & Weisman (1967), from a study of phagocytosis in *Acanthamoeba*, have suggested that there is an optimal size for the formation of a phagocytic vesicle. They found that latex beads with diameters of 1.305, 1.9 or 2.68 μm were always phagocytosed singly while smaller beads accumulated at the surface of the cell until they reached a 'critical volume'. The process of phagocytosis by the digestive cell of *Cardium* was observed only in association with graphite particles and did not occur sufficiently frequently to enable a detailed study of the stages involved in the process to be undertaken. In those cases that were observed, particles ranging from 0.05 to 0.3 μm in diameter were involved and these were invariably phagocytosed singly. There was no evidence of phagocytosis of accumulations of smaller particles although such particles were present, together with the larger graphite particles, in the type 1 macrovesicles; they were presumably ingested by pinocytosis. Thus it would appear that, compared with *Acanthamoeba*, the digestive cell of *Cardium* phagocytoses single particles of a much smaller size, i.e. the 'critical volume' for phagocytosis is lower. The upper size limit for phagocytosis is not known since 0.3 μm represents the largest particles of graphite present in the lumen of the tubule.

The experiments carried out in this preliminary study were such that it is possible to do little more than speculate on the subsequent fate of the ingested material. Of the remaining

macrovesicles, types 3 and 3*a* would seem the most likely candidates for residual bodies representing the end-point of intracellular digestion (de Duve & Wattiaux 1966). The contents are invariably distributed in electron-dense clumps round the periphery of the vesicle and they tend to occur with increasing frequency in blood-fed animals over the first 12 h after feeding. Of the remaining macrovesicles, type 2, with its diffuse contents, would appear to be intermediate between types 1 and 3, and the main site of digestion. The role of the type 4 macrovesicles in this sequence of intracellular digestion is difficult to suggest. They possess a characteristic structure which appears to bear little relationship to any of the other three types. Nevertheless, they do give positive results to tests for acid phosphatase activity and also on occasions contain exogenous material derived from the lumen of the tubule, although generally in lower concentrations than the other three types. One possibility is that they are derived from the Golgi vesicles and as already indicated function as primary lysosomes which operate in conjunction with the type 2 phagolysosomes to produce type 3 residual bodies.

A general picture of the structure and function of the digestive tubule of *C. edule* can now be drawn. The epithelium lining the tubule consists of at least two types of mature cells, secretory basiphil cells and digestive cells. There are also flagellated, basiphil cells which appear to be immature and may serve to replace one or both types of mature cell. The role of the digestive cells is clearly to ingest and further process material which has reached the lumen of the tubules from the stomach. The role of the basiphil secretory cells is less clear, particularly in the absence of any knowledge concerning the nature of the secretion. It is almost certainly a protein and probably enzymic. It is possible that these cells are the source of the weak proteolytic actvity that has been recorded in the gastric juice of various species of bivalves (Reid 1965).

To what extent this pattern of the digestive tubule of *C. edule* is repeated in other bivalve remains to be seen. It is anticipated that this essentially morphological study will provide a basis for further experimental work, particularly on the uptake and intracellular digestion of materials more closely related to the natural food of these animals than those used in the present study.

List of abbreviations

av	autophagic vesicle	*li*	lipid
bb	basal body	*lu*	lumen
bc	basiphil cell	*m*	microvilli
cf	circular muscle fibre	*MBC*	mature basiphil cell
cr	flagellar rootlet	*mf*	muscle fibres
cs	collagen sheath	*mi*	mitochondrium
dc, *DC*	digestive cell	*mv*, *mvl*	microvesicle
f	flagellum	*n*	nucleus
g	graphite particle	*p*	phagosome
Ga	Golgi apparatus	*p*1–*p*4	macrovesicles (digestive spheres)
Gb	Golgi body	*pv*	pinocytic vesicles
ger	granular endoplasmic reticulum	*r*	ribosome
gl	glycogen	*s*	coat of type 4 macrovesicles
Gs	Golgi saccule	*sd*	septate desmosome
Gv	Golgi vesicle	*sv*	secretory vesicle
IBC	immature basiphil cell	*tb*	terminal bar
lf	longitudinal muscle fibre		

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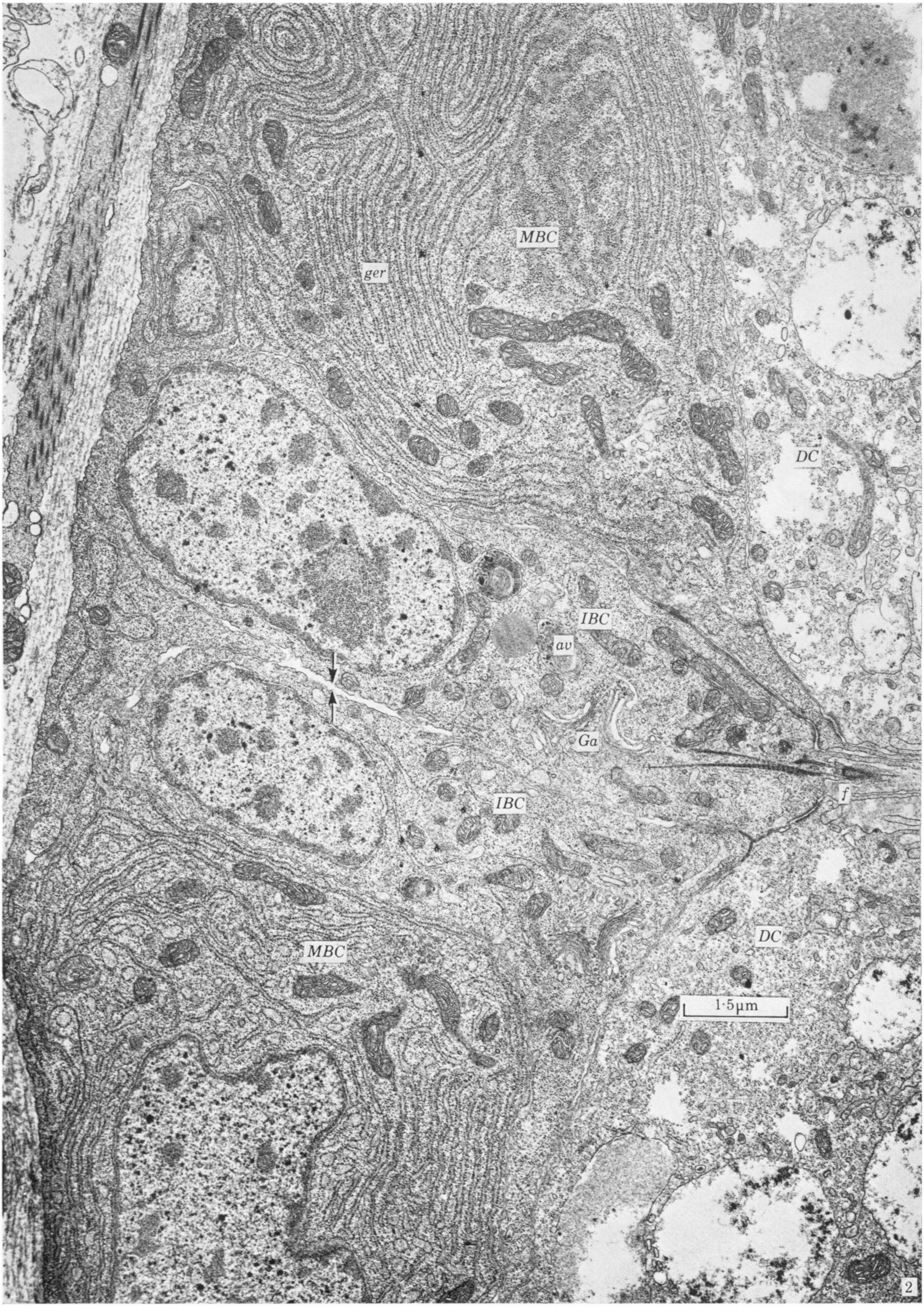
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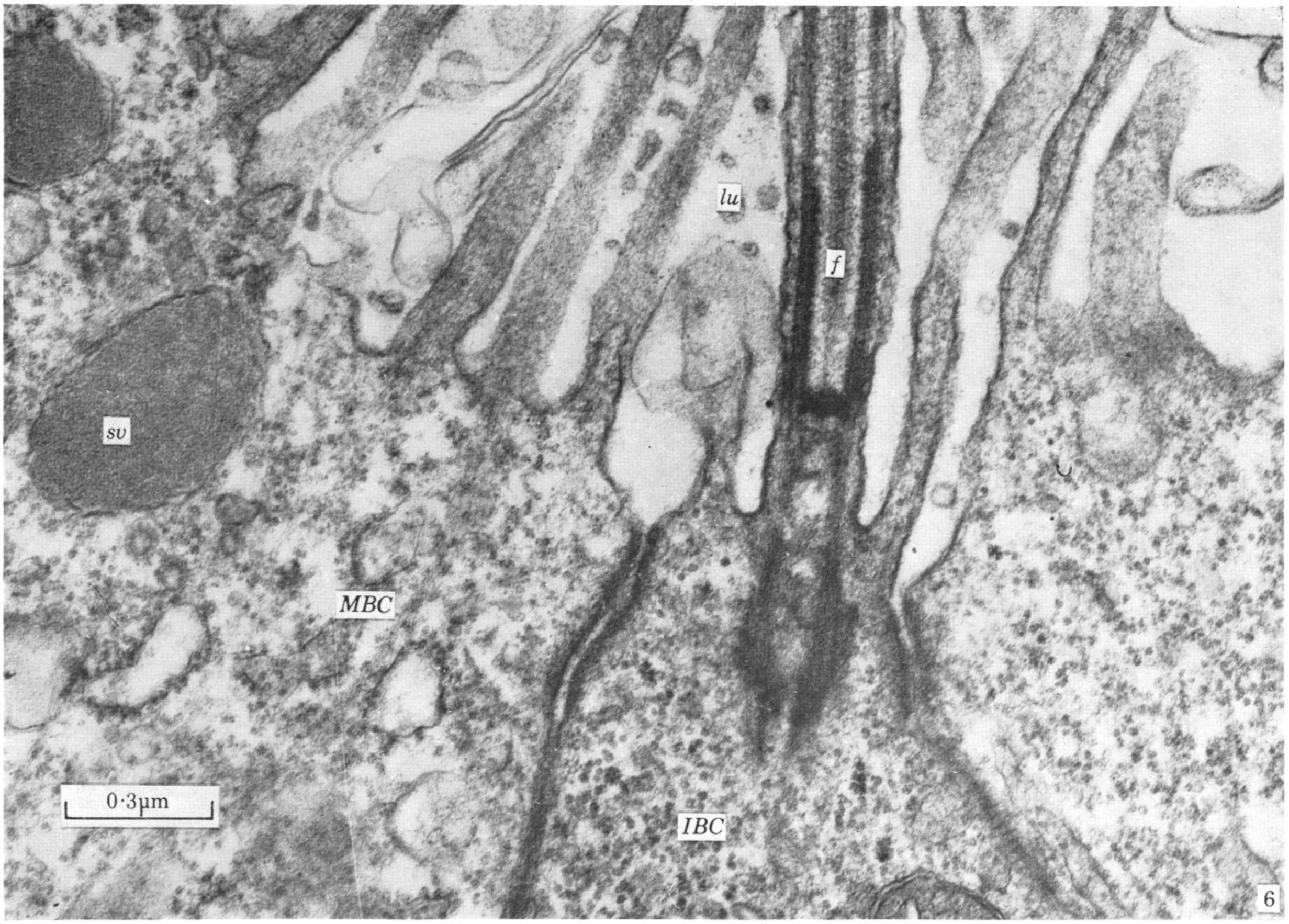
MBC
DC
1·5μm
2

