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The membrane relationships of smooth muscles: an electron microscope study

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INTRODUCTION

Smooth muscles have been classified physiologically into two groups, multi-unit muscles such as the nictitating membrane and constrictor pupillae, and those muscles which act as a single unit, as in the gastro-intestinal tract and ureters (Bozler, 1948). Single unit muscles exhibit spontaneous rhythmic activity, which is conducted from cell to cell and is independent of nervous influence although it is modified by it. Multi-unit muscles are characterized by the absence of myogenic conduction, and their excitation is purely nervous.

The light microscope has not revealed any differences in structure between the two types of muscle which could explain their different physiological properties. Electron microscope studies of muscles of the single unit type have been made by Mark (1956), Caesar, Edwards & Ruska (1957), Thaemert (1959), Prosser, Burnstock & Kahn (1960) and Taxi (1961). These authors attempted to determine the structures responsible for myogenic conduction in smooth muscle, four possible theories being considered:

(1) Mechanical pull from cell to cell.

(2) A morphological syncytium, through the existence of intercellular protoplasmic bridges.

(3) Electrical spread of excitation by means of low resistance contacts between the membranes of adjacent cells.

(4) A chemical transmitter acting between the fibres.

Thaemert reported that protoplasmic continuity could be demonstrated between smooth muscle cells, in anastomotic intercellular bridges. Mark also described discontinuities in the cell membranes between smooth muscle cells, which could be interpreted as showing a syncytial relationship between them, but he did not rule out the possibility that they were breaks due to preparation artefacts. Bergman (1958) also described intercellular bridges, but with the important qualification that these protoplasmic 'bridges' between adjacent cells are always interrupted by membranes. Bergman's findings were supported by Prosser and by Taxi.

The purpose of the present work was to examine a smooth muscle of the single unit type, giving attention to inter-cell relationships and the presence or absence of protoplasmic continuity, and to contrast this with two typical multi-unit type muscles.

The amnion musculature was chosen as an extreme example of a single unit type, because it is completely nerve free, and is known to transmit a contraction wave

(Evans, Schild & Thesleff, 1958). The nictitating membrane was chosen to contrast with the amnion because of the rich adrenergic nerve supply in the former. The constrictor pupillae was chosen as a second muscle of the multi-unit type having by contrast a cholinergic nerve supply.

MATERIALS AND METHODS

Amnion. Chick eggs incubated for 8–14 days were used. A window was cut from the shell and 1 % osmium tetroxide in Ringer's solution buffered at pH 7·4 in veronal acetate was poured on the surface of the amnion. The membrane was then removed, cut into small pieces and placed in fresh fixative at 4° C. for 3 hr. After dehydration in graded ethanols the specimens were placed in 1 % phosphotungstic acid in absolute ethanol for three hours. Araldite embedding was used, and sections were examined with a Siemens 1b electron microscope.

The *constrictor pupillae* and *nictitating membrane* muscles were obtained from cats and rabbits anaesthetized with nembutal. The constrictor pupillae was exposed by making a circular incision in the eye-ball at the ciliary margin and the anterior part of the eye-ball containing the iris was inverted and osmium tetroxide fixative poured into it. The iris was then cut in the fixative into narrow strips by making radial incisions 1 mm. apart. The medial muscle of the nictitating membrane was exposed, removed and transferred to fixative. It was cut into pieces about 1 mm. cube.

Subsequent treatment for both the nictitating membrane and constrictor pupillae followed that described for the amnion.

OBSERVATIONS

Amnion. Pl. 1 shows a low-power view of an amnion after 8 days incubation. Lining the amniotic cavity there is a layer of epithelium one or two cells thick, which is separated from the muscle coat (*m.c.*) by a thin layer of loose connective tissue (fibroblasts and collagen). On the chorionic surface of the muscle coat there is another layer of loose connective tissue. The muscle coat is invaded from both surfaces by invaginations of the connective tissue. After 8–10 days incubation the coat is four or five cells thick but growth of the embryo produces gradual stretching and by about 16 days it is only one cell thick.

Pl. 2 shows a transverse section of part of the muscle coat at higher magnification. A thin basement membrane (*b.m.*), often faint and apparently discontinuous in places, lines both surfaces of the muscle coat. This is about 100–150 Å. thick and it follows the invaginations of the connective tissue into the larger intercellular spaces (*b.m.*[1]). Each muscle cell is completely bounded by its surface membrane, and extensive search failed to reveal evidence of protoplasmic continuity from cell to cell. The relationship between individual cells is irregular. As can be seen, in places the cells are separated by large intercellular zones containing collagen fibrils and bounded by a basement membrane. In other places the individual muscle cells are separated by narrow intercellular channels about 200–250 Å. wide, which contain no basement membrane (*x*), the basement membrane being reflected over the mouths of these channels. These close relationships may involve an extensive

border of adjacent cells, or may be confined to small areas where the tips of finger-like processes are apposed to neighbouring cells.

There are two types of membrane specialization in these narrow intercellular channels:

(1) Small heavily staining electron dense areas occur facing each other on apposed surface membranes (Pl. 3, fig. 3). The intercellular zone between these areas often shows an increase in density (*d*) but there is usually no narrowing. These intercellular connections have the appearance of desmosomes or attachment plaques but they do not show clearly the arrangement of cytoplasmic tonofibrils running into the dense zone which is usually associated with desmosomes.

(2) Occasionally the surface membranes show regions of extremely close apposition One of these is shown at low magnification in Pl. 3, fig. 4. At higher magnification (Pl. 3, fig. 5) it can be seen to be a quintuple-layered system composed of three dense laminae separated by two clear zones. This is interpreted as a region of almost or complete obliteration of extracellular space, the central lamina representing the outer surfaces of the apposed membranes (see Robertson, 1959; Karrer, 1960; Gray, 1961; Peters, 1962).

In longitudinal section the muscle cells are elongated with tapering, lobulated, centrally placed nuclei. The nuclei have the normal envelope with 'pores' and aggregations of chromatin around the periphery. There is also a peri-nuclear cluster of mitochondria and endoplasmic reticulum which is especially prominent at the poles of the nuclei.

The greater part of the cell is composed of myofilaments orientated in the longitudinal plane (Pl. 2). The majority of these are fine and lightly staining but some are larger and conspicuously electron dense. Mitochondria, Golgi apparatus (*G.a.*) and granular reticulum are seen in the cytoplasm especially at the margins of the cells. Multivesicular bodies (*m.b.*) and vesicles ranging from 200–800 Å. in diameter occur near the cell border (Pl. 2). The cell membrane frequently shows infoldings forming cisterns extending into the cytoplasm (Pl. 3, fig. 6). Some of these cisterns are closed, others appear to open at the cell membrane. Some are studded with ribosomes.

