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Uptake of ferritin into neurosecretory terminals

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[Plates 69 to 73]

Introduction

The release of neurosecretory products from terminals in the corpus cardiacum has been described in a few insect species (Scharrer 1963, 1968; Johnson 1966; Bowers & Johnson 1966; Normann 1965; Smith & Smith 1966; Smith 1970). Exocytosis has been suggested as the mechanism of release in *Calliphora erythrocephala* (Normann 1965, 1969) and in *Carausius morosus* (Smith & Smith 1966; Smith 1970), whereas fragmentation into 'synaptoid' regions has been indicated in *Periplaneta americana* (Scharrer 1963, 1968) and in *Myzus persicae* (Johnson 1966; Bowers & Johnson 1966). It is clear that structures representing both these features exist and differences in interpretation may depend on the sequence of events contributing to release. Using an electron-dense marker, an attempt has been made to determine the origin of the small vesicles which occur in neurosecretory axons and to throw some light on the dynamics of the release process.

Materials and methods

Corpora cardiaca from adult females of the stick insect, *Carausius morosus*, were exposed as described previously (Smith 1970) and fixed in ice-cold 0.25 mol/l glutaraldehyde in 0.05 mol/l cacodylate buffer at pH 7.4 and containing 0.17 mol/l sucrose. After washing in several changes of buffered 0.34 mol/l sucrose, they were placed in 1 % OsO_4 in veronal acetate buffer, dehydrated in an ethanol series and embedded in Araldite (A.R.L. (Ciba) Ltd, Duxford, England) via propylene oxide. Sections were cut on an LKB Ultrotome III and examined in a Phillips EM 200. For routine microscopy, sections were double-stained using lead citrate (Reynolds 1963) and saturated uranyl acetate in 50 % ethanol, ferritin-treated material was stained only in lead citrate for 3 min or examined unstained.

The introduction of ferritin, prepared as a 25 % solution in Ringer (Wood 1957), was achieved by direct immersion of exposed corpora cardiaca for periods ranging between 1 and 10 min. The corpora cardiaca were transferred to glutaraldehyde and processed for electron microscopy as described above.

Results

Fine structural examination of the corpus cardiacum reveals a complex organization including cell bodies of intrinsic secretory cells and their terminals, as well as the terminals of extrinsic neurosecretory cells whose perikarya are situated in the brain, glial elements and basement membrane material (Smith & Smith 1966). The brain neurosecretory cells terminate as swollen bulbs (figure 1).

The intrinsic cells of the corpus cardiacum produce secretory granules (approximately 200 nm in diameter) which appear to be elaborated in the Golgi region (figure 4).

Evidence for the release of neurosecretory products by exocytosis has been described previously (Smith & Smith 1966) and is illustrated for the electron-opaque (figure 5) and the electron-lucent neurosecretory vesicles (figure 2). It is suggested that, in regions devoid of glial insulation, fusion takes place between the limiting membrane of the neurosecretory storage vesicle and the axon membrane, followed by breakdown of the apposed membranes at this point, subsequently resulting in the release of the contained secretory product into the extracellular space. For the electron-opaque material evidence can be found for non-membrane-bounded secretion droplets in the extracullular space, occurring close to axon terminals which show other morphological signs of active release. The number of extracellular droplets increases after electrical stimulation of the cardiacum (figure 5). A striking feature of axon terminals of both types is the presence of a population of smaller vesicles approximately 30 nm in diameter. It should be stressed that these small vesicles are electron-lucent not only in axons containing electron-lucent (figure 2), but also in those containing electron-opaque (figure 5) products. These small vesicles are most frequently encountered in, if not restricted to, the axon endings, and are particularly prominent at sites which show other morphological indications of secretory activity (i.e. exocytotic profiles or presumed sites of granule release, and coherent extracellular non-membrane-bounded droplets).

The distribution of the electron-dense marker ferritin is illustrated in figures 6 to 10. Ferritin particles (diameter 11 nm) penetrate readily through the basement membrane sheath of the corpus cardiacum and permeate the extracellular space throughout the organ. Its distribution in relation to the axon endings is absolutely specific and circumscribed; it occurs only in the following situations:

(1) Large open depressions (exocytotic profiles) (figure 8).

(2) Large electron-lucent vesicles presumed to be confluent with the extracellular space at a level outside the plane of section in axons containing electron-lucent (figure 7), and in axons containing electron-opaque contents (figure 6).

(3) Small electron-lucent vesicles in axons containing electron-lucent (figure 9) products. Small electron-lucent vesicles in axons containing electron-opaque (figure 10) products.

(4) These small vesicles in the process of formation (figure 9).

It should be stressed that ferritin is never found free in the neuronal cytoplasm and is never found in 'complete' storage granules.

Discussion

Evidence for the origin of the various components of the corpus cardiacum has been discussed previously (Smith & Smith 1966). It is quite clear that cells synthesizing, transporting and releasing each of the types of secretory granule exist and that one cell is not a different stage of the other. For instance, the suggestion that the electron-opaque granules are the storage form and that the electron-transparent vesicles represent 'empty' shells after release (Scharrer 1963) is not consistent with the tracing of the production of each type within the Golgi bodies of distinct cells. The intrinsic cells of the cardiacum seem to produce solely electron-opaque contents, and are not strictly 'neuronal'. However, their secretory nature has been documented (Scharrer 1963; Smith & Smith 1966) and these cells are produced into elongated axon-like extensions and are partially invested by glia (Smith 1968).

Electron microscopic evidence believed to represent release of vesicular material has been presented for cells of both extrinsic (figures 2, 3) and intrinsic (figure 5) origin. It has been

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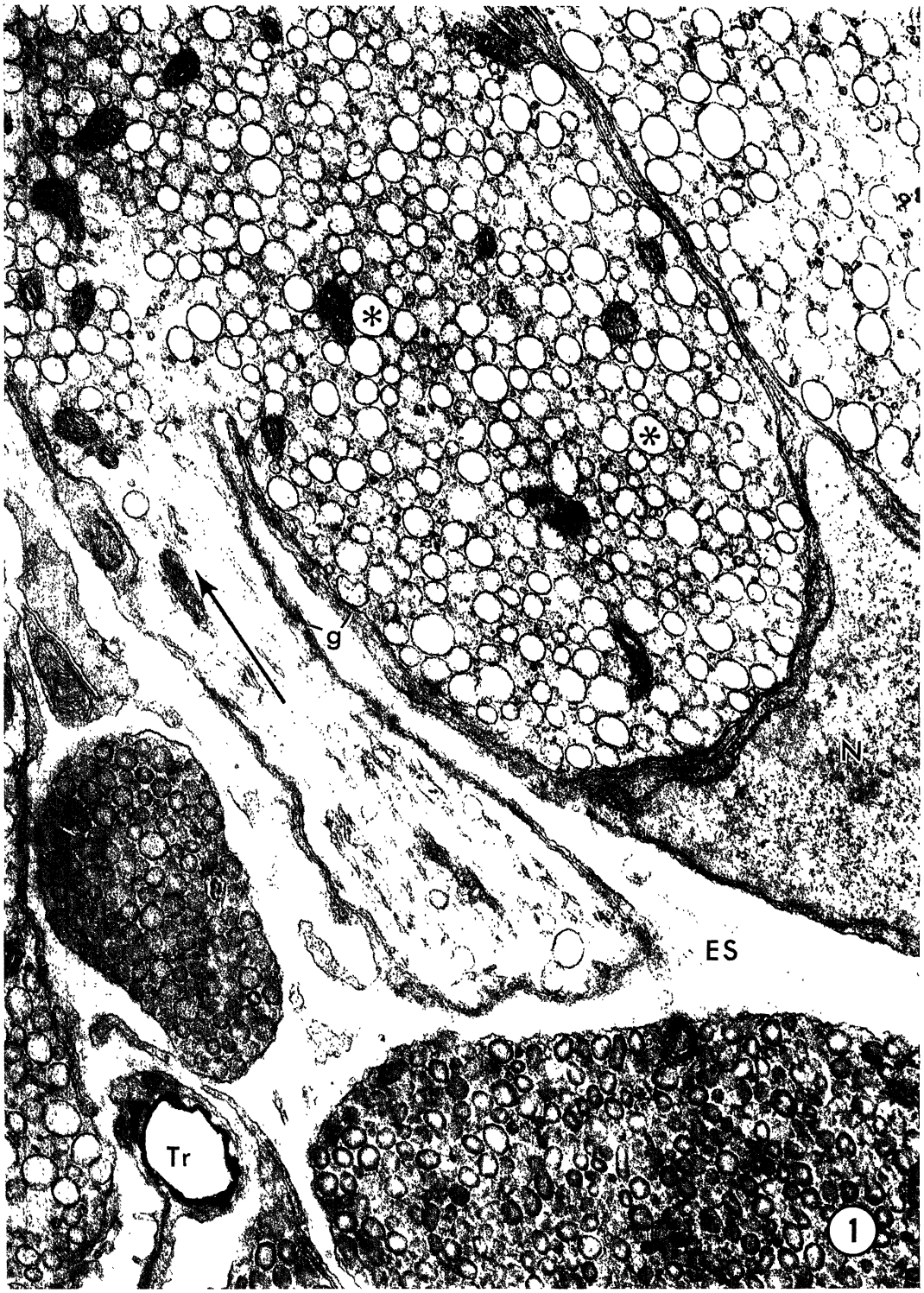