lu
SU
SU
Gv
Gb
sv
gl
1·0μm
Gb
3
0·4μm
lu
sd
sv
tb
sv
4



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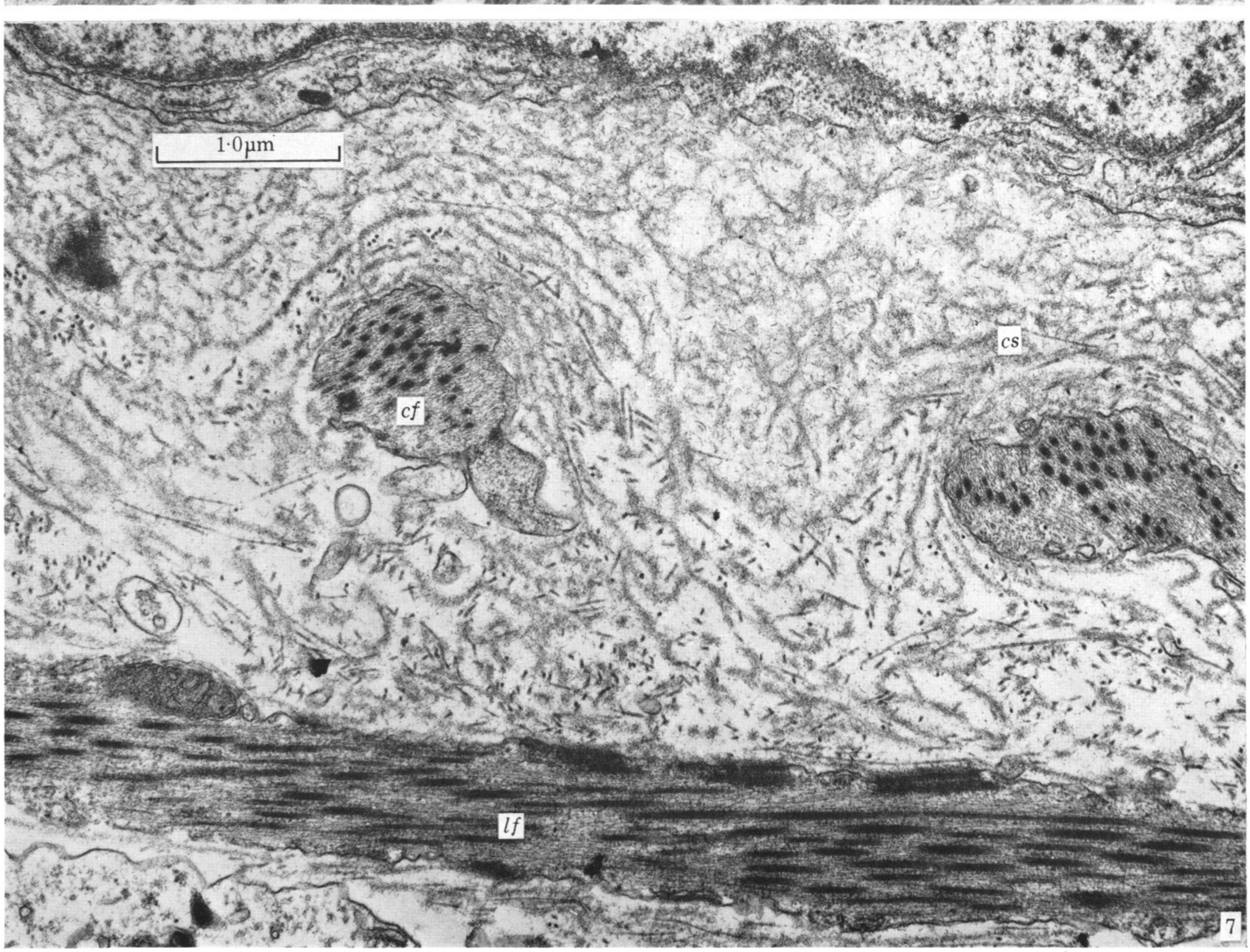

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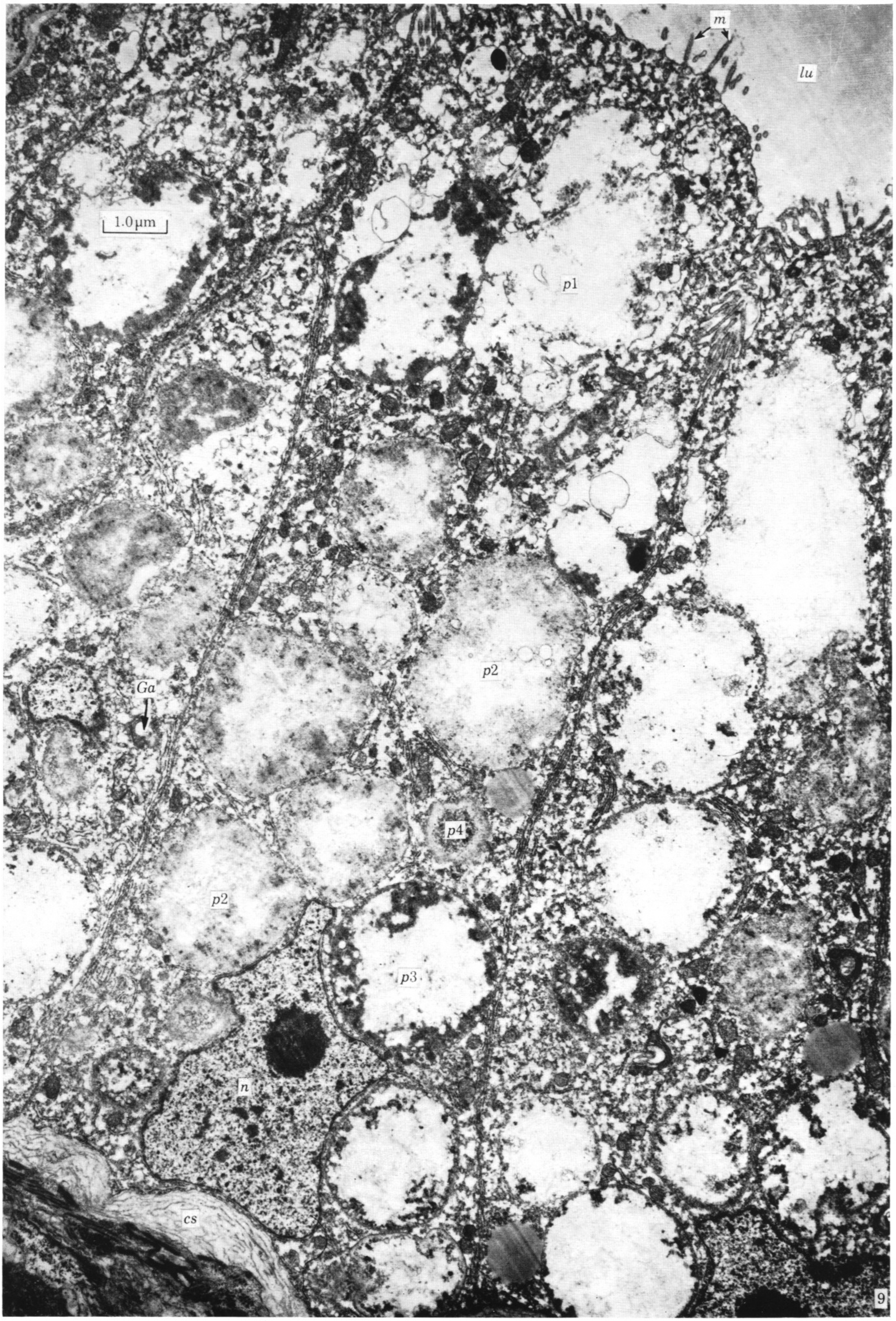


FIGURE 9. Electron micrograph of a digestive cell showing the general columnar form of the cell and the four types of macrovesicle ($p1$ to $p4$) typically present.