No nerves, or isolated axons or presynaptic bags were observed in any of the numerous sections examined.

Nictitating membrane. Pl. 4 shows a low-power transverse section through the medial muscle of the nictitating membrane. The muscle fibres are tightly packed together and much more regular in shape than those of the amnion. They run in small bundles separated by connective tissue consisting of a dense concentration of collagen fibres (*col*). In some of the muscle fibres the plane of section has passed through the nucleus (*nuc.*). At this low magnification the opposed boundaries of the muscle cells appear simply as thick dense lines and the myofilaments (*m.f.*) are indistinct. Fine nerves, too small to be identified at this magnification, are embedded in the extracellular channels.

Pl. 5 is a higher-power photograph showing the disposition of the contractile elements, which form a prominent feature of the muscle cells of the nictitating membrane. Numerous lightly staining myofilaments with a minority of thicker darkly staining myofilaments together form a sleeve round the nucleus and

perinuclear sarcoplasm (*sl of mf*), from which processes extend to the cell membrane. Mitochondria and vesicles are found near the surface membrane between the projections of myofilaments; they are seen at greater magnification in Pl. 6. The vesicles (*ves*), which are round or oval in shape appear to be arising from the surface membrane or opening into the cleft between muscle cells, presumably in the process of pinocytosis. At this magnification it can be seen that each cell is surrounded by a distinct basement membrane, which is 150–200 Å. thick and lies about 200 Å. from the muscle surface membrane. In some regions the basement membranes between two muscle fibres remain separated by a clear zone (e.g. at *k* and *l*). In other regions the basement membranes lie parallel and in contact (e.g. at *n*) and sometimes they appear to be completely fused (e.g. at *o*). Careful search of many sections revealed no evidence of protoplasmic continuity from cell to cell, nor of any kind of attachment plaque between cells, and everywhere the individual muscle cells were separated from each other by a basement membrane. A few sections showed small processes apparently invaginated into larger processes with no intervening basement membrane. Without serial sections it is not possible to state whether these are from the same or from different cells.

Higher power photographs show nerve fibres running singly or in small groups in the connective tissue septa which separate the bundles of muscle fibres (Pl. 7, fig. 10). The axons (*ax*), which are ensheathed within Schwann cells contain the usual components of tubules 200 Å. in diameter (*ax* 1) and mitochondria and bundles of neurofilaments which in cross section appear as dots 100 Å. in diameter (*ax* 2). Some axons show a mixture of both tubules and neurofilaments (see Elfvin, 1961). Schwann cell cytoplasm appears at this level to consists of very fine folds containing granular material. The axons send extremely fine branches into small clefts which penetrate between individual muscle cells. These small clefts usually also contain a sparse number of collagen fibrils. Axons running in these clefts are often no larger than the mitochondria of surrounding muscle cells. They may be only partially covered by extensions of Schwann cell cytoplasm (e.g. *S.c.* in Pl. 7, figs. 11 and 13) or they may be covered only by basement membrane (Pl. 7, fig. 12) or they may not even have basement membrane (Pl. 7, fig. 12, ax_3). In this terminal part of their course the axons show dilatations which contain mitochondria and numerous vesicles of uniform size (approximately 500 Å.), some of which contain electron dense material. These vesicles presumably can be termed synaptic vesicles and the electron dense material in some of them might represent catechol amines since the terminals in which they are found are adrenergic (see Milofsky, 1957; De Robertis & Pellegrino de Iraldi, 1961; Grillo & Palay, 1962; Wolfe, Axelrod, Potter & Richardson, 1962; Von Euler, 1958). In spite of observations of numerous sections at various levels on no occasion has the vesicle-containing dilatation been observed lying between the basement membrane and surface membrane of the muscle fibre. Thus apart from the vesicles no other specialization which might indicate that this is a synaptic junction has been observed.

Constrictor pupillae. This muscle has so far been studied in less detail than the amnion and nictitating membrane. The muscle cells have elongated, lobulated, centrally placed nuclei, at the poles of which mitochondria can be seen, together with numerous vesicles. A layer of myofilaments surrounds the nucleus, with

mitochondria and vesicles near the surface membrane. The basement membrane (Pl. 8, fig. 14) is a prominent feature in the constrictor pupillae. It covers the whole surface of most of the cells except for occasional areas of close apposition where it is reflected (Pl. 8, fig. 15), and the extracellular channel is reduced to a width of 200 Å. (*x*). Occasionally even this cleft is completely closed in the form of a quintuple-layered contact similar to that described in the amnion. Such a contact is shown at *z*, and in Pl. 8, fig. 16, another from this muscle is shown at high magnification. Attachment plaques similar to those of the amnion also occur in the region where the basement membrane is missing.

Axons embedded in Schwann cells cytoplasm are seen in the intercellular zones between muscle fibres; presynaptic bags naked of Schwann cell cytoplasm occur in both transverse and longitudinal section. These contain synaptic vesicles 300–600 Å. and numerous very dense mitochondria (Pl. 8, fig. 14). The presynaptic bags are partially covered by basement membrane (*b.m.*) except for the area in close contact with muscle where the surface membrane of the presynaptic bag is separated from the surface membrane of the muscle cell by a narrow extracellular cleft 150–200 Å. wide.

DISCUSSION

The smooth muscles of the amnion and the nictitating membrane have contrasting physiological properties in that the amnion has no nerves and excitation is purely myogenic, whereas in the nictitating membrane excitation is provided by nerve fibres running parallel to the muscle cells; the amnion is spontaneously active while the nictitating membrane shows no spontaneous activity; the amnion responds to stretch and the nictitating membrane does not. The present investigation sought a morphological basis to account for these differences.

The muscle cells of the nictitating membrane are regular in shape and completely surrounded by a well marked basement membrane, which separates individual cells by gaps of not less than 600 Å. In contrast the muscle cells of the amnion are irregular in contour and have a less definite basement membrane, and extensive areas of close apposition of cell membranes where the basement membrane is deficient altogether. Specialized regions of even closer apposition (the quintuple-layered systems) occur within these areas. It seems probable that myogenic conduction takes place through these areas of close apposition.

