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Chromogranins in Sympathetic Nerves

P. Banks and K. B. Helle

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Chromogranins in sympathetic nerves

By P. Banks and K. B. Helle
Department of Biochemistry, Sheffield University and Department of Physiology, Bergen University, Norway

[Plates 52 and 53]

In post-ganglionic sympathetic neurons, biochemical and electron-microscopical evidence indicate that noradrenaline is stored within granules which, although smaller, resemble adrenal medullary chromaffin granules in some respects. This resemblance between neuronal and adrenal medullary granules has been extended by immunological and enzymic investigations which show that sympathetic nerve granules contain the proteins chromogranin A and dopamine β-hydroxylase which characteristically occur in chromaffin granules.

Introduction

The observation that most of the catecholamines of the adrenal medulla are located in chromaffin granules which can be separated from other cell organelles by centrifuging, led, in 1956, to the discovery of similar particles storing noradrenaline in bovine splenic nerves (von Euler & Hillarp 1956). Since then much work has been devoted to describing and comparing the properties of adrenal chromaffin granules and splenic nerve granules (for reviews see Stjärne (1964) and Smith (1968)); in general, advances in understanding chromaffin granules have acted as a spur to further investigation of the nerve granules. The similarities between the two types of particle are very striking, for example both contain catecholamine and ATP in a molar ratio of about 4:1 (but see De Potter this volume, p. 313); both display a Mg^{2+}-ATP-dependent uptake of catecholamines and both carry the enzyme dopamine β-hydroxylase. On the other hand, there are marked differences in their osmotic fragilities, rates of loss of catecholamines at 37 °C and responses to treatment with drugs such as reserpine and phenoxybenzamine. The nerve granules are also considerably smaller than chromaffin granules. Furthermore, while about 80 % of the total catecholamines of the adrenal medulla can be recovered regularly in chromaffin granules, it is unusual to recover more than 40 % of the total noradrenaline of splenic nerve in the nerve granules. Hence it is not clear whether the relative importance of the nerve granules as a noradrenaline store is as great as that of the chromaffin granules. However, Stjärne (1966) has reported that under some circumstances about 60 % of splenic nerve noradrenaline can be recovered in a granular fraction. It seems likely therefore that most nerve noradrenaline is bound to particles *in vivo* but that disruption of the nerve trunk leads to a disturbance of the normal balance between release and uptake of noradrenaline by the nerve granules and results in a net loss of amine from the storage vesicles. This view agrees with the observation that noradrenaline is lost at a high rate from nerve granules incubated at 37 °C *in vitro* without ATP. It is probable that a pure preparation of nerve granules has yet to be obtained since all appear to consist of a heterogeneous population of particles in the electron microscope (see Roth, Stjärne, Bloom & Giarman 1968). Dense-cored vesicles, the strongest candidates for noradrenaline storage granules, account for only a small proportion of the total population of vesicles that can be isolated from splenic nerves. A similar heterogeneous population of vesicles showing marked variation in the electron density of their contents has been

found to accumulate in constricted splenic nerves (see Banks, Mangnall & Mayor 1969). Some of this variation may be related to the degree to which noradrenaline storage particles are laden with their contents.

Lysis of chromaffin granules by osmotic shock disrupts the lipoprotein membranes and releases almost all the catecholamines and ATP and about 70 % of the total protein into solution. The major protein component of the water-soluble fraction has been purified (Smith & Winkler 1967) and given the name chromagranin A (Schneider, Smith & Winkler 1967). Recently evidence has been obtained by immunological techniques which suggests that this protein is also a major constituent of the water-insoluble fraction of the granules (Helle & Serck-Hanssen 1969*a*). The form of chromogranin A present in the granule membranes and released from them by treatment with detergent is associated with phospholipids in a complex that can be distinguished from the water-soluble chromogranin A by its slightly lower electrophoretic mobility. However, this phospholipid-rich chromogranin, now termed chromogranin A_I, cross-reacts with antichromogranin sera to give a pattern of complete identity with the water-soluble chromogranin A. Dopamine β-hydroxylase activity of the granules has also been detected in a soluble as well as in a membrane-bound form (Laduron & Belpaire 1968*a*; Viveros, Arqueros, Connett & Kirshner 1969).

Other characteristic constituents of the water-insoluble membrane fraction of the chromaffin granule are a Mg^{2+}-ATPase and several electron transfer proteins, i.e. dopamine β-hydroxylase, a *b*-type cytochrome and flavoprotein(s). The latter is responsible for the high NADH:(acceptor)oxidoreductase (EC 1.6.99.3) activity of the membranes for which ferricyanide, 2,6-dichlorophenol-indophenol, bovine heart ferricytochrome *c* as well as the endogenous ferricytochrome b_{561} serve as electron acceptors (T. Flatmark, O. J. Terland & K. B. Helle 1970, unpublished observations).