p2

Gv

li

p3

p2

p3

1·0μm

li

n

10

Ga

s

p4

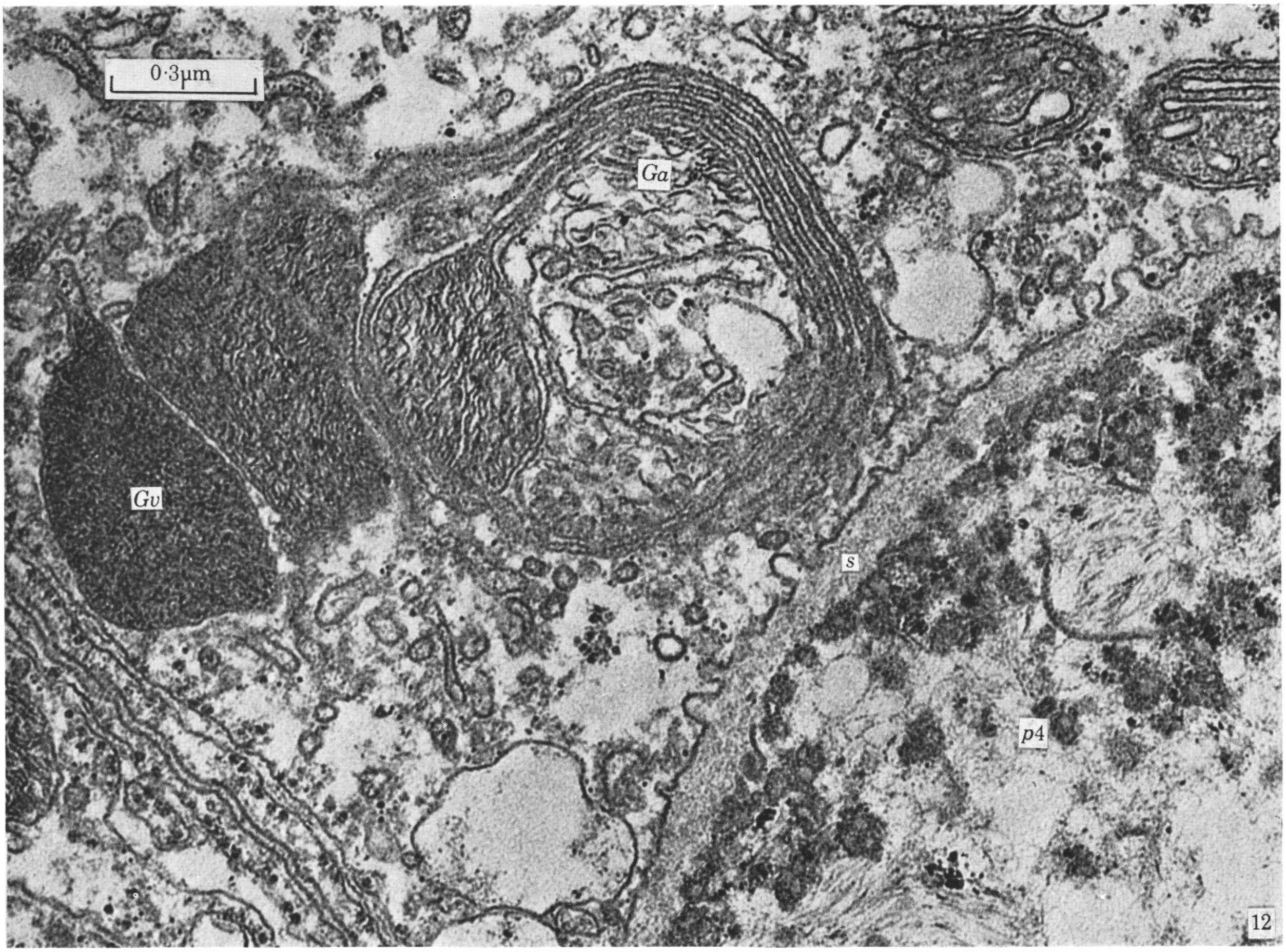
0·5μm

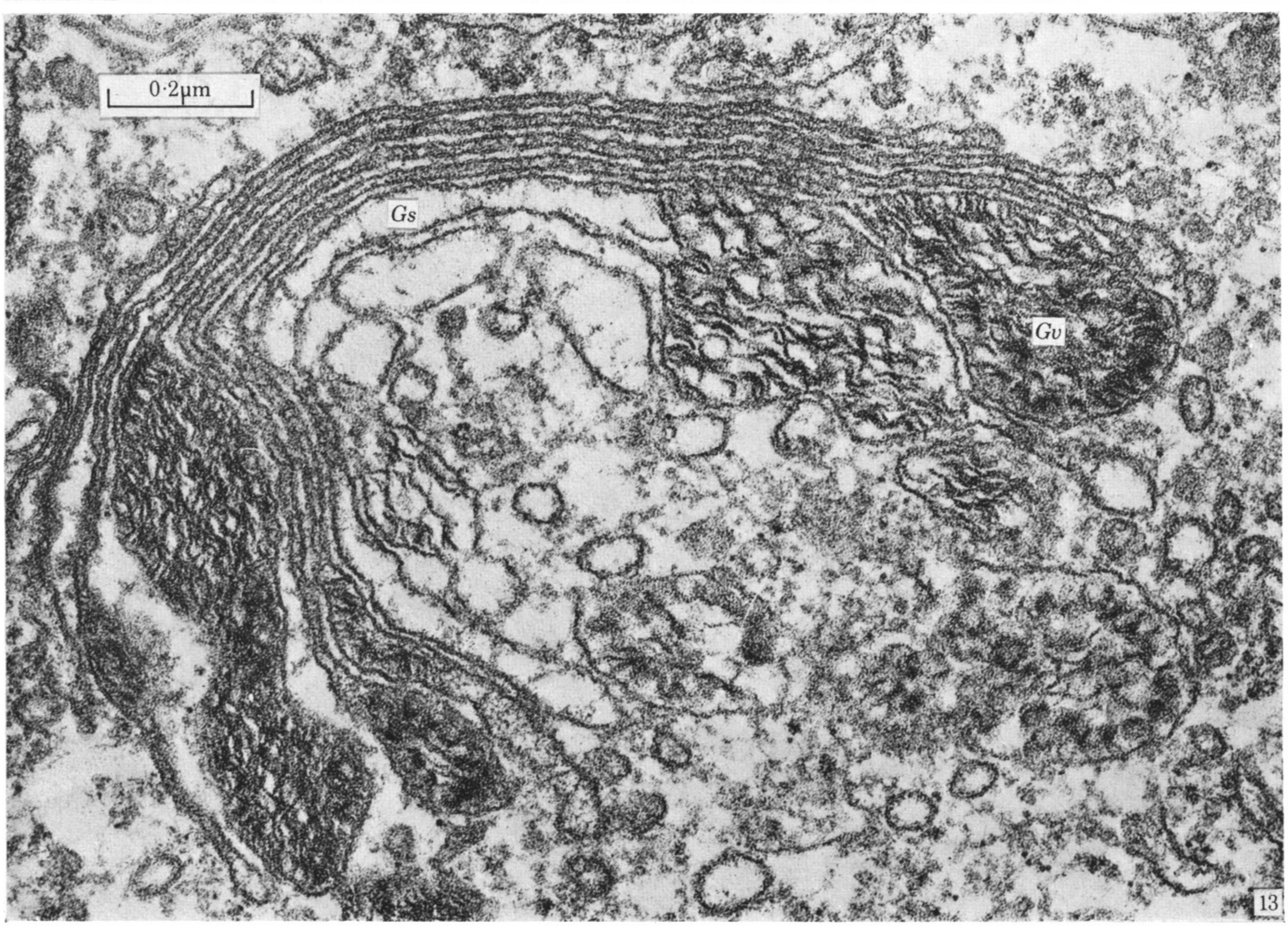
li

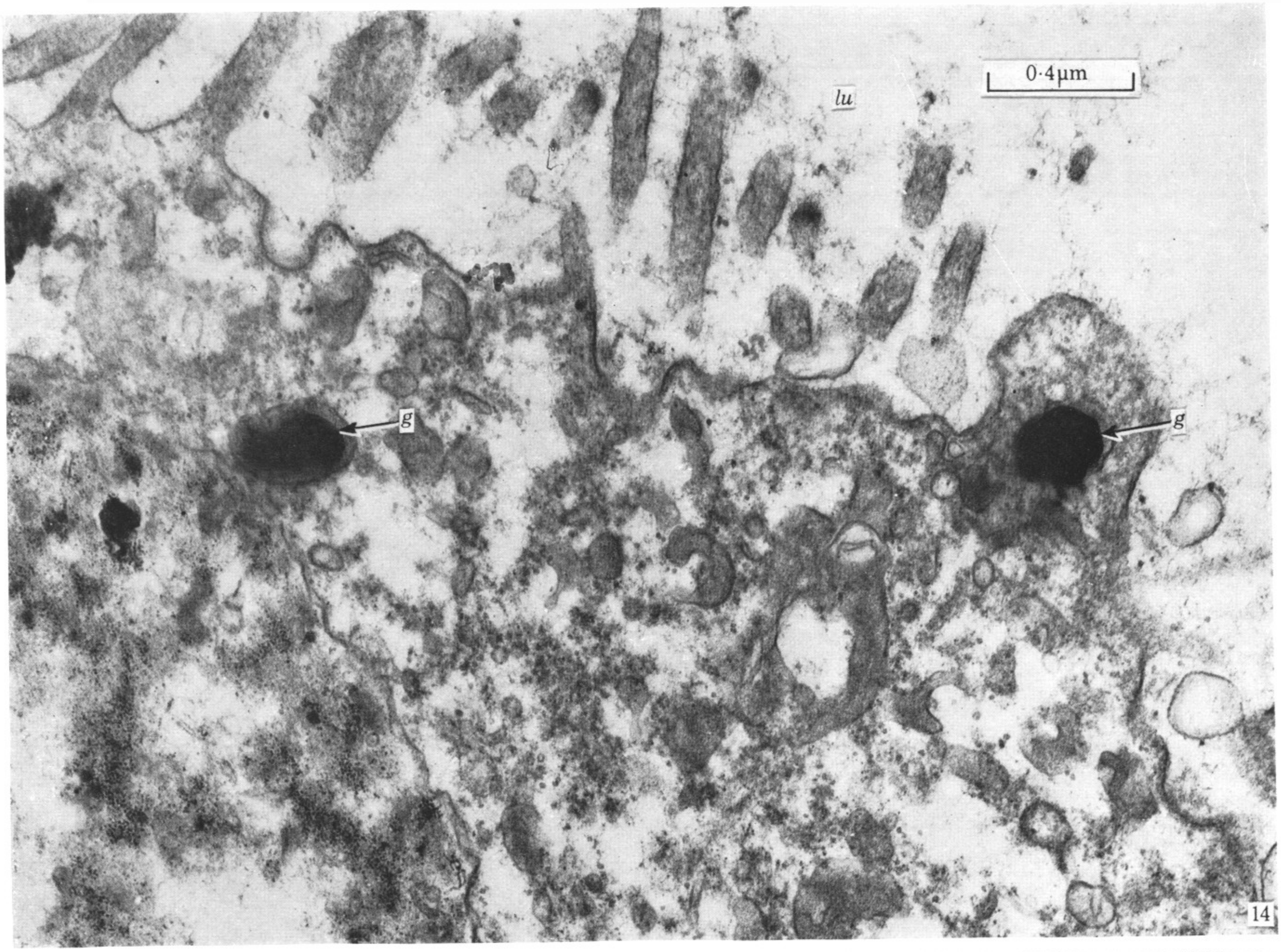
11

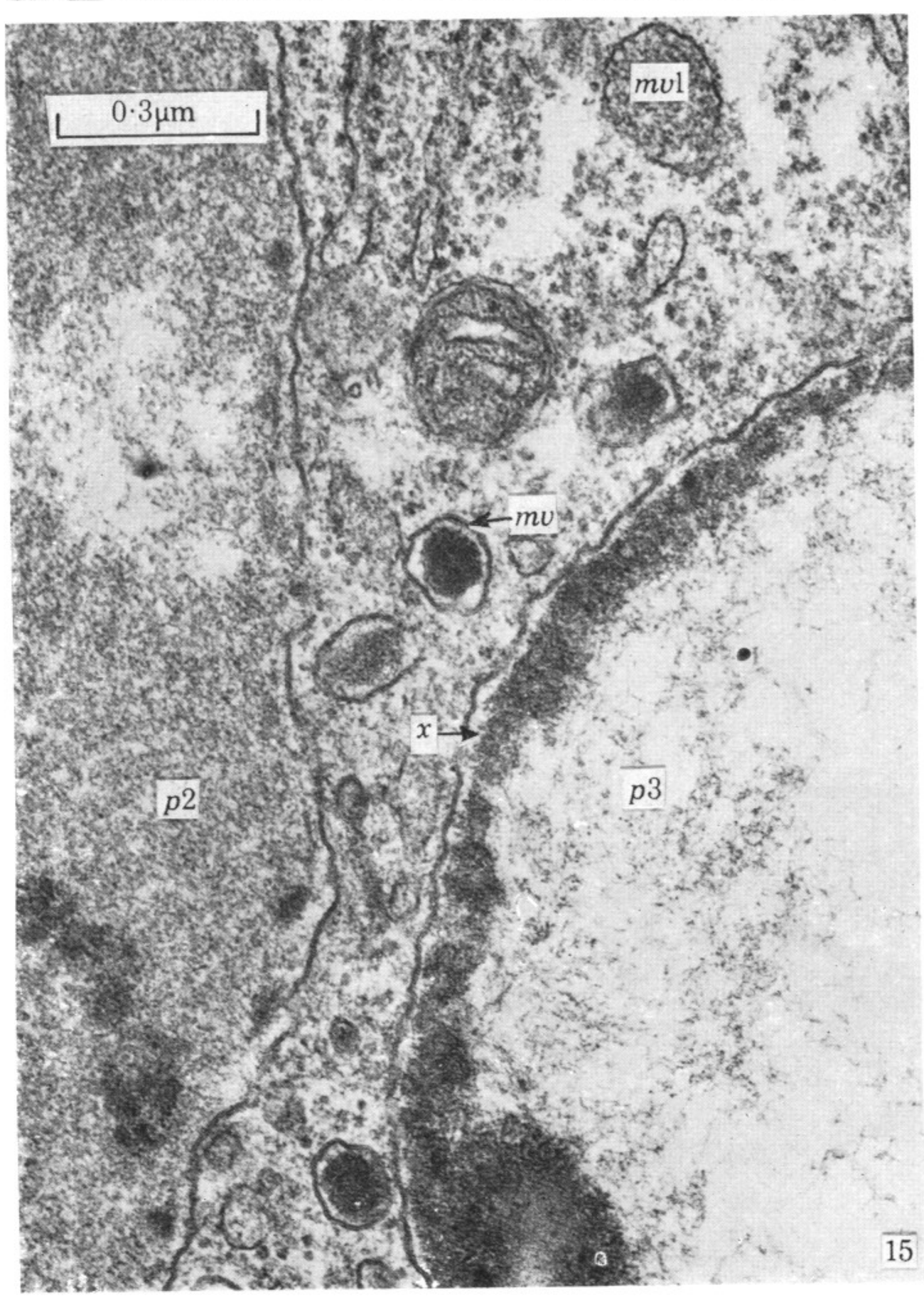
FIGURE 10. Electron micrograph of the mid-region of a digestive cell showing details