It is known that smooth muscle cells of the visceral or single unit type act as a functional syncytium, and previous workers have reported seeing a protoplasmic syncytium at an electron microscope level. Mark (1956) working on gravid and non-gravid rat uteri and gravid human uteri presented evidence of protoplasmic continuity through intercellular junctions in uterine smooth muscle, but he did not observe myofilaments passing through these junctions. Thaemert (1959), working on the gastro-intestinal tracts of rats described intercellular bridges occurring between the lateral aspects of cells. He considered these bridges to be links of muscle protoplasm. However, in our work on chick amnion we were not able to find any evidence of syncytial connexion between smooth muscle cells, and it seems improbable that syncytial connexions have any function in the conduction of excitation from cell to cell. The amnion was specially chosen as a muscle which shows

brisk spontaneous activity propagated by purely myogenic conduction, and protoplasmic continuity should be easily demonstratable in such a muscle if conduction of excitation in smooth muscle takes place through the medium of protoplasmic continuity.

The areas of close apposition of surface membranes, where the basement membrane is absent, may allow the passage of excitation from one smooth muscle cell to another. A membrane need not necessarily be a barrier to the spread of excitation, as has been demonstrated in the giant fibres of the earthworm (Adey, 1951; Bullock, 1945; Eccles, Granit & Young, 1933; Kao & Grundfest, 1957; Prosser, 1952) and in the axonal contacts of the giant nerve fibres of the crayfish (Johnson, 1924; Wiersma, 1947; Kao & Grundfest, 1956; Furshpan & Potter, 1959). These segmental septa do not show the usual attributes of synaptic junctions, since transmission is electrical and not chemical. Morphologically they resemble the areas of close apposition of surface membrane observed by us in the amnion.

The quintuple-layered systems seen at localized regions within these regions of apposition have been interpreted as areas where excitation passes from cell to cell. Kareer & Cox (1960) have found them in the musculature of pulmonary veins and suggested that they might be of significance in conduction of excitation; Robertson (1961) has also found them in the median-to-motor giant synapse of the crayfish. Dewey & Barr (1962) have observed these structures in the jejunum of the dog and have described them by the term nexus, taken to mean a region where the plasma membranes of two excitable cells are fused. They considered that the resulting fusion of the outer surfaces of the membranes without any microscopically resolvable intervening extracellular space allows electronic spread of current from one cell membrane to another in a way similar to its spread along an axon. These quintuple-layered systems have, however, been reported in situations where they cannot be considered to conduct excitation, e.g. in uterine epithelium (Karrer, 1960) and between neuroglial cells (Gray, 1961; Peters, 1962). They have also been seen by us in contacts between capillary endothelial cells.

Another way in which the quintuple-layered systems could be involved in the spread of excitation is purely mechanical, since these structures are points of strong attachment between cells and have been shown to be resistant to mechanical disruption (Whittaker & Gray, 1962). The desmosomes observed along the path of the narrow intercellular channels are also points of strong attachment. They are generally accepted as functioning in securing cohesion between cells.

The rich system of vesicles seen at the borders of the smooth muscle cells seems to lend some support to a theory of humoral transmission, especially since it is easy to find situations as in Pl. 3, fig. 6, where apparently orderly systems of vesicles are lined up on either side of regions of apposition of cell membranes. However, unlike synaptic vesicles these are very variable in size and shape and are not preferentially concentrated at these regions. Also they are even more prominent in the nictitating membrane than in the amnion where myogenic conduction is so marked.

From our observations on the constrictor pupillae it has not been possible to determine whether all fibres are innervated although nerve endings are frequently to be seen. The greater part of the surface membrane of the muscle cells is covered by a dense basement membrane, but areas of close apposition, containing quintuple-

layered systems together with desmosomes, are also found. This suggests that there may be some degree of myogenic conduction in the constrictor pupillae. Unfortunately this point has not been tested physiologically.

By contrast with both the amnion and the constrictor pupillae the nictitating membrane shows no morphological evidence of myogenic conduction at all, and this is in accordance with electro-physiological evidence. It seems probable that each individual muscle fibre in the nictitating membrane is reached by one at least of the many fine axons which can be seen running in the extracellular spaces, and in minute clefts between individual fibres. In the absence of serial sections it is impossible to be dogmatic on this point, however.

Finally, our finding, of large vesicles containing dense granules in the nerve endings of the nictitating membrane supports the observations of workers who have associated such vesicles with adrenergic endings (Milofsky, 1957; Von Euler, 1958; De Robertis & Pellegrino de Iraldi, 1961; Grillo & Palay, 1962; Richardson, 1962; Wolfe *et al.* 1962).

SUMMARY

1. The fine structure of the amnion nictitating membrane and constrictor pupillae has been examined with special reference to intercellular connexions, cell membranes, and the presence or absence of intercellular anastomosis or special conduction systems. The most striking difference between the amnion and the nictitating membrane is the presence in the latter muscle of basement membrane around every cell, while in the amnion there are large areas where the surface membrane of one cell is separated from the surface membrane of a neighbouring cell only by a narrow extracellular zone where basement membrane is absent.

2. Attachment plaques and quintuple-layered systems occur at localized regions along these narrow channels.

3. No nerve fibres could be found in the amnion.

4. The nerve supply to the nictitating membrane and constrictor pupillae is described briefly.

5. The role of the channels of close apposition between surface membranes, and of the quintuple-layered systems is discussed with reference to myogenic conduction.