FIGURE 1. Axon (arrow) of an extrinsic brain neurosecretory cell terminating as a swollen bulb which contains electron-lucent neurosecretory vesicles (*) (diameter approximately 200 nm). The terminal is surrounded by a thin coat of glia (*g*); a glial nucleus is seen at N. Tracheole (Tr), extracellular space (ES). (Magn. × 23 000.)

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

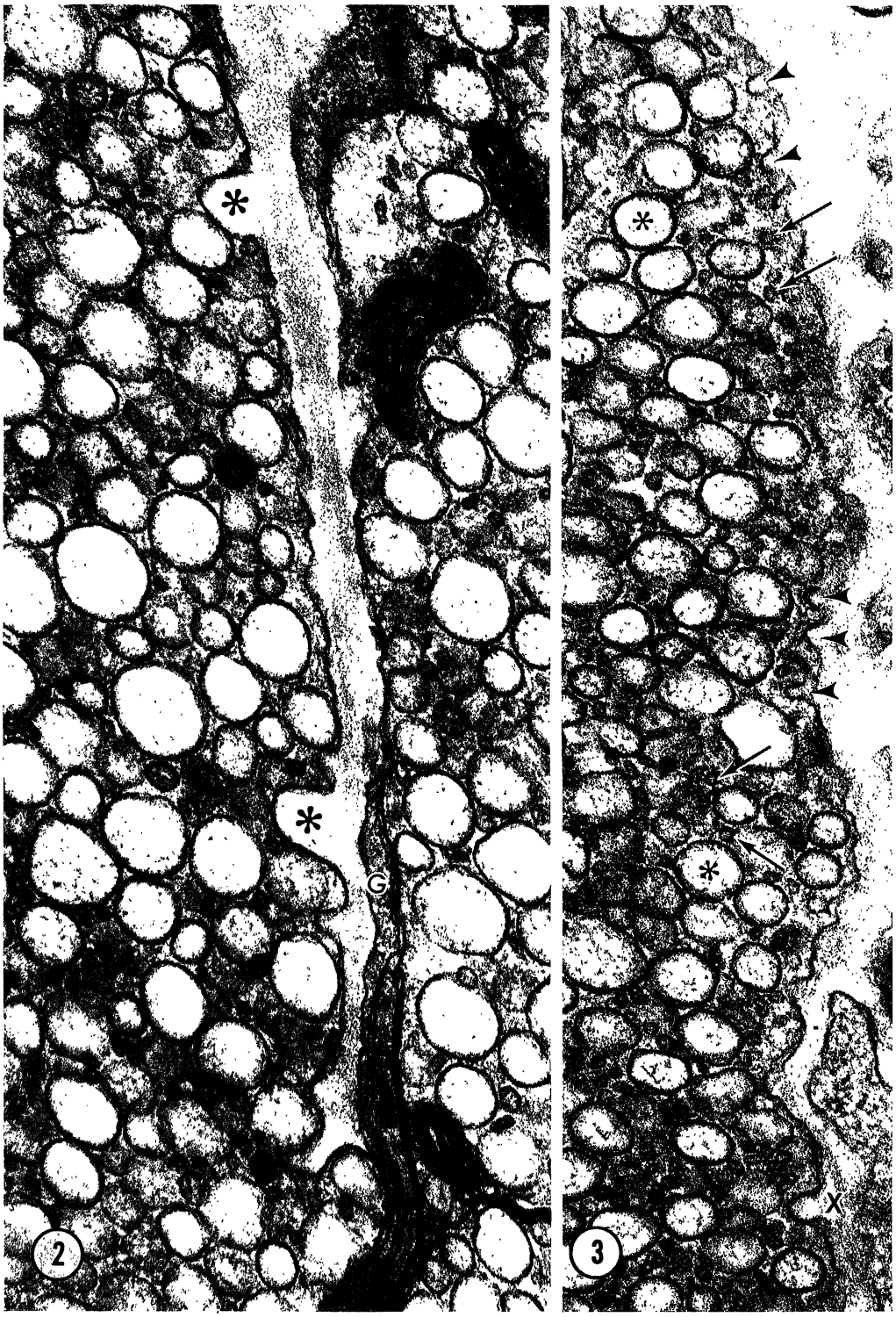

FIGURE 2. Profile of two extrinsic neurosecretory axon terminals containing electron-lucent products. A glial finger (G) partially extends over one surface. In regions where the glial coat is absent, two Ω shaped depressions (*) can be seen. It is suggested that these represent sites of the release of neurosecretory products by a process of fusion between the limiting membrane of the neurosecretory droplet and the axon membrane. The presence of small electron-lucent vesicles should be noted. (Magn. × 58 000.) (From Smith & Smith, *J. Cell Sci.* **1**, 1966.)

FIGURE 3. Portion of an axon terminal containing electron-lucent products bordering on the extracellular space. The axon contains large (150 to 200 nm) electron-lucent storage vesicles (*) as well as a population of smaller (30 nm) vesicles (arrows). The plasma membrane of the terminal abuts directly on the extracellular space (ES) and is frequently indented (arrowheads) in regions which may represent active pinocytosis and which may give rise to the smaller vesicles. On the basis of size, it is suggested that the depression (×) is the site of release of vesicle contents by exocytosis (compare figure 2). (Magn. × 38 000.)