of two types of macrovesicles (*p*2, *p*3) which may represent phagolysosomes and residual bodies respectively.

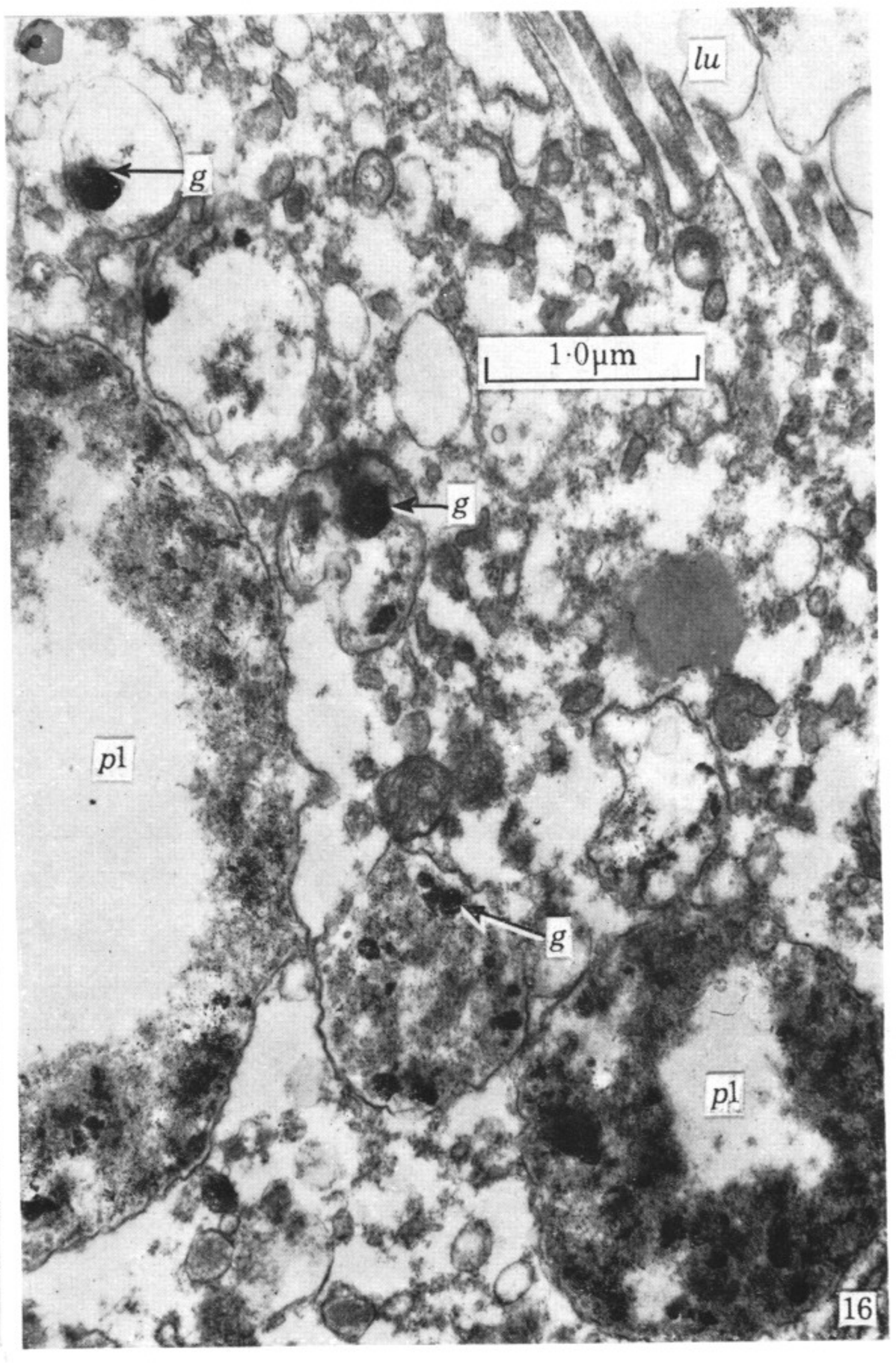
FIGURE 11. Electron micrograph of a type 4 macrovesicle, showing the characteristic coat which surrounds this type of vesicle; note the adjacent Golgi body (*Ga*).