The discovery of readily solubilized proteins in chromaffin granules in addition to catecholamines and ATP led to the idea that these three materials formed some kind of storage complex within the granules. In consequence, interest focused upon the supposed storage role of the major soluble protein, chromogranin A. Little evidence concerning interactions between chromogranin A, ATP and amines has accumulated but it is now well established that chromogranin A, ATP-catabolites and catecholamines are released together during the secretory response of the adrenal medulla (for review see Douglas 1968). The relative proportions of catecholamines to ATP-catabolites and chromogranin A in perfusates collected from stimulated adrenal medullae do not differ significantly from the proportions of catecholamines to ATP and chromogranin A in soluble lysates prepared from chromaffin granules by osmotic shock.

The discovery that chromogranin A was released during the Ca^{2+}-dependent secretion of catecholamines (Banks & Helle 1965; Kirshner, Sage, Smith & Kirshner 1966; Blaschko *et al.* 1967), although there was no release of phospholipids or lactic dehydrogenase, strongly supported the view of De Robertis & Vaz Ferreira (1957) that catecholamine secretion from chromaffin granules takes place by exocytosis (see Schneider *et al.* 1967; Kirshner & Kirshner, this volume p. 279).

In view of the similarities between chromaffin and splenic nerve granules and of the common embryological origin of the adrenal medulla and post-ganglionic sympathetic neurons, it was of interest to attempt to locate chromogranin A in the nerve granules. Furthermore, since the release of noradrenaline from nerve endings is a Ca^{2+}-dependent process similar to the exocytotic release of noradrenaline from the chromaffin cell, it is possible that if nerve granules contain

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chromogranin A, this protein may also be released at the nerve endings following stimulation. Thus a search for chromogranin A in noradrenergic neurons could provide valuable information for the understanding of synaptic transmission.

Chromogranin A in splenic nerve granules

A preparation of nerve granules containing 5 nmol noradrenaline per milligram protein and having an NA/ATP molar ratio of about 5:1 was obtained from the 600 g supernatant of a press juice of bovine splenic nerves by centrifuging at 20000*g* for 20 min. It was shown that after lysis of the granules with 0.5% deoxycholate the granule lysate cross-reacted, on Ouchterlony double-diffusion plates, with an antiserum prepared against chromogranin A purified from bovine adrenal medullary chromaffin granules (figure 1, plate 52) (Banks, Helle & Mayor 1969). Estimates of the ratio of noradrenaline (nmol) to chromogranin (mg) in these granules, using the double diffusion technique, gave values of about 80. Using the double-diffusion technique Helle finds that only 10 to 20% of the chromogranin A present in splenic nerve granules can be detected immunologically unless the granules have been solubilized with a detergent. One of the solubilized proteins exhibits an electrophoretic mobility identical to chromogranin A_I, the membrane-bound, detergent-soluble form of chromogranin A found in medullary granules.

Using splenic nerve granules equilibrating in 1.0 to 1.2 mol/l sucrose during density gradient centrifugation and having 10 to 20 nmol NA/mg protein, K. B. Helle & G. Serck-Hanssen (1969, unpublished observations) have found values of 620 to 1670 nmol NA/mg chromogranin A_I. De Potter, De Schaepdryver, Moerman & Smith (1969), using the microcomplement fixation technique, have obtained values of 5000 nmol NA/mg chromogranin A for bovine splenic nerve granules prepared by differential centrifugation. This value, which is about half that found in adrenal chromaffin granules, is some ten times greater than the highest value found by Helle & Serck-Hanssen for granules prepared by differential centrifugation (120 to 490 nmol NA/mg chromogranin A_I). However, the particles prepared by De Potter *et al.* (1969) contained about 10 nmol NA/mg protein which compares well with particles prepared on a density gradient by Helle & Serck-Hanssen (1969, unpublished observations). It is possible that the microcomplement fixation technique when applied to lysates of nerve granules prepared by osmotic shock and freezing and thawing, detects only the water-soluble form of chromogranin A. If this is so comparison of the noradrenaline: chromogranin A ratios obtained by the two different immunological assays suggest that the water soluble chromogranin accounts for only 10 to 20% of the total chromogranin A of the nerve granules.

However, it is clear that preparations of splenic nerve granules containing noradrenaline also contain a protein that is immunologically indistinguishable from chromogranin A, but differs from it in respect to solubility in water. What is not so clear is whether all the noradrenaline is associated with the water-soluble fraction of chromogranin A, at a ratio of 5000 nmol of noradrenaline/mg chromogranin A, or whether the noradrenaline in the nerve granule is held in a water-insoluble lipoprotein matrix containing chromogranin A at a ratio of about 1000 nmol noradrenaline/mg chromogranin A.