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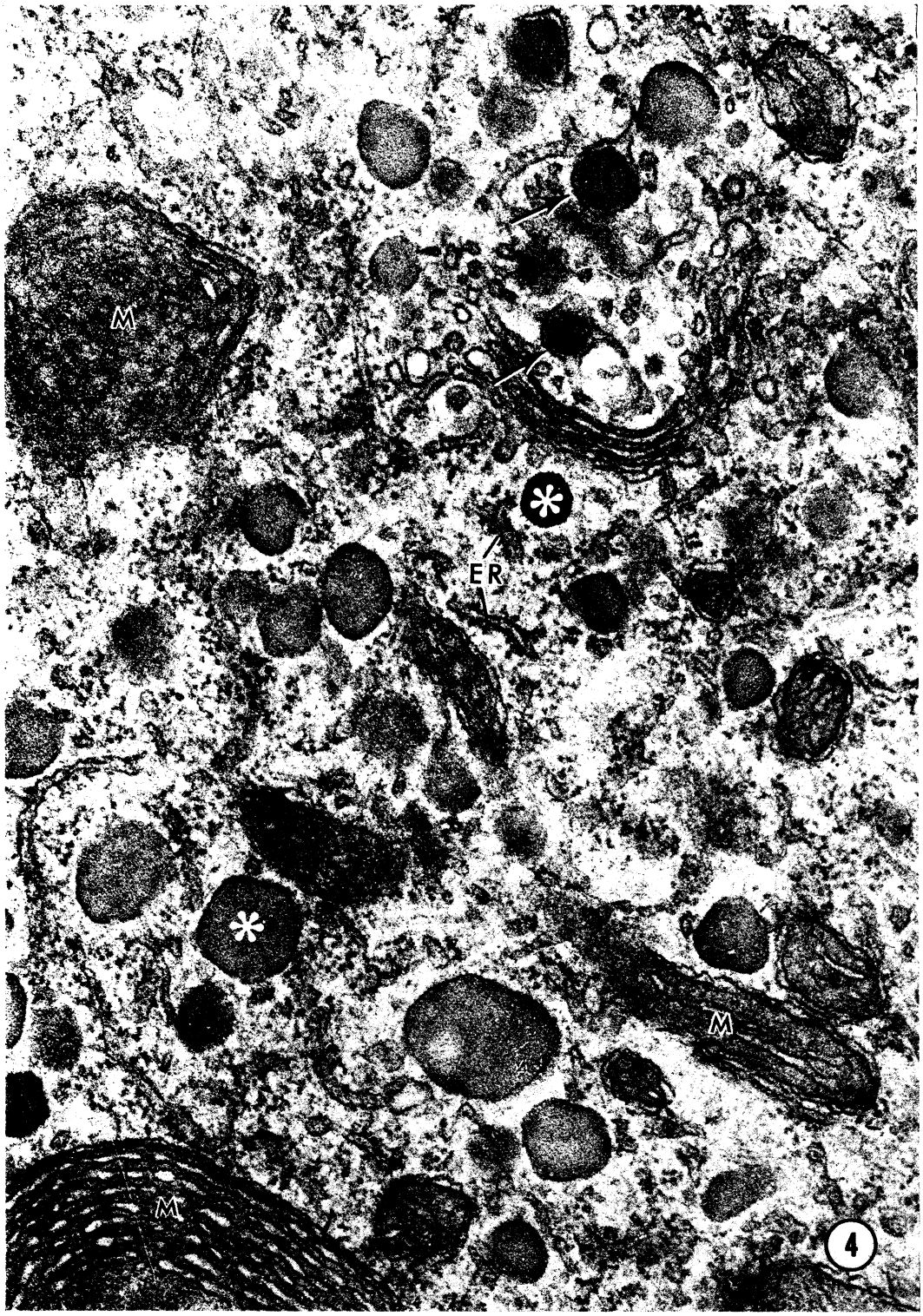

FIGURE 4. Portion of the cell body of an intrinsic corpus cardiacum secretory cell showing mitochondria (M), some of which display concentrically arranged crista membranes and rough-surfaced endoplasmic reticulum (ER). Numerous electron-opaque secretory granules are present (*) which are originally elaborated in association with the Golgi bodies (G). During formation, the agranular limiting membrane of the droplets can easily be distinguished (arrows). (Magn. × 54 000.)

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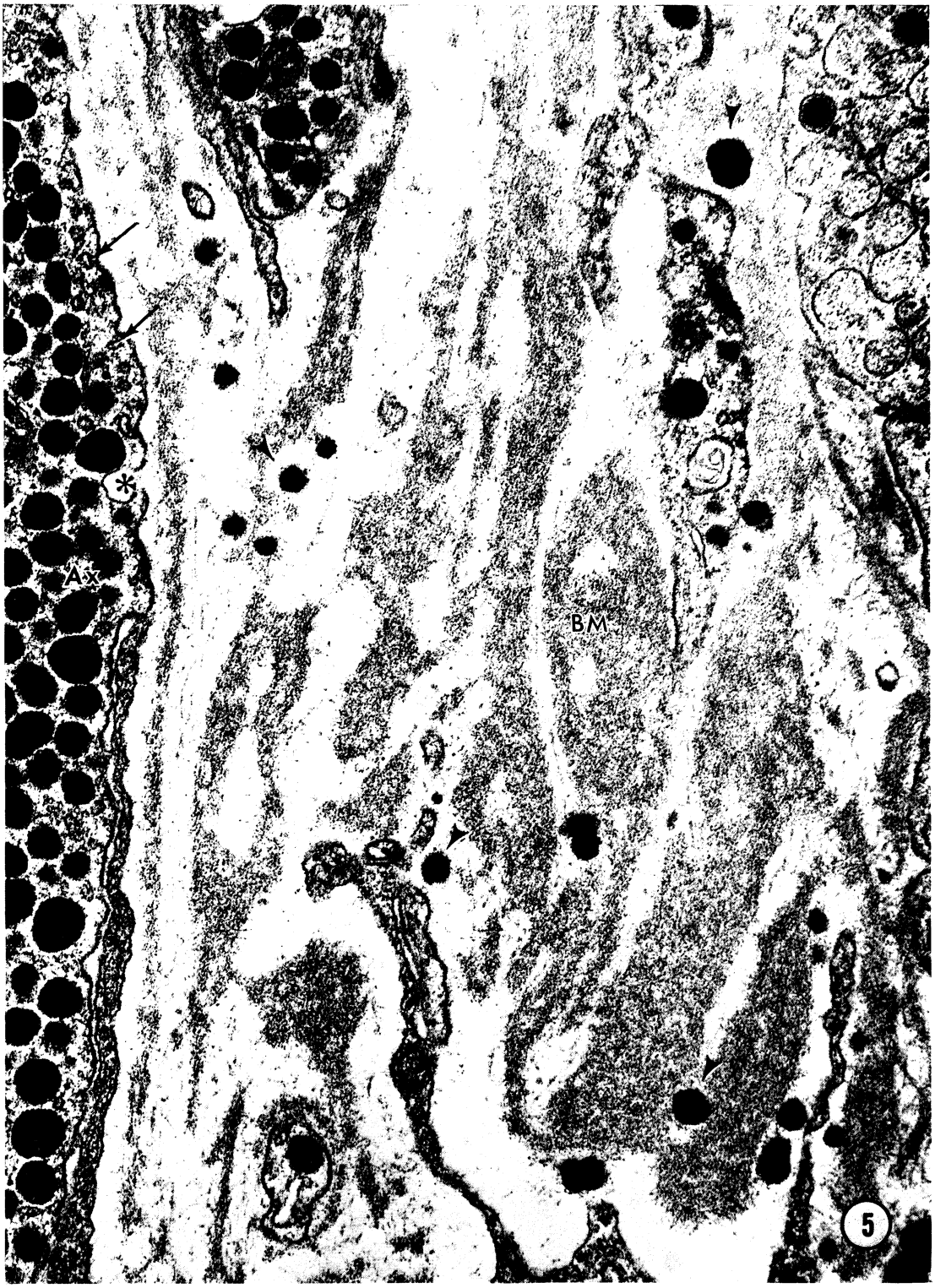

FIGURE 5. Field taken from a corpus cardiacum which had been electrically stimulated (suction electrodes, square pulses, 30/s for 2 min) showing profile of axon terminal bordering directly on extracellular space. The latter contains a finely fibrillar basement-membrane-like material (BM). The extracellular space contains numerous non-membrane-bounded droplets (arrowheads) similar in size and electron-density to those membrane-limited components contained within the axon ending (Ax). Small electron-lucent vesicles are indicated by arrows. It is suggested that the profile marked by an asterisk represents the limiting membrane of a neuro-secretory vesicle after fusion with the plasma membrane and release of the contained product. (Magn. $\times 53\,000$.)