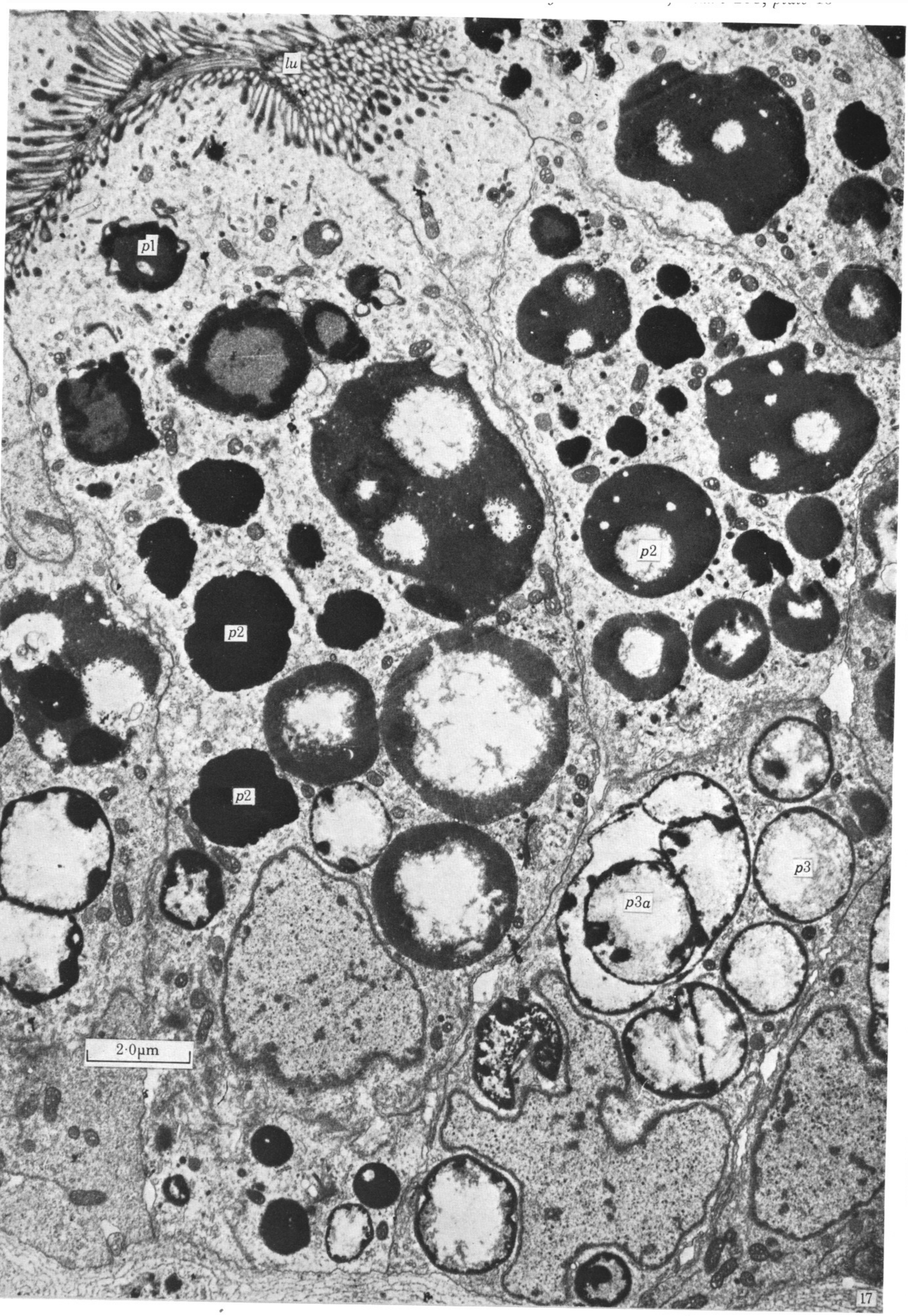

0·3μm
Ga
Gv
s
p4
12

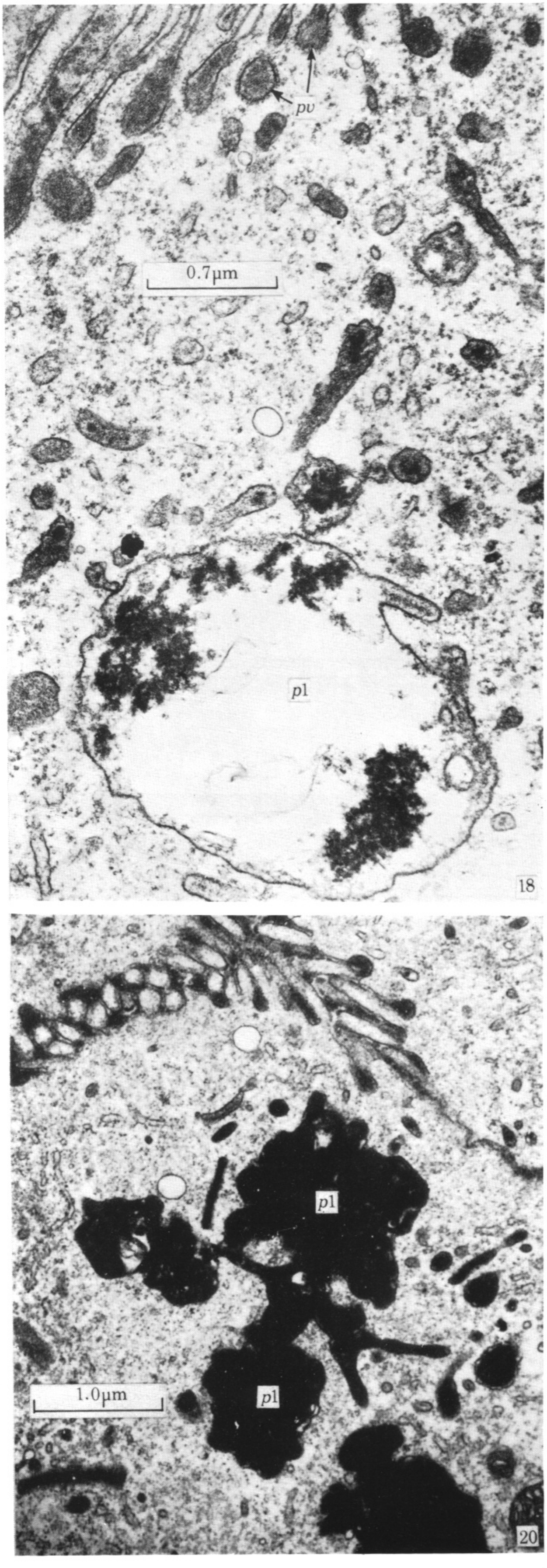

0·2μm
Gs
Gv
13


0·4μm
lu
g
g
14


0·3μm
mvl
mv
x
p2
p3
15


lu
g
1·0μm
g
pl
g
pl
16


lu
pl
p2
p2
p2
p2
p3a
p3
2·0μm



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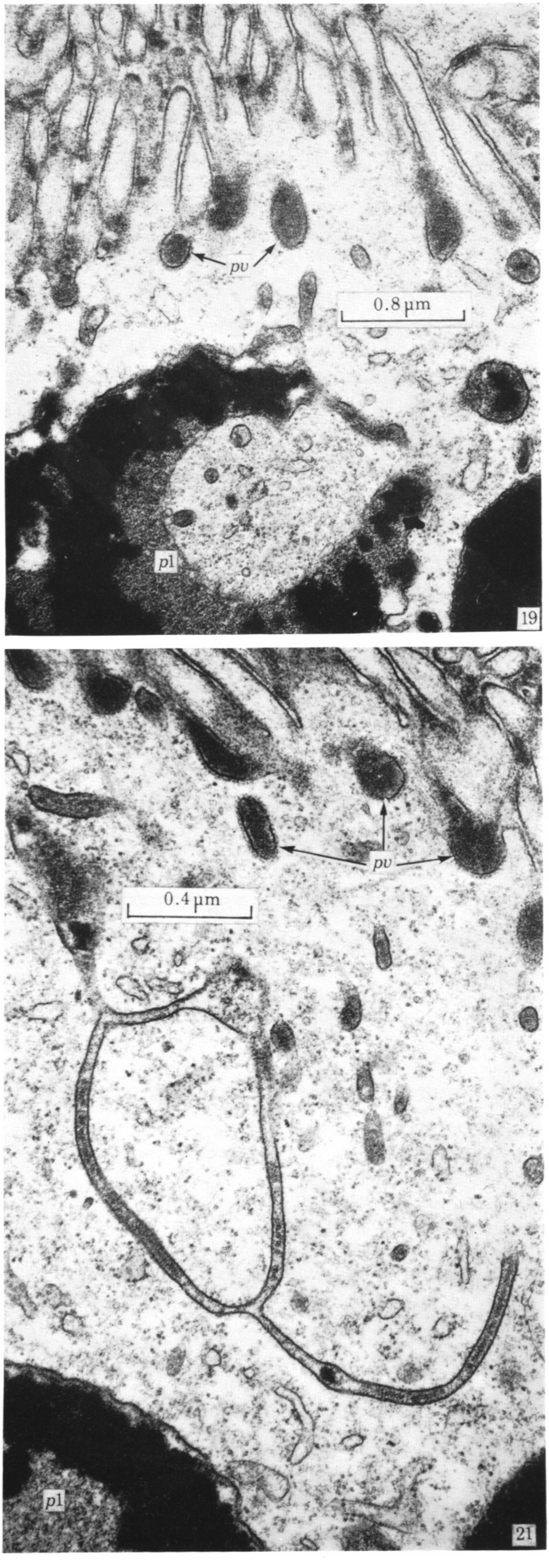

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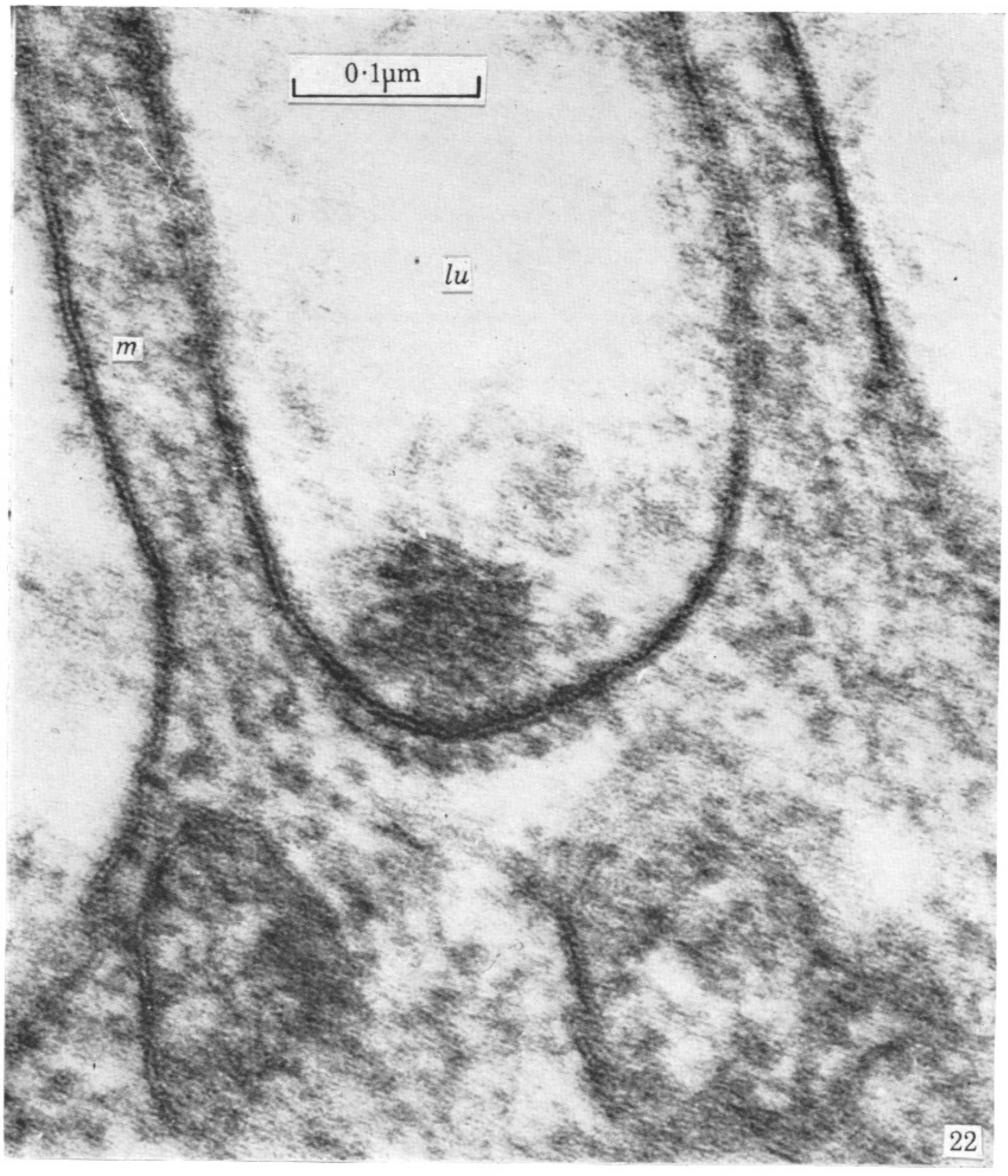