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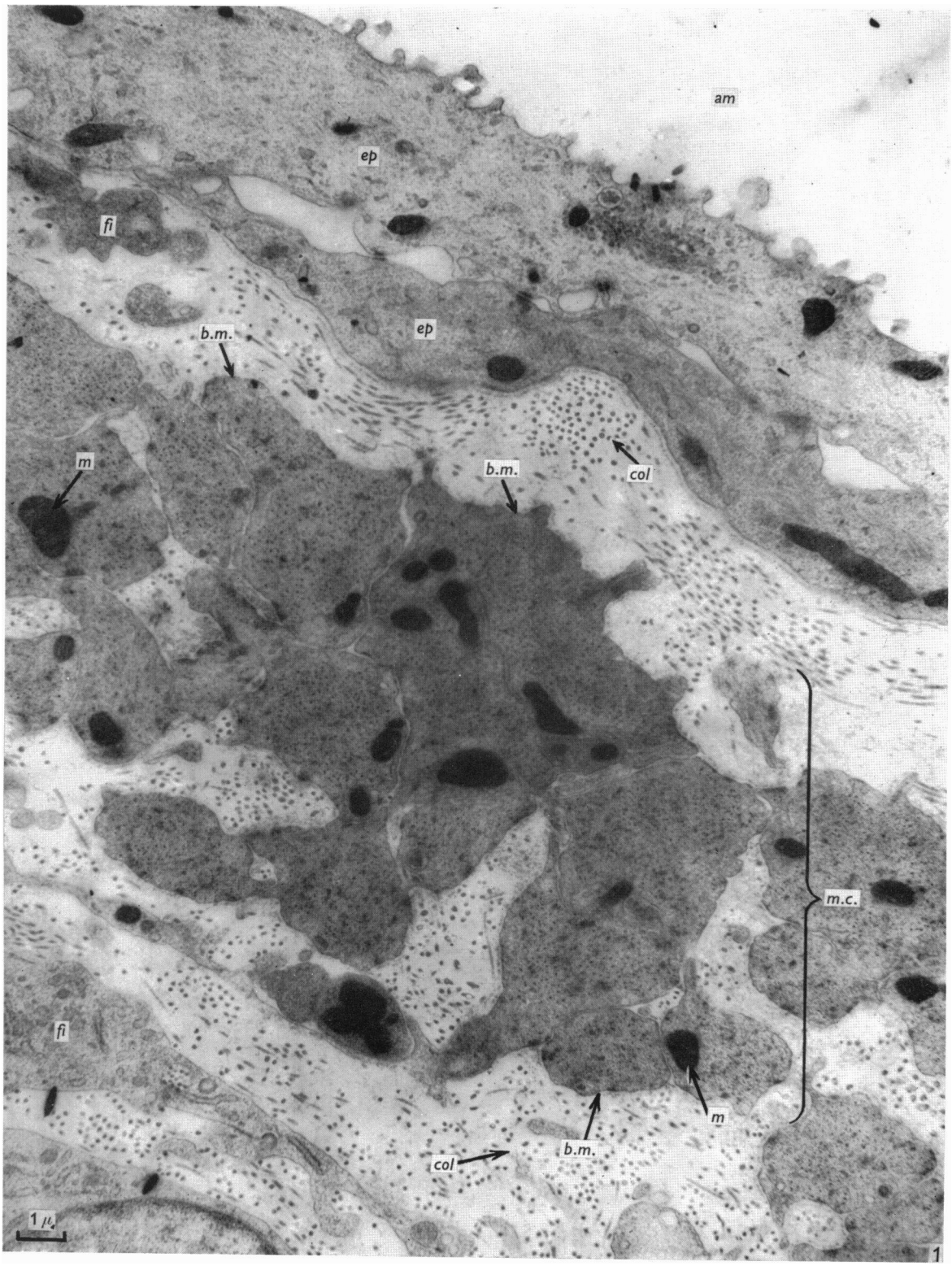

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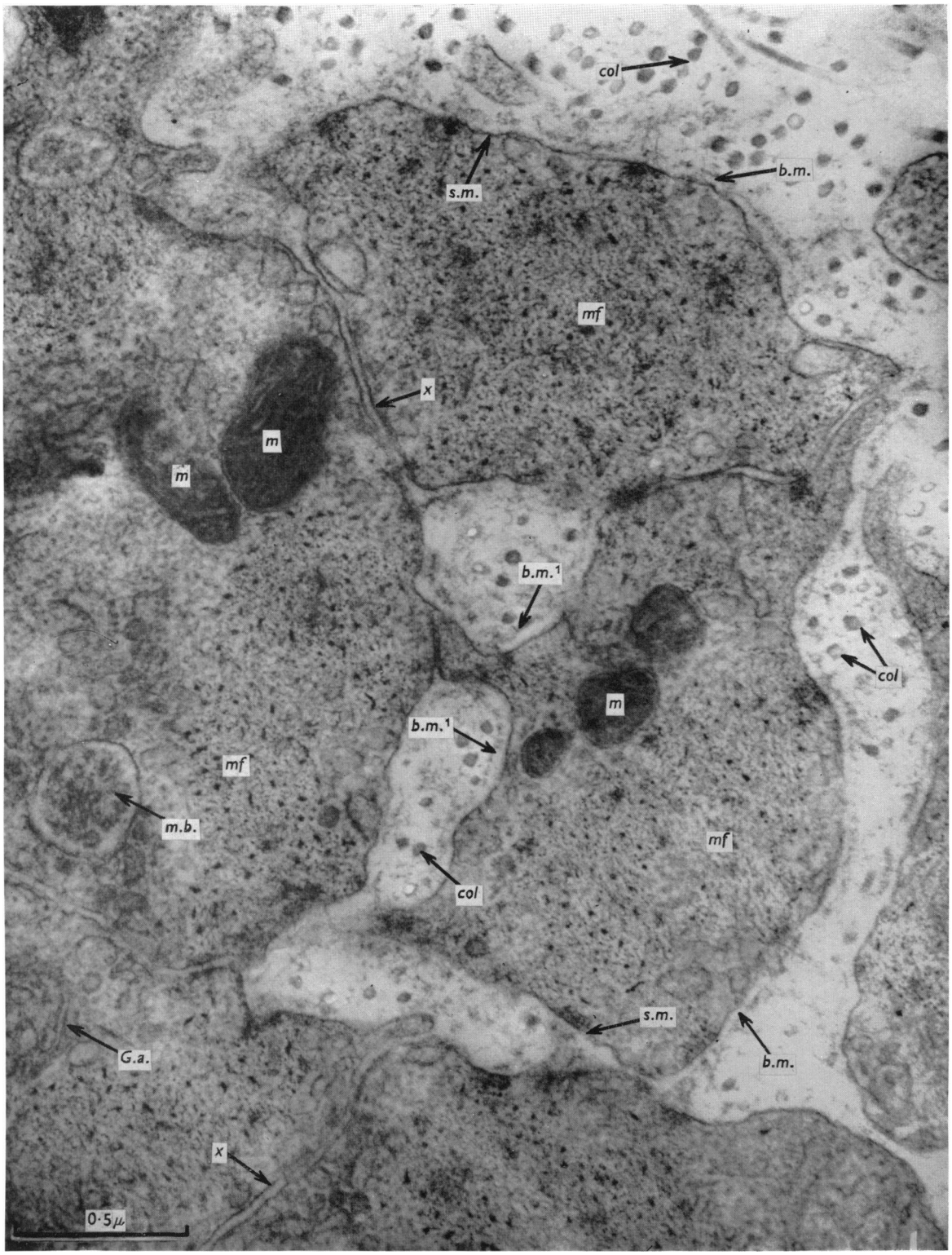