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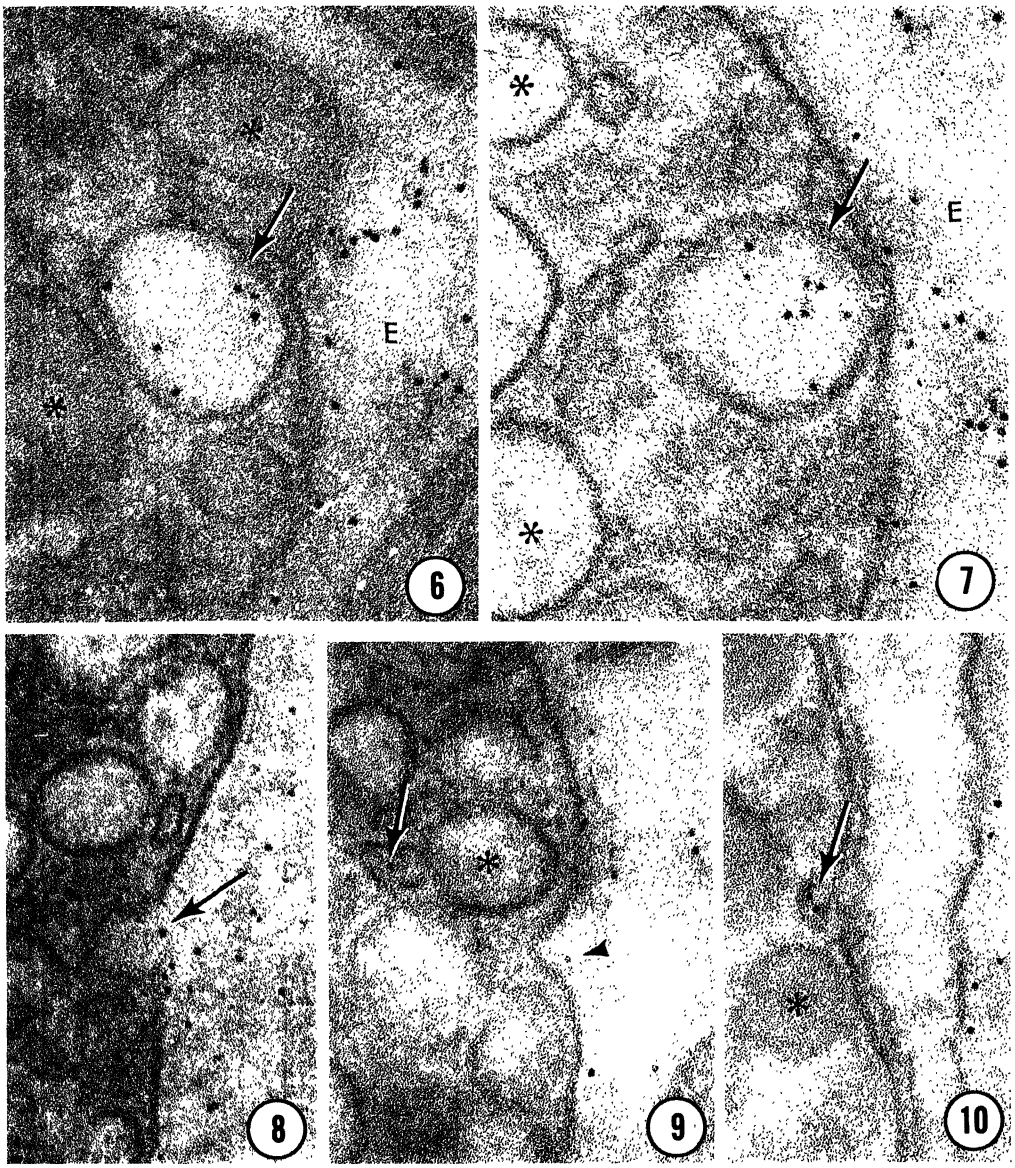

Figures 6–10 illustrate the results of experiments in which corpora cardiaca were exposed to a 25% solution of ferritin in Ringer for 5 min. Figures 6–9 are reproduced from Smith (1970).

FIGURE 6. Ferritin particles can be seen permeating the extracellular space (E) within the corpus cardiacum. Ferritin can also be found in the larger (200 nm) electron-lucent vesicles (arrow) which adjoin the cell membrane, and which are presumably confluent with the extracellular space at a level out of the plane of section. This figure illustrates an axon containing electron-opaque neurosecretory products (*); it is significant that ferritin is not seen elsewhere in the cytoplasm. (Magn. × 190 000.)

FIGURE 7. Ferritin is seen in a large electron-lucent vesicle at the surface of the axon (arrow) which is presumably in communication with the extracellular space (E). This figure illustrates features similar to those shown in figure 6, but in an axon containing electron-lucent products. (Magn. × 173 000.)

FIGURE 8. Large vesicle (arrow) in direct communication with extracellular space containing ferritin particles. This profile may represent the limiting membrane of a neurosecretory vesicle after fusion with the cell membrane. (Magn. × 140 000.)

FIGURE 9. The small depression in the cell membrane containing a ferritin particle (arrowhead) may represen a pinocytotic vesicle in the process of formation. A complete vesicle containing a ferritin particle (arrow) can be seen within the cell. This axon contains electron-lucent storage vesicles (*). (Magn. × 140 000.)

FIGURE 10. Two ferritin particles within a small electron-lucent vesicle (arrow) in an axon containing electron-opaque neurosecretory material (*). (Magn. × 190 000.)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

suggested (Smith & Smith 1966) that release occurs by a process of 'reverse pinocytosis' (de Robertis & Vaz Ferreira 1957) or 'exocytosis' (de Duve 1963) which is a well-known cellular mechanism for the externalization of stored secretory products (Palade 1959). This involves collision, fusion and subsequent breakdown of the limiting membrane of the neurosecretory vesicle and the axon membrane, thus allowing a free passage of the contained products to the exterior.

However, as with all purely morphological observations derived from static electron micrographs of fixed material, it is not possible to comment on the dynamics of a process and the mere presence of a membrane depression, while indicating the bulk transfer of material across a cell membrane, gives no evidence of direction.

As circumstantial evidence favouring the exocytosis hypothesis, the following points should be mentioned:

(*a*) The presence, in the case of electron-opaque products, of non-membrane-bounded material outside the axon of the same size and density as the membrane-bounded constituents within the cell. It is not possible to say how long the secretory products retain their integrity outside the cell or whether these images represent only a protein carrier or result from glutaraldehyde fixation.

(*b*) The complete absence of large electron-lucent vesicles in cells producing electron-opaque secretion products. Large electron-lucent vesicles would be obtained if the omega-shaped depressions (exocytotic profiles) were sites of incipient ingoing vesicles, or if the secretory products were to 'leak' out of the cell after rupture of the vesicle membrane.