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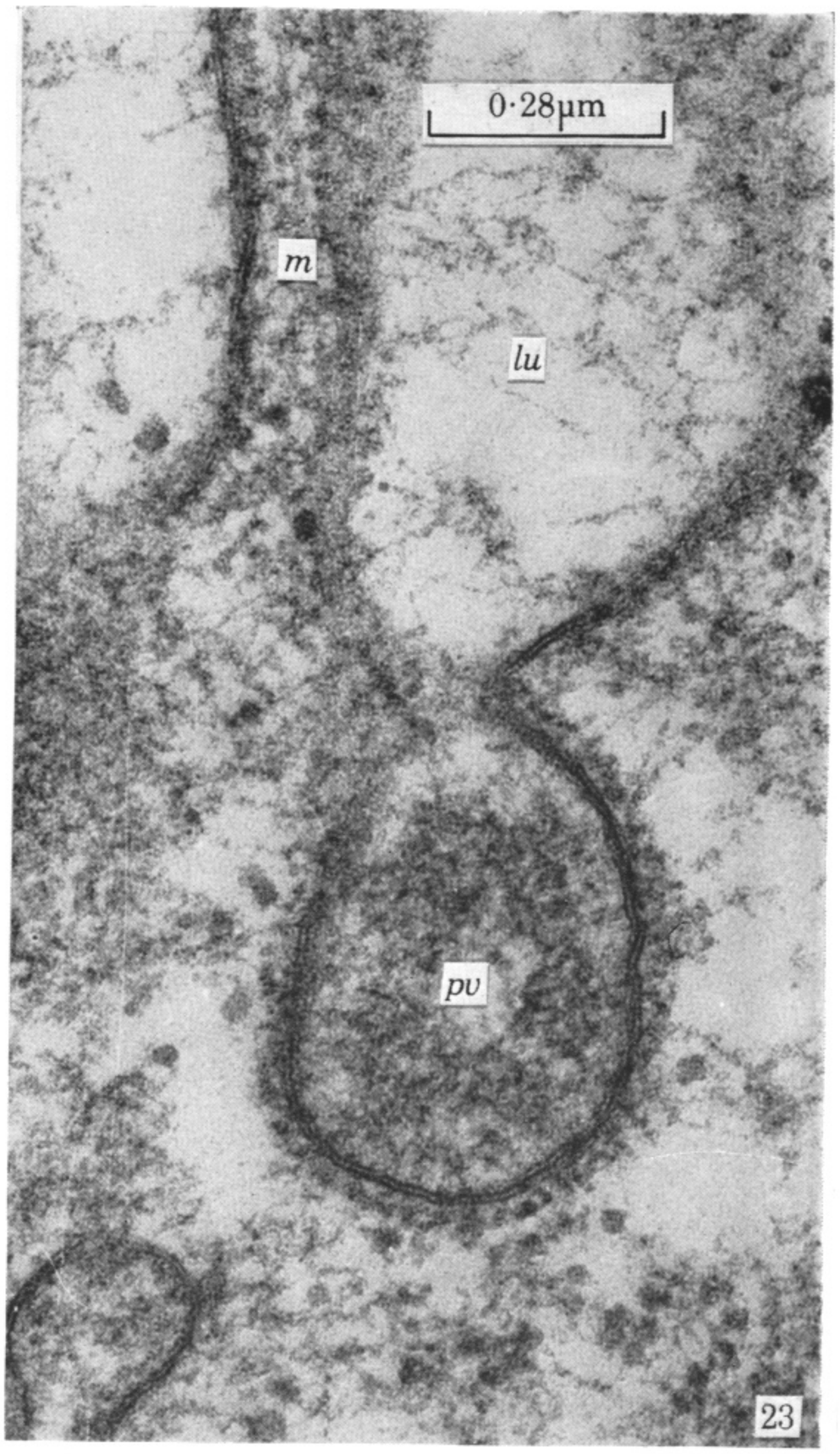

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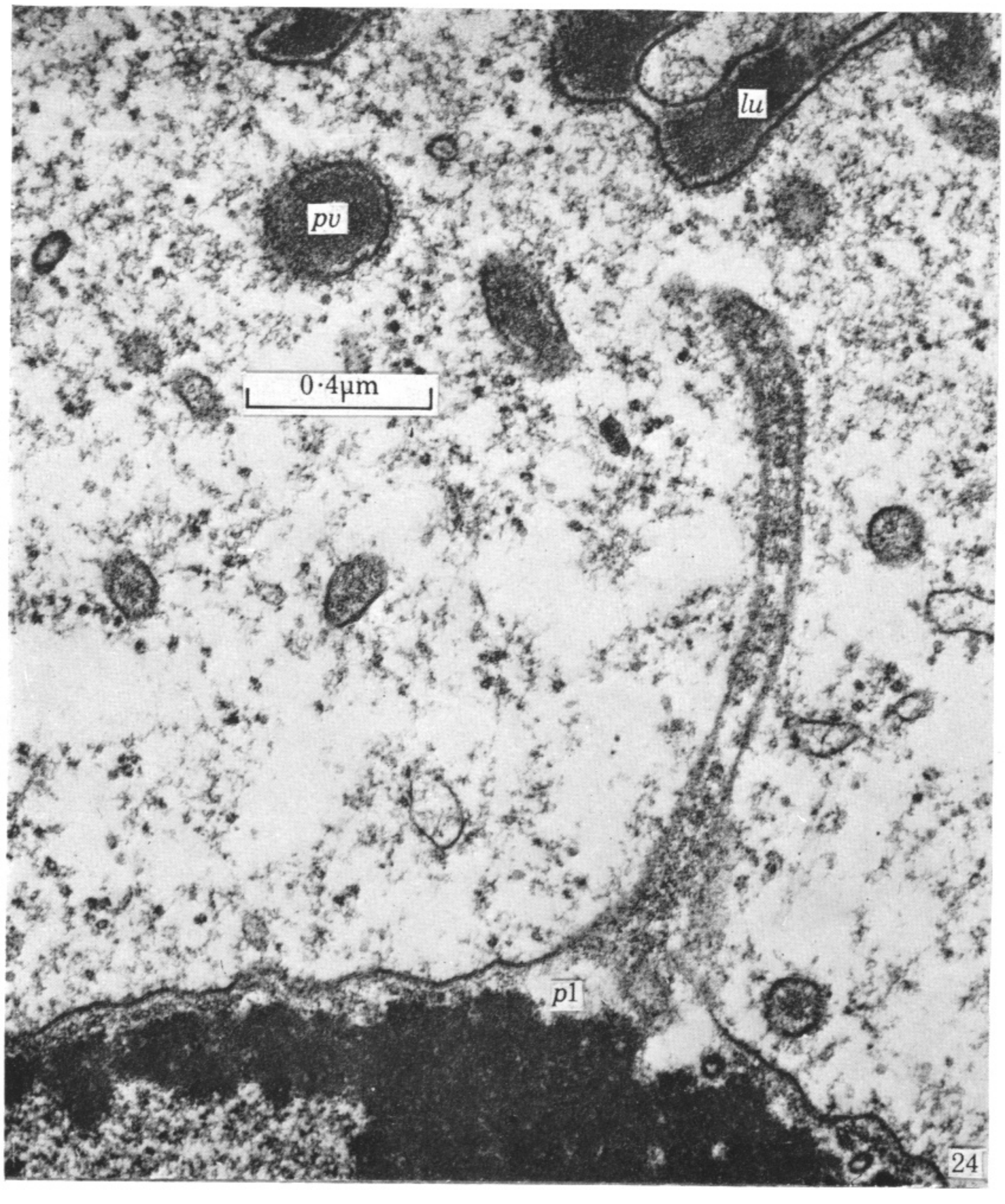