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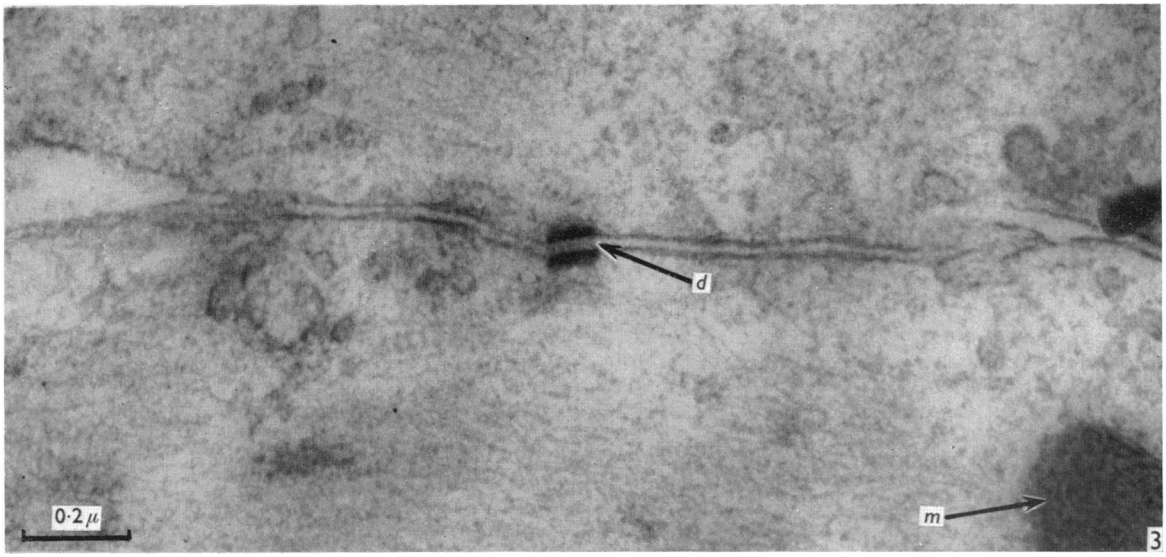
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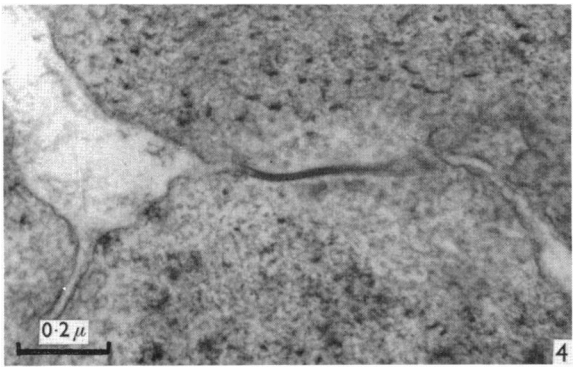
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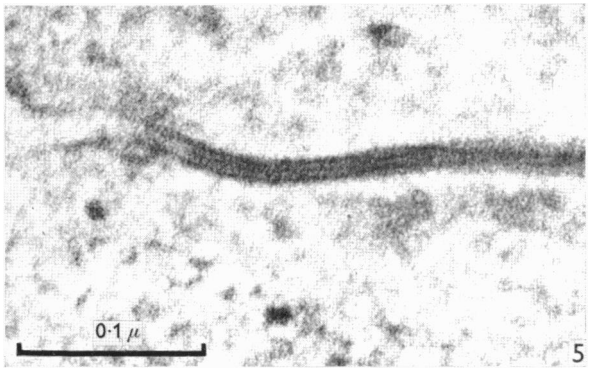
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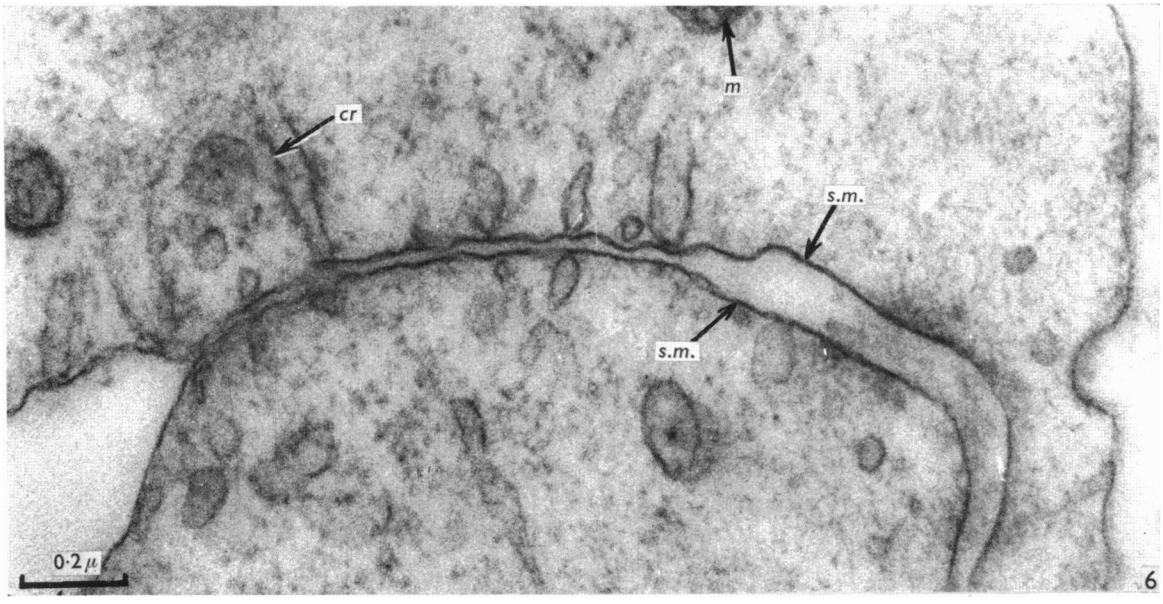
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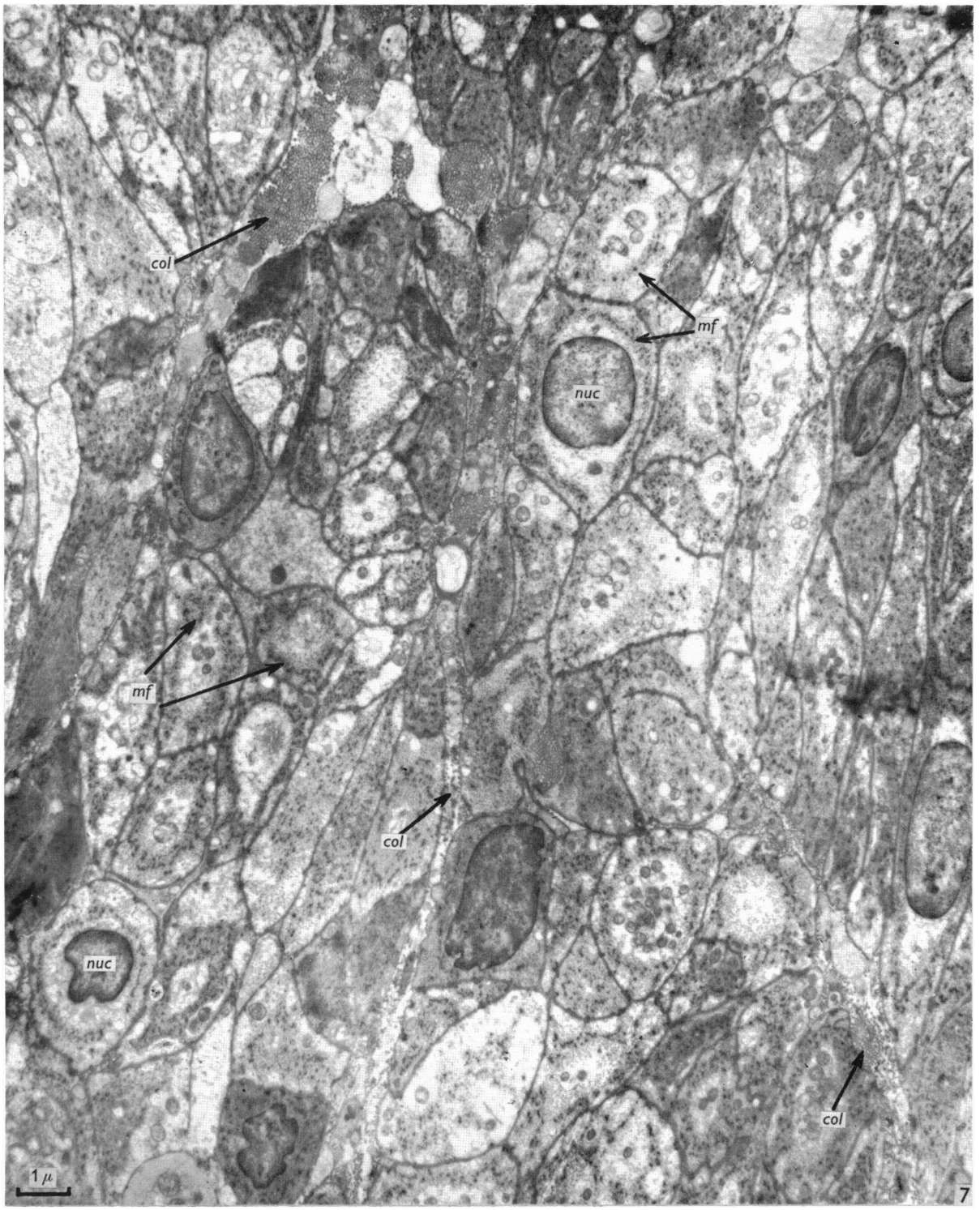
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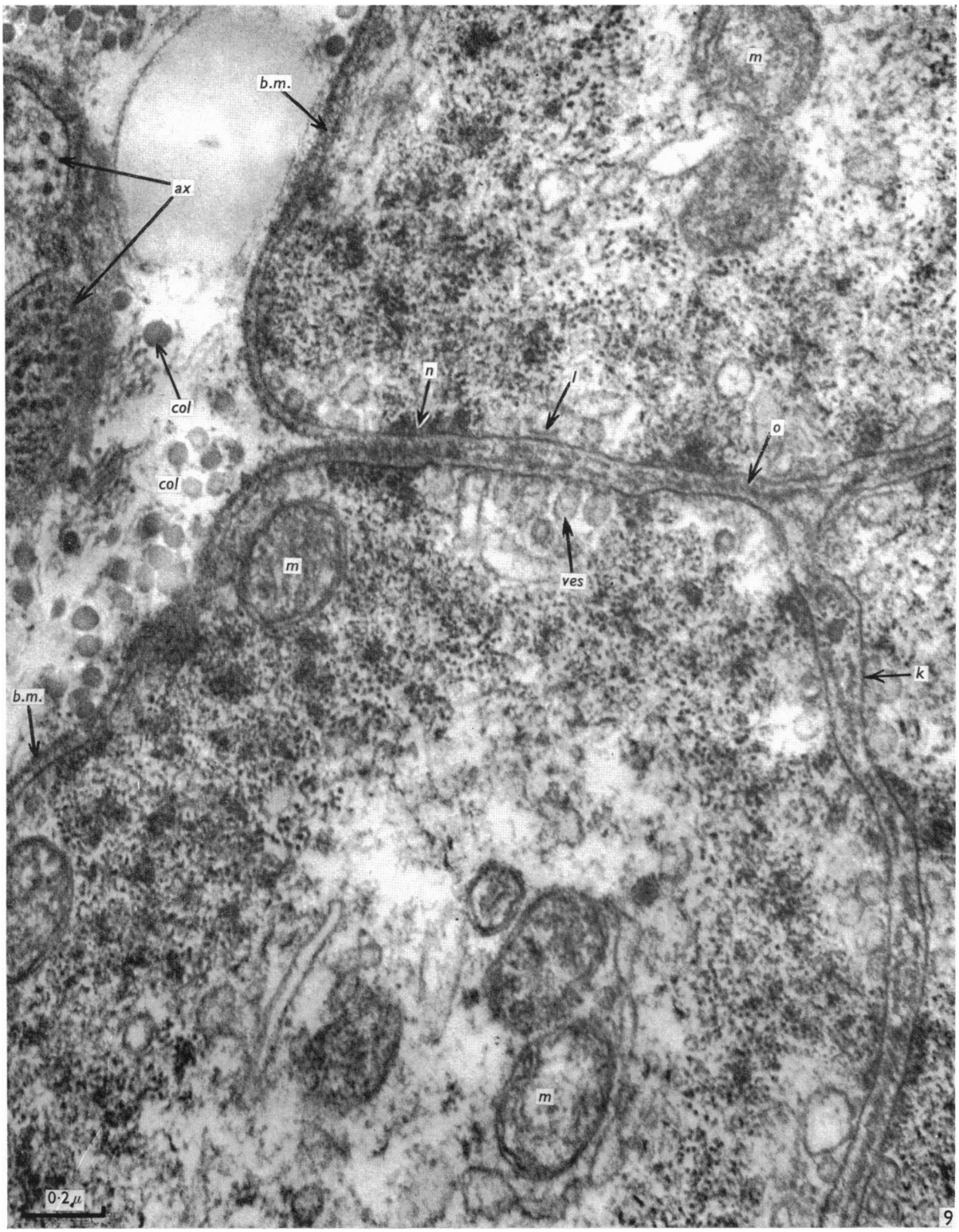
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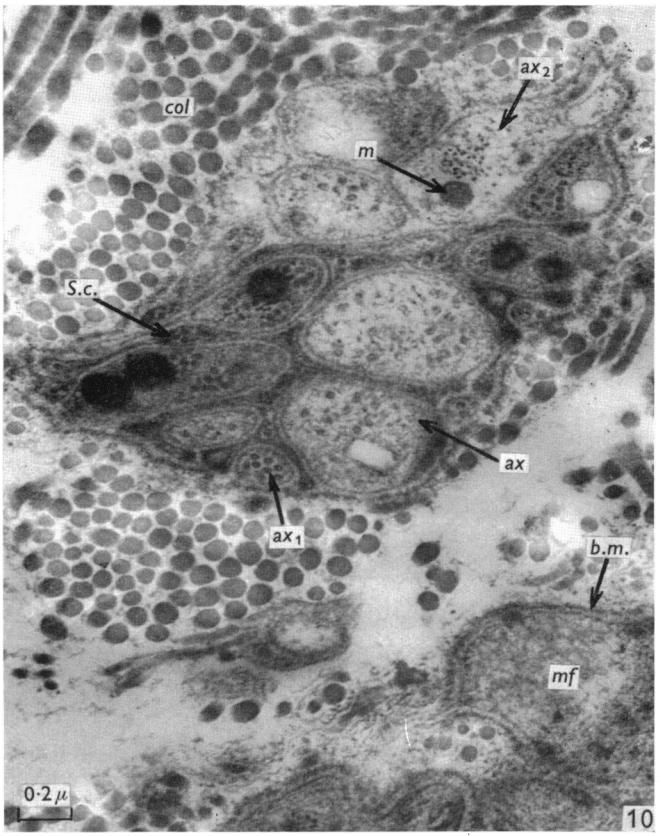
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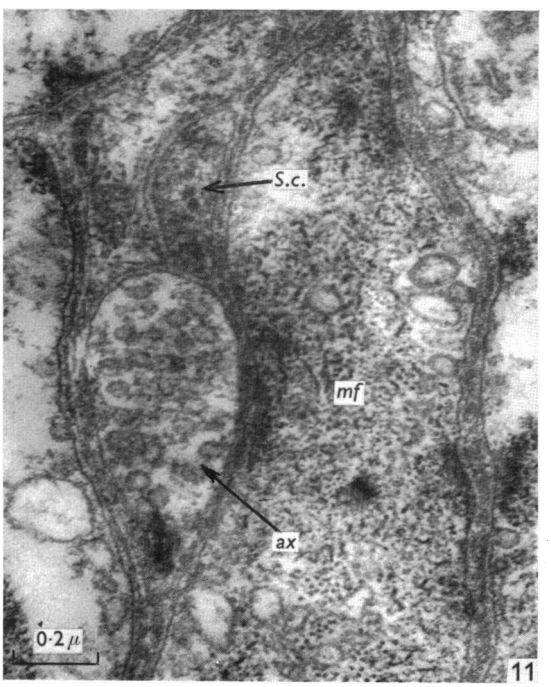
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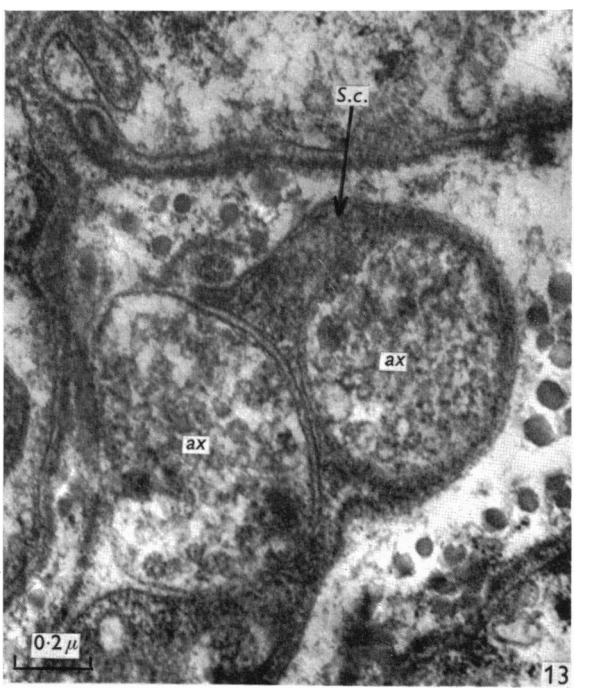
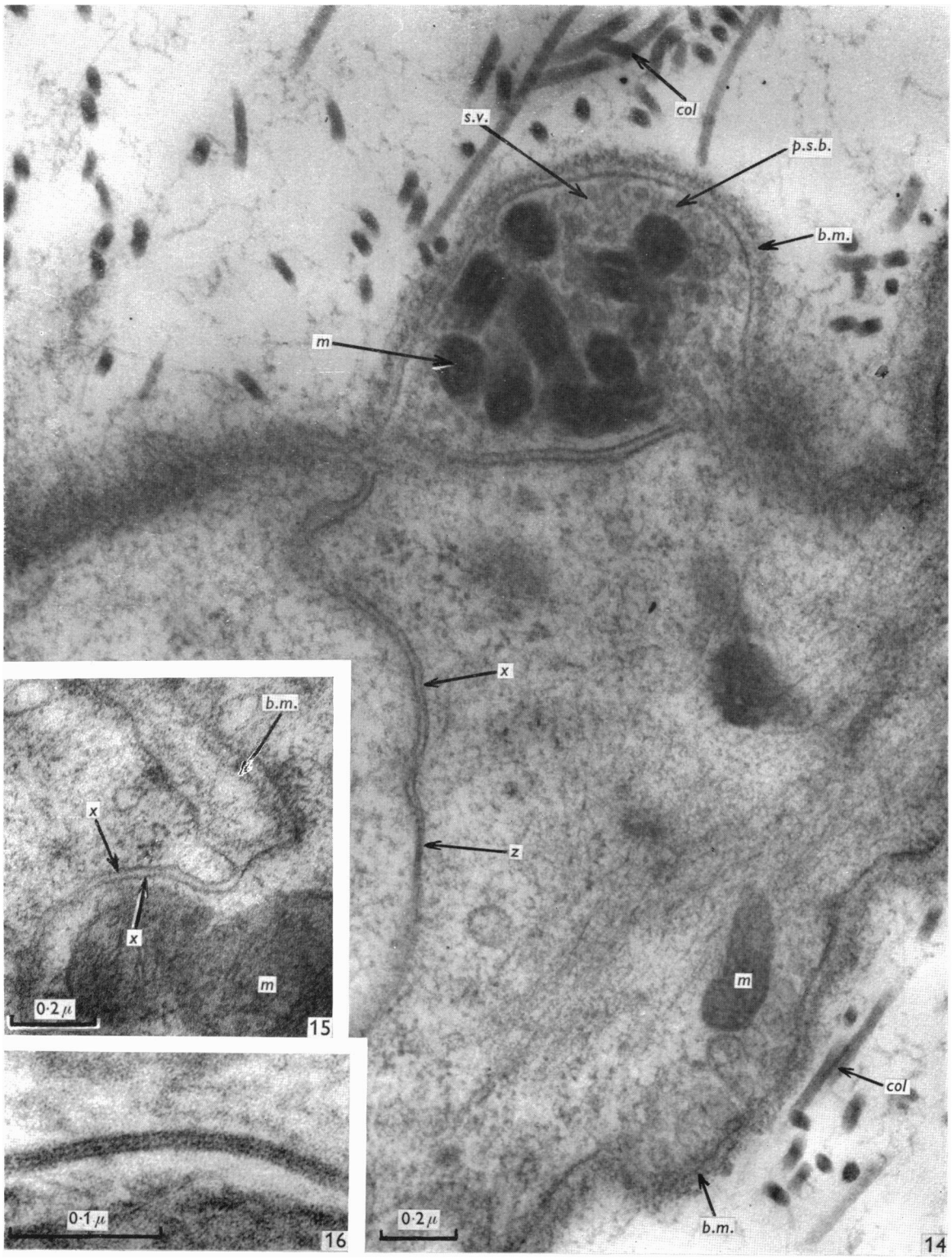

S.c.
ax
ax
0·2 μ
13


col
s.v.
p.s.b.
b.m.
m
x
z
m
col
b.m.
0·2 μ
14
b.m.
x
x
m
0·2 μ
15
0·1 μ
16

EXPLANATION OF PLATES

PLATE 1

Amnion, 12 days' incubation. Low-power view showing the epithelial (*ep.*) and muscular (*mc*) layers.

PLATE 2

Amnion, 12 days' incubation. Muscle cells and their myofilaments (*mf*) are cut transversely. A basement membrane (*b.m.*) invests the muscle coat.

PLATE 3

Fig. 3. Amnion. 10 days' incubation. Longitudinal section of two smooth muscle cells showing area of close apposition of plasma membranes, with no intervening basement membrane. An attachment plaque is shown at (*d*).

Fig. 4. Amnion. 11 days' incubation. Transverse section showing quintuple-layered system.

Fig. 5. Amnion. 11 days' incubation. An enlarged view of the quintuple-layered system shown in Fig. 4. The three dense laminae are separated by clear zones.

Fig. 6. Amnion. 10 day's incubation. Area of close apposition of muscle cells showing round and oval vesicles and cisterns, some studded with ribosomes, at cell margins.

PLATE 4

Nictitating membrane. Cat. Low power transverse section of smooth muscle.

PLATE 5

Nictitating membrane. Cat. Transverse section through smooth muscle showing annular disposition of myofilaments.

PLATE 6

Nictitating membrane. Cat. Transverse section of smooth muscle fibres showing membrane relationships. The basement membrane is present between individual muscle cells, in places as a double membrane, (*n*), elsewhere as a fused membrane, (*o*), and occasionally there is a gap between the basement membrane of apposed cells (*k*).

PLATE 7

Fig. 10. Nictitating membrane. Cat. Connective tissue and Schwann cell (*S.c.*), containing several axons, lying between adjacent muscle bundles.

Fig. 11. Nictitating membrane. Cat. Enlarged view of intercellular cleft showing one fine axon (*ax*) containing vesicles.

Fig. 12. Nictitating membrane. Cat. Axons in their terminal course between muscle cells. One has a basement membrane. Two are devoid of basement membrane (ax_3).

Fig. 13. Nictitating membrane. Cat. Nerve ending in intercellular cleft. Two axons are shown accompanied by fragments of Schwann cell, and surrounded by basement membrane.

PLATE 8

Fig. 14. Constrictor pupillae. Cat. Nerve ending (*p.s.b.*) containing synaptic vesicles (*s.v.*) in direct contact with smooth muscle cell, without intervening basement membrane.

Fig. 15. Constrictor pupillae. Cat. Contact between two smooth muscle cells, showing narrow intercellular channel (*y*) and reflection of basement membrane over the outer surface of the muscle cells. (*b.m.*)

Fig. 16. Constrictor pupillae. Cat. Quintuple-layered structure in intercellular channel between two smooth muscle cells, at higher magnification.

KEY TO LETTERING

am	Amniotic cavity	*nuc*	Nucleus
ax	Axon	*n*	Area where basement membrane is in contact
b.m.	Basement membrane		
col	Collagen	*o*	Area where basement membrane appears to be fused
cr	Cistern studded with ribosomes		
d	Dense zone	*p.s.b.*	pre-synaptic bag
ep	Epithelium	*S.c.*	Schwann cell
fi	Fibroblast	*sl of mf*	Sleeve of myofilaments
G.a.	Golgi apparatus	*s.m.*	Surface membrane
k	Clear zone	*s.v.*	Synaptic vesicles
l	Clear zone	*ves.*	Vesicles
m	Mitochondria	*x*	Area of close apposition, basement membrane being absent
m.b.	Multivesicular body		
m.c.	Muscle coat	*z*	Quintuple-layered system
mf	Myofilament		