(*c*) The small vesicles are electron-lucent whether they are contained in axons bearing electron-opaque or electron-lucent neurosecretory granules; in the former case, the loss of electron-opacity is not explained by the hypothesis of fragmentation into 'synaptoid' vesicle clusters before release (Scharrer 1968) but is consistent with release by exocytosis followed by pinocytotic uptake (Smith 1970).

An inevitable outcome of exocytosis, which involves fusion of the vesicle membrane to the cell membrane, would be the continual addition of membrane material to the nerve terminal. This could in theory be counteracted by immediate reclosure and withdrawal of the storage vesicle—there is no morphological evidence for this in the corpus cardiacum, or, alternatively, by micropinocytosis either of undifferentiated cell membrane material or of the original vesicle membrane into a series of smaller vesicles, i.e. fragmentation of the vesicle membrane *after* release into a cluster of small vesicles. The smaller the vesicle, the greater the amount of membrane material that could be resorbed for the smallest uptake of extracellular environment. The smallest theoretical size for a unit membrane limited vesicle is 30 nm (Stein 1967) which corresponds exactly to the observed diameter of small vesicles in neurosecretory axons. It is quite clear, from the intracellular distribution of ferritin, that small vesicles can on no account be derived from storage vesicles before release nor are they synthesized within the cell since ferritin is never found in storage vesicles or elsewhere in the neuronal cytoplasm. The uptake of ferritin solely into small vesicles shows that they are derived either from the plasma membrane or from the vesicle membrane which becomes continuous with it after release. On ultrastructural grounds alone, these two possibilities cannot be distinguished; however, evidence from other systems seems to favour the latter. Winkler (this volume) suggests that in the adrenal medulla the retrieval of membrane is more likely to occur by resorption of the original chromaffin granule membrane which has biochemical properties not shared by the remainder of the plasma

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

membrane. Furthermore, specific metabolic activities have been shown to be restricted to endothelial pinocytotic vesicle membranes which, though confluent with the plasma membrane, remain functionally distinct (Smith & Ryan 1970*a*, *b*, 1971). In experimentally induced exocytosis, Normann (1969) has observed an increase in the number of small vesicles which he suggests are derived from the remnants of granule membranes. Similar results have been obtained in preliminary experiments with electrically stimulated corpora cardiaca of *Carausius* which also show an increase in the numbers of extracellular droplets (figure 5).

Exocytosis has been invoked to explain the release of a number of secretory products synthesized for export from the cell. Neurosecretion may be defined as the product of neuronal glandular activity and in this connexion it is significant that exocytosis has been suggested for the release of neurotransmitters (see papers by B. Katz and A. D. Smith in this volume) and for the secretion of chromaffin granules from the adrenal medulla (see papers by N. and A. G. Kirshner, p. 279 and O. Grynszpan-Winograd, p. 291, in this volume).

It may be that the explanation for the different interpretations of the release of neurosecretory material from the corpus cardiacum lies in the sequence of events. While fragmentation of vesicles may occur, it seems certainly to occur after release. It is suggested that release takes place by exocytosis and that small vesicles may represent a mechanism for the retrieval of membrane material.

It is a pleasure to thank Mrs Juana Alvarez and Miss Sharon Hedden for technical assistance and Dr D. S. Smith for use of the Philips EM 200.

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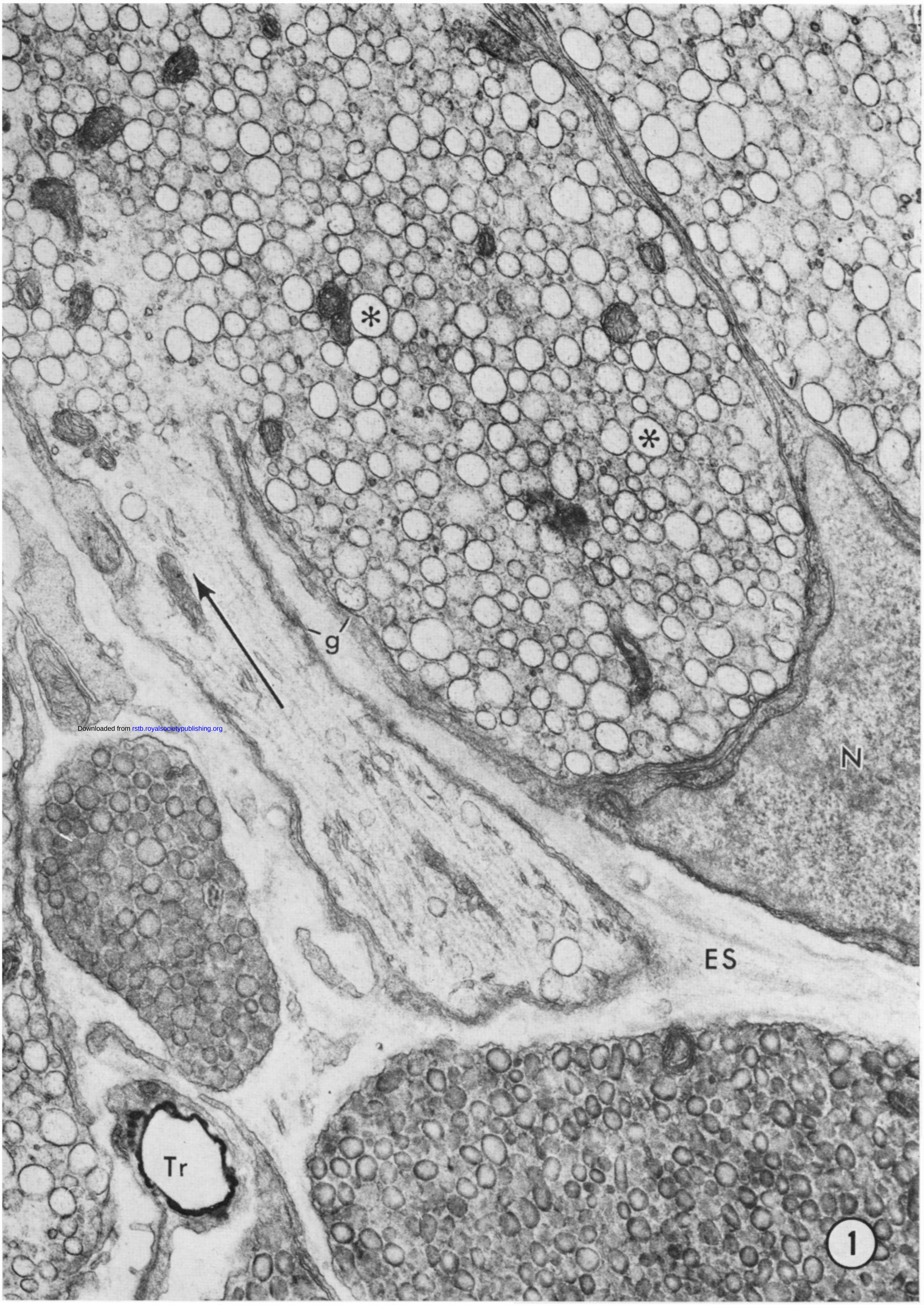

FIGURE 1. Axon (arrow) of an extrinsic brain neurosecretory cell terminating as a swollen bulb which contains electron-lucent neurosecretory vesicles (*) (diameter approximately 200 nm). The terminal is surrounded by a thin coat of glia (*g*); a glial nucleus is seen at N. Tracheole (Tr), extracellular space (ES). (Magn. × 23 000.)