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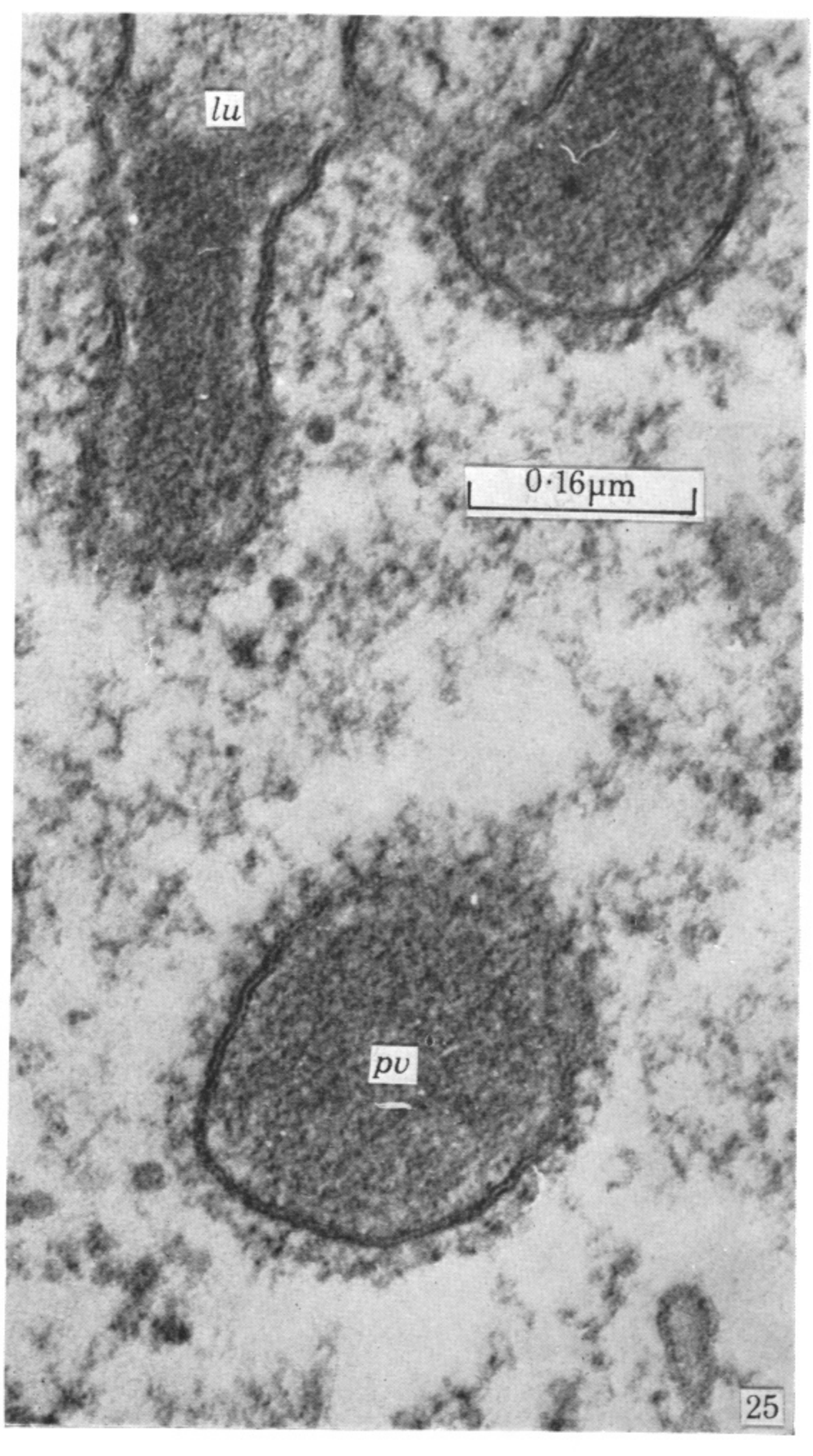

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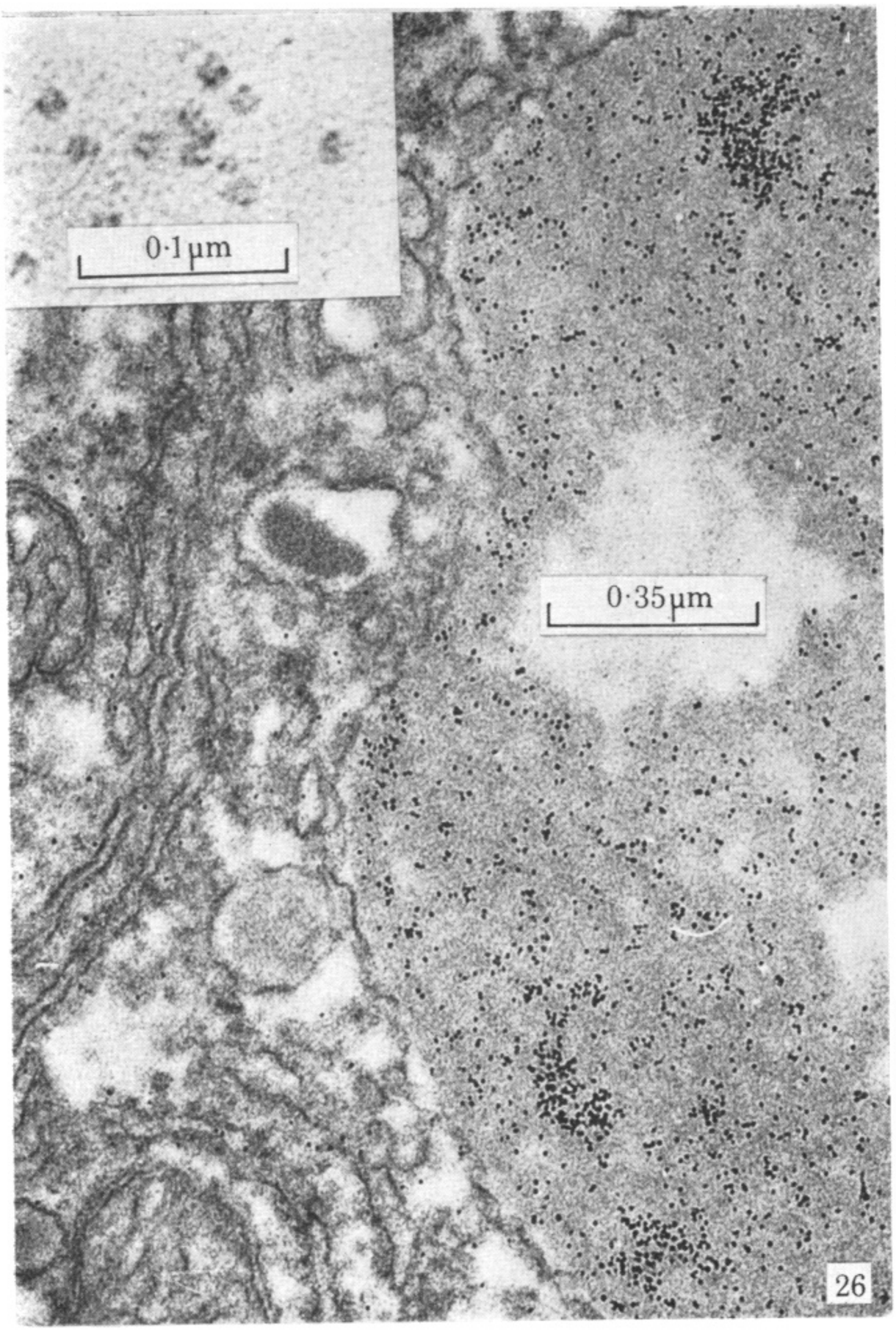

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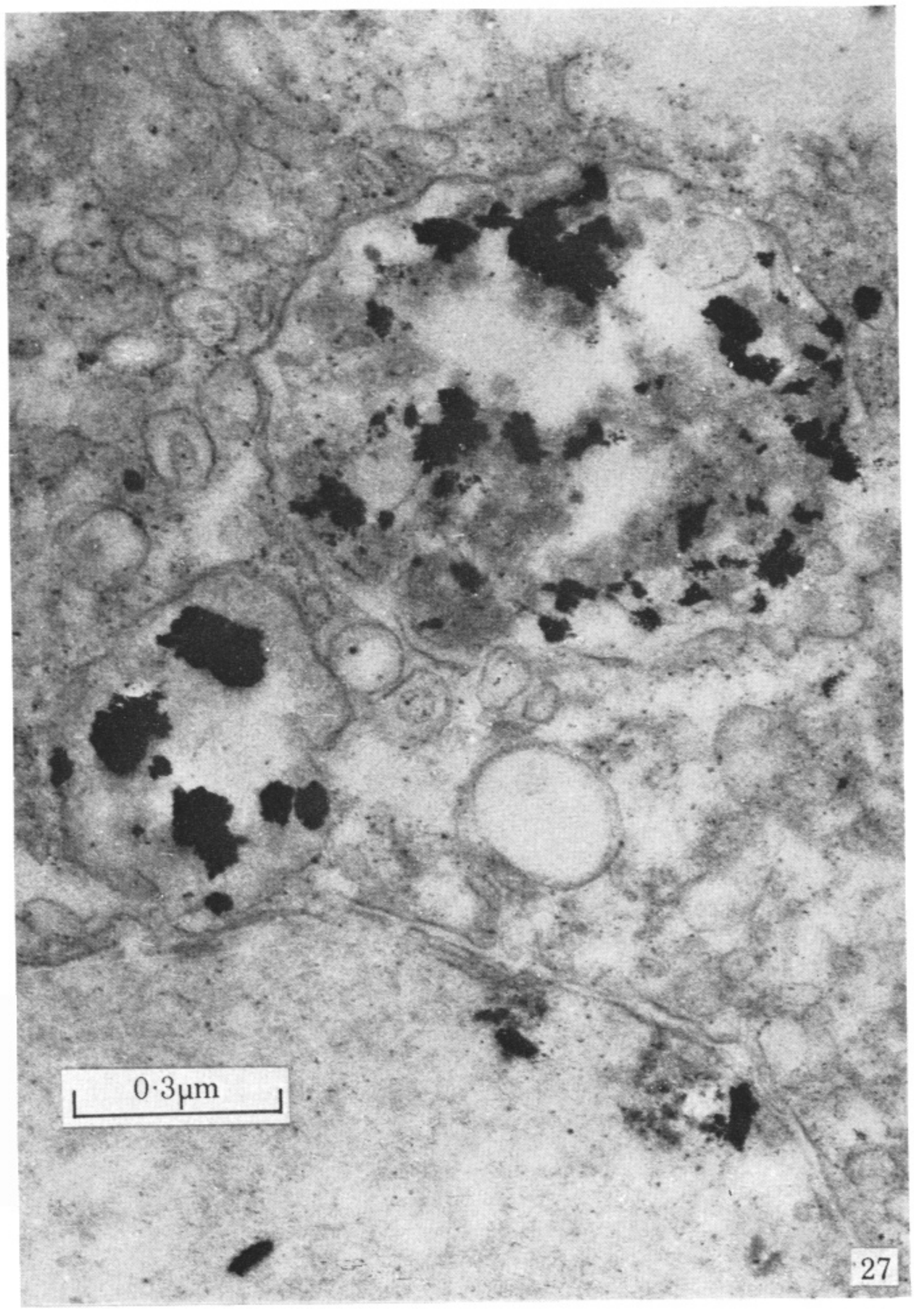