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The distribution of chromogranin in constricted noradrenergic nerves in relation to the distribution of noradrenaline containing vesicles

Evidence that noradrenaline is stored in dense-core vesicles can be obtained from studies on ligated noradrenergic nerves. When a ligature is tightly applied to a postganglionic sympathetic nerve trunk such as the hypogastric nerve, noradrenaline accumulates on the proximal or ganglionic side of the ligature but not on the distal side of the ligature. The noradrenaline accumulation can be located by the fluorescence histochemical technique of Falk (1962) or by the quantitative fluorimetric method of Häggendal (1963) and is seen to be confined to a region extending about 2.5 mm from the ligature on the proximal side. The amount of noradrenaline accumulating increases linearly with time over a period of about 4 days (Banks *et al.* 1969) and thereafter declines (D. Tomlinson 1970, unpublished observations). When the regions of nerves immediately adjacent to the constriction are investigated with the electron microscope at different times after applying the ligature, only the axons immediately proximal to the constriction contain an increased population of dense-cored vesicles (figure 2, plate 53). Dense-cored vesicles do not accumulate on the distal side of the constriction. Other cell organelles such as mitochondria and agranular vesicles accumulate on both sides of the ligature as does the mitochondrial enzyme cytochrome oxidase (Banks *et al.* 1969; Geffen & Ostberg 1969).

That the dense-cored vesicles are the probable storage sites for noradrenaline can be shown by correlating the effects of drugs upon both the noradrenaline content and the ultrastructure of the segment of nerve immediately proximal to the ligature. Treating a cat with reserpine about 16 h before death entirely depletes the accumulation of noradrenaline proximal to the ligature and the number of dense-cored vesicles is drastically reduced. On the other hand, when cats are treated with a monoamine oxidase inhibitor, such as iproniazid, the accumulation of noradrenaline is greater than without the drug and there is a concomitant increase in the number of dense-cored vesicles found in the region (Banks, Kapeller & Mayor 1969). Recently we have been able to show that about 45 % of the noradrenaline accumulating on the proximal side of the nerve construction, 48 h after operation, can be recovered on a sucrose density gradient and must therefore be associated with particles (D. Tomlinson, D. Mayor & P. Banks 1970, unpublished observations). It seems very probable, therefore, that the dense-cored vesicles, seen to accumulate proximal to the ligatures and also to be constituents of nerve granule preparations, are the chief storage sites for noradrenaline in postganglionic sympathetic neurons. If this is so, then dopamine β-hydroxylase and chromogranin A would be expected to be distributed within constricted postganglionic axons in the same manner as noradrenaline and dense-cored vesicles.

Laduron & Belpaire (1968*b*) found that dopamine β-hydroxylase, measured enzymically, accumulated on the proximal side but not on the distal side of a ligature applied to canine splenic nerves and A. D. Smith, D. Mayor & P. Banks (1970 unpublished observations) have recently confirmed this observation using cat splenic and hypogastric nerves. Using immunohistochemical techniques Geffen, Livett & Rush (1969*a*) have shown that following ligation of ovine splenic nerves, chromogranin A and dopamine β-hydroxylase accumulate only on the proximal side of the ligature.

These experiments strongly suggest that noradrenaline, chromogranin A and dopamine

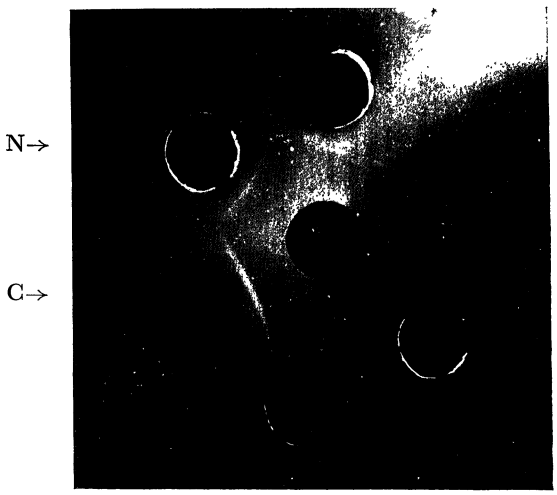

FIGURE 1. Cross-reaction of nerve granule protein with anti-chromogranin A serum. Immunodiffusion was carried out on an Ouchterlony plate in which the centre well contained antiserum against chromogranin A. Well C contained purified chromogranin A and well N contained Triton-solubilized protein obtained from the noradrenaline-rich particles isolated on the sucrose gradient at the level of 1.0 mol/l sucrose.

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