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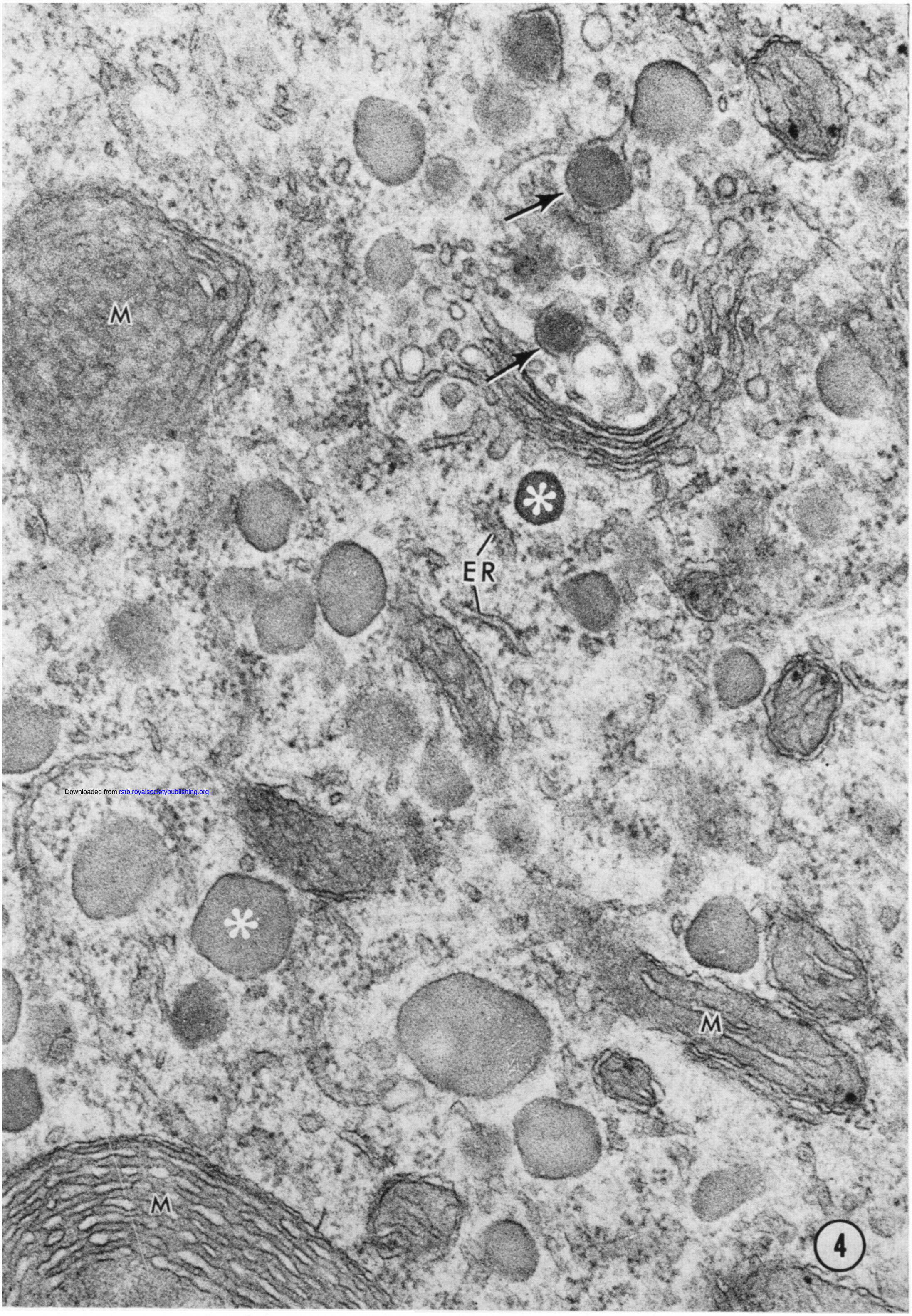

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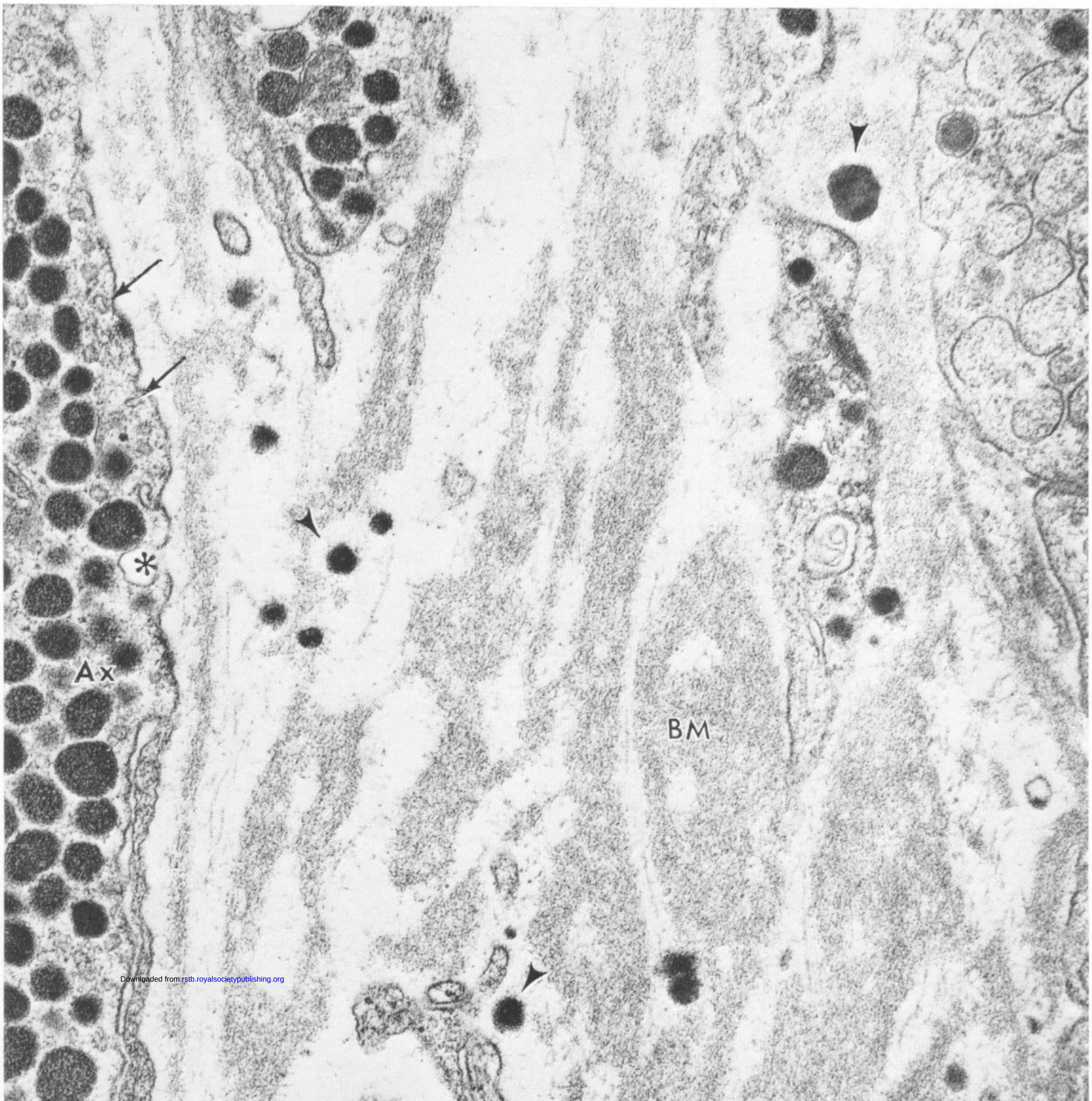

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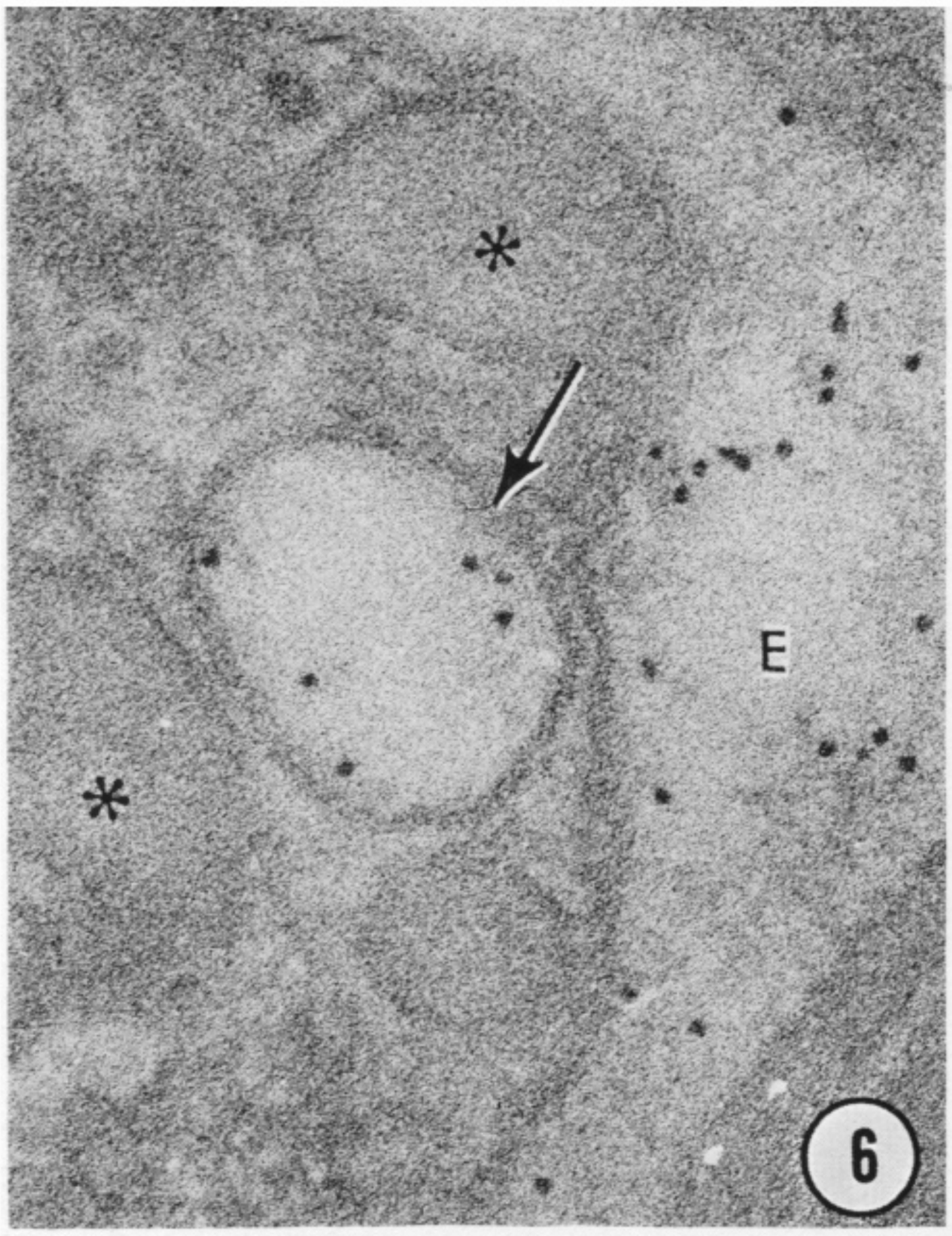

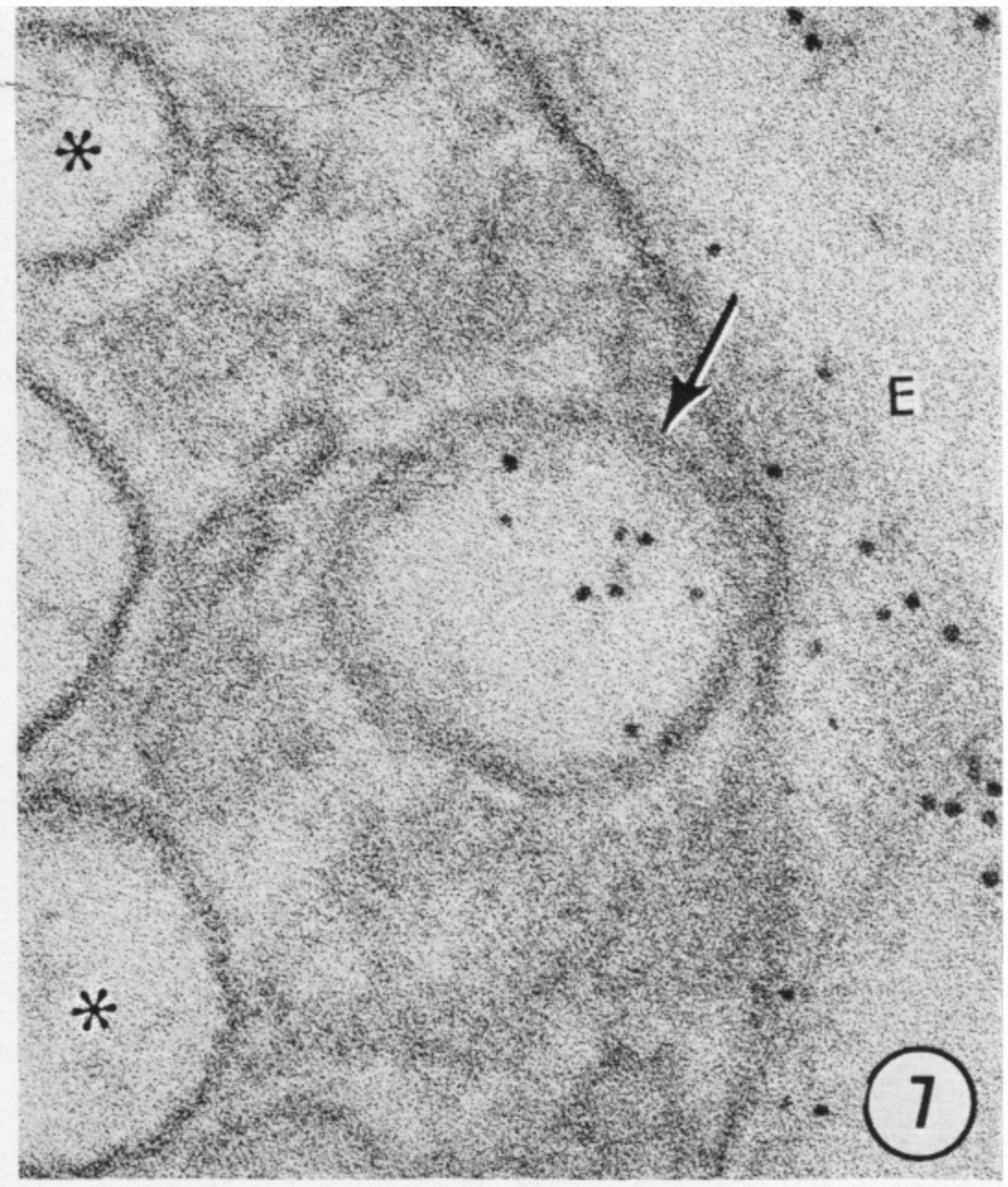

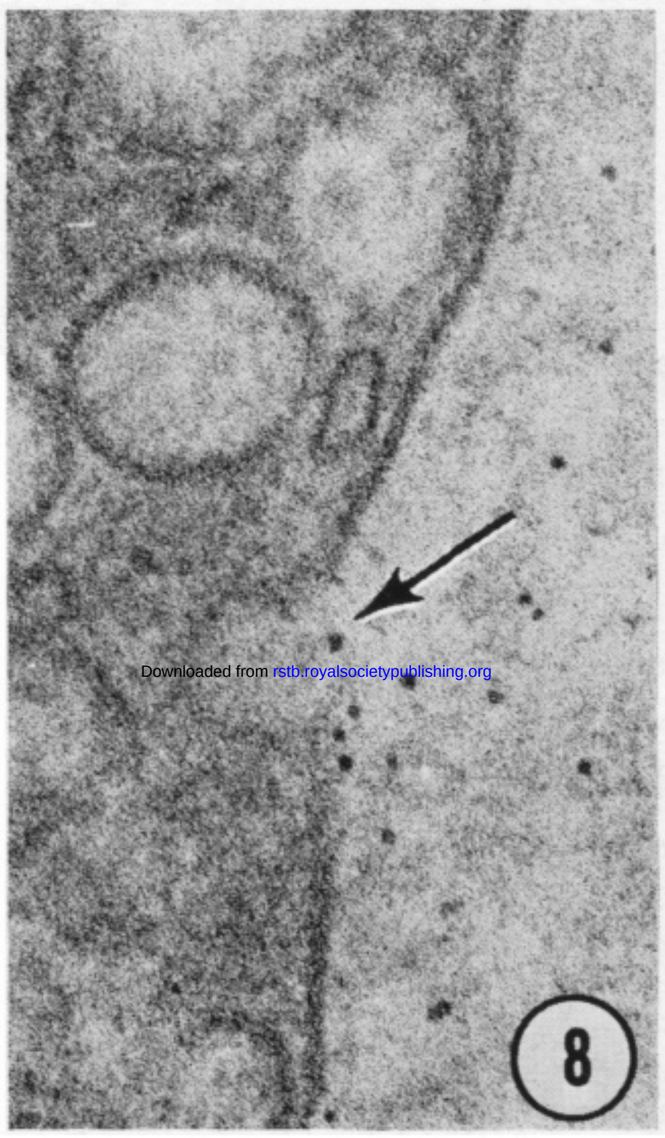

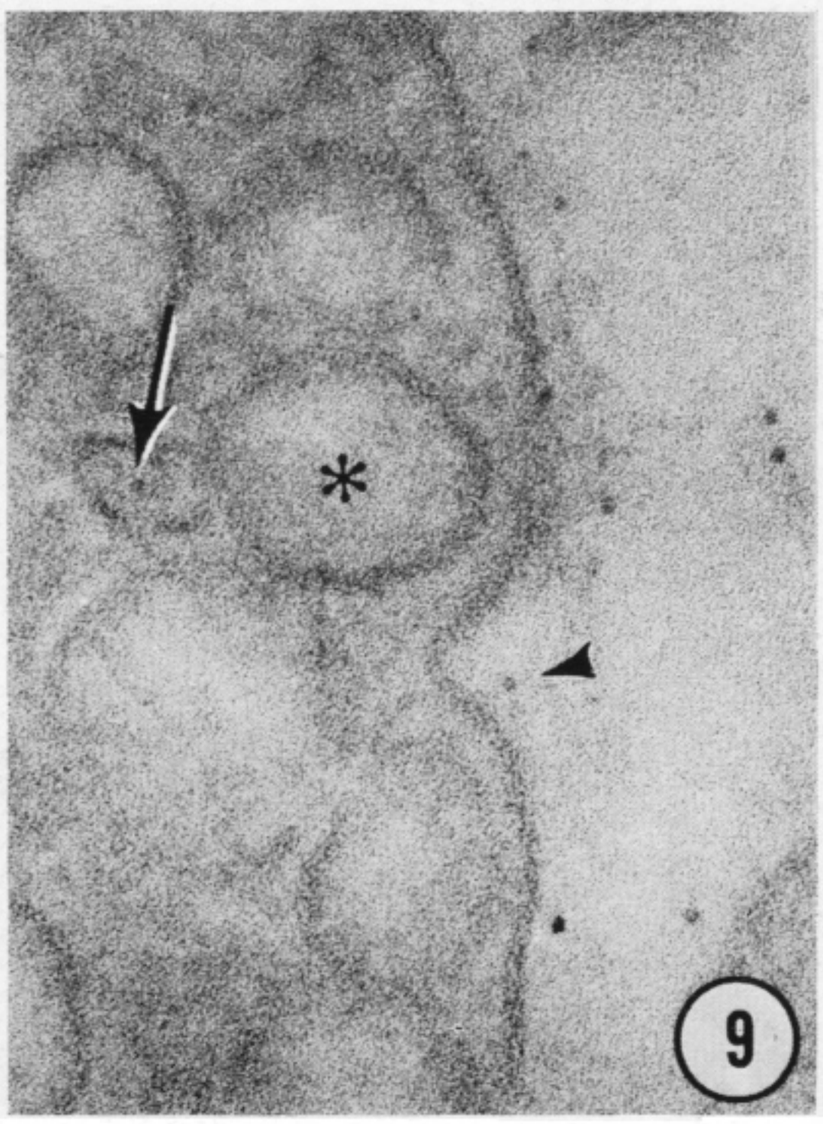

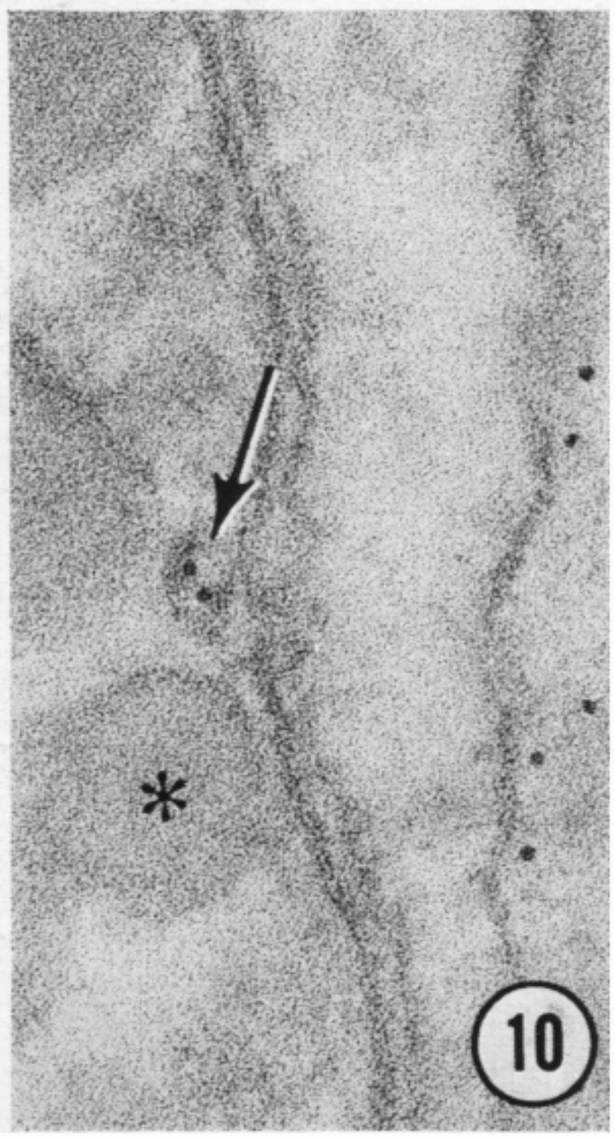

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