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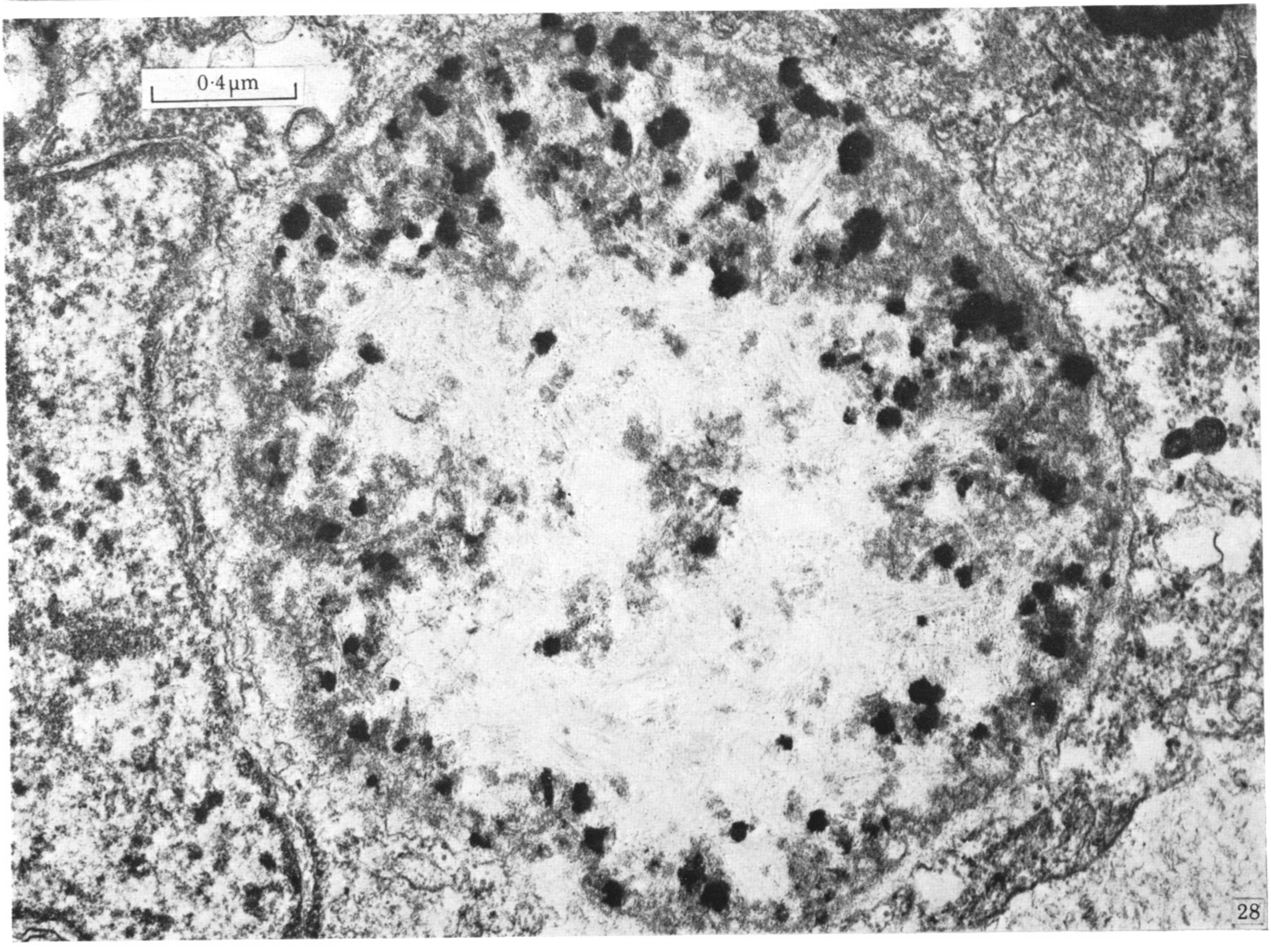

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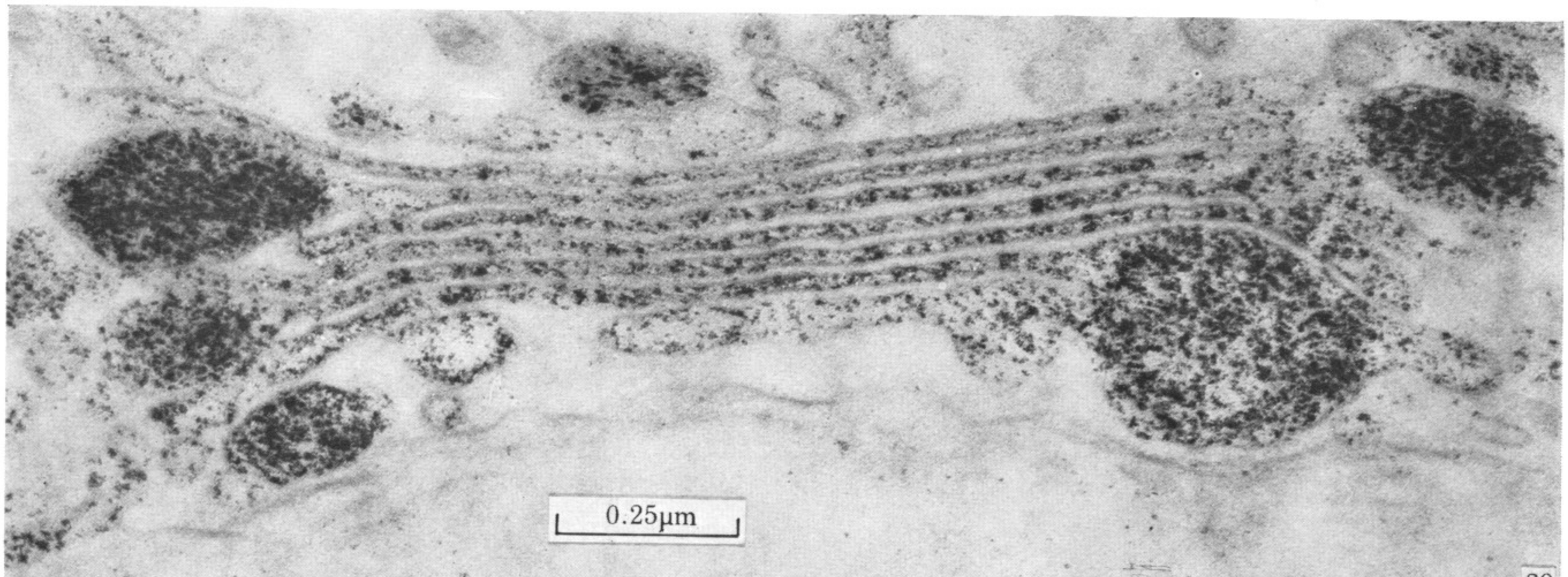
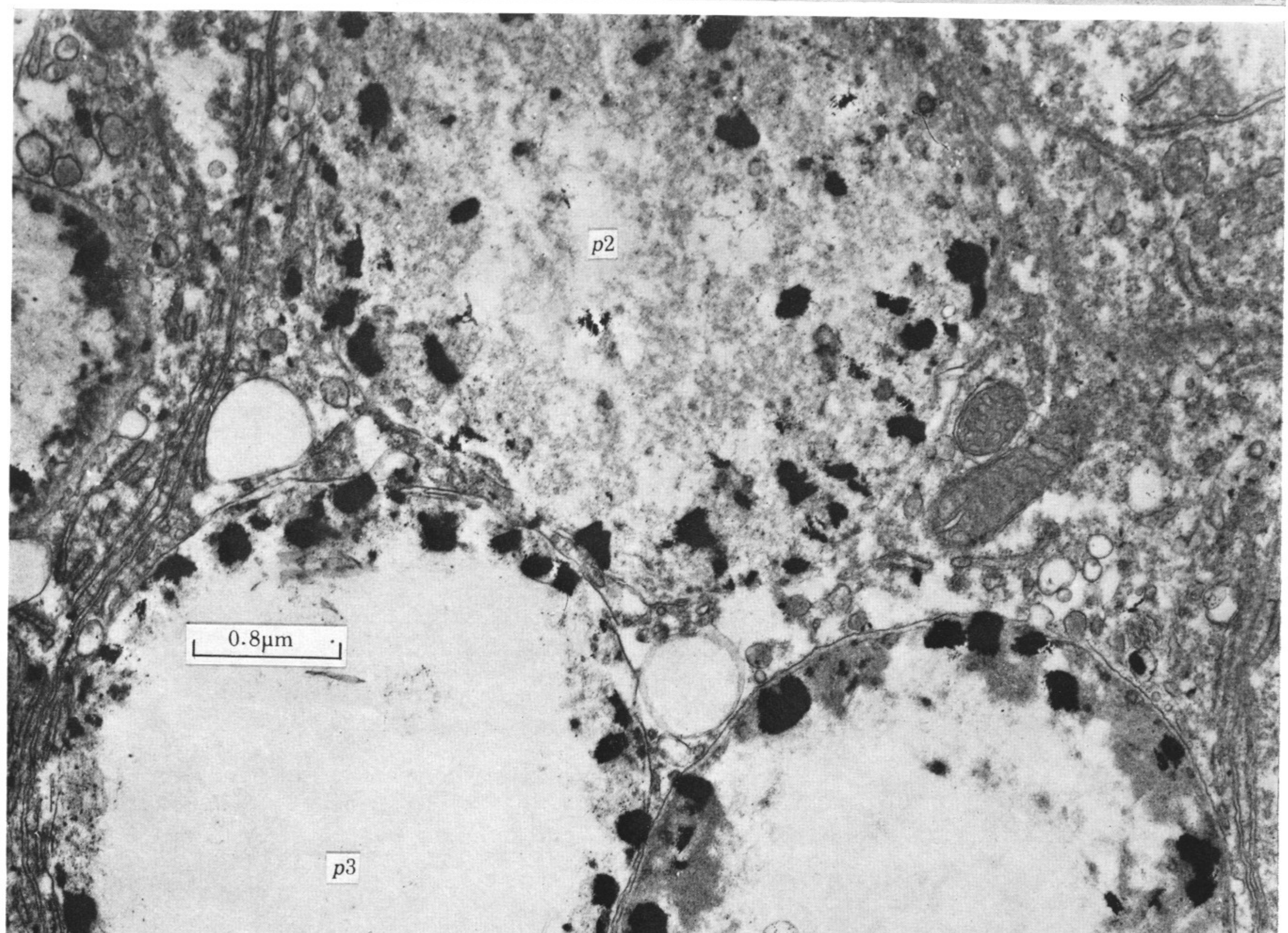

0.25μm


p2
0.8μm
p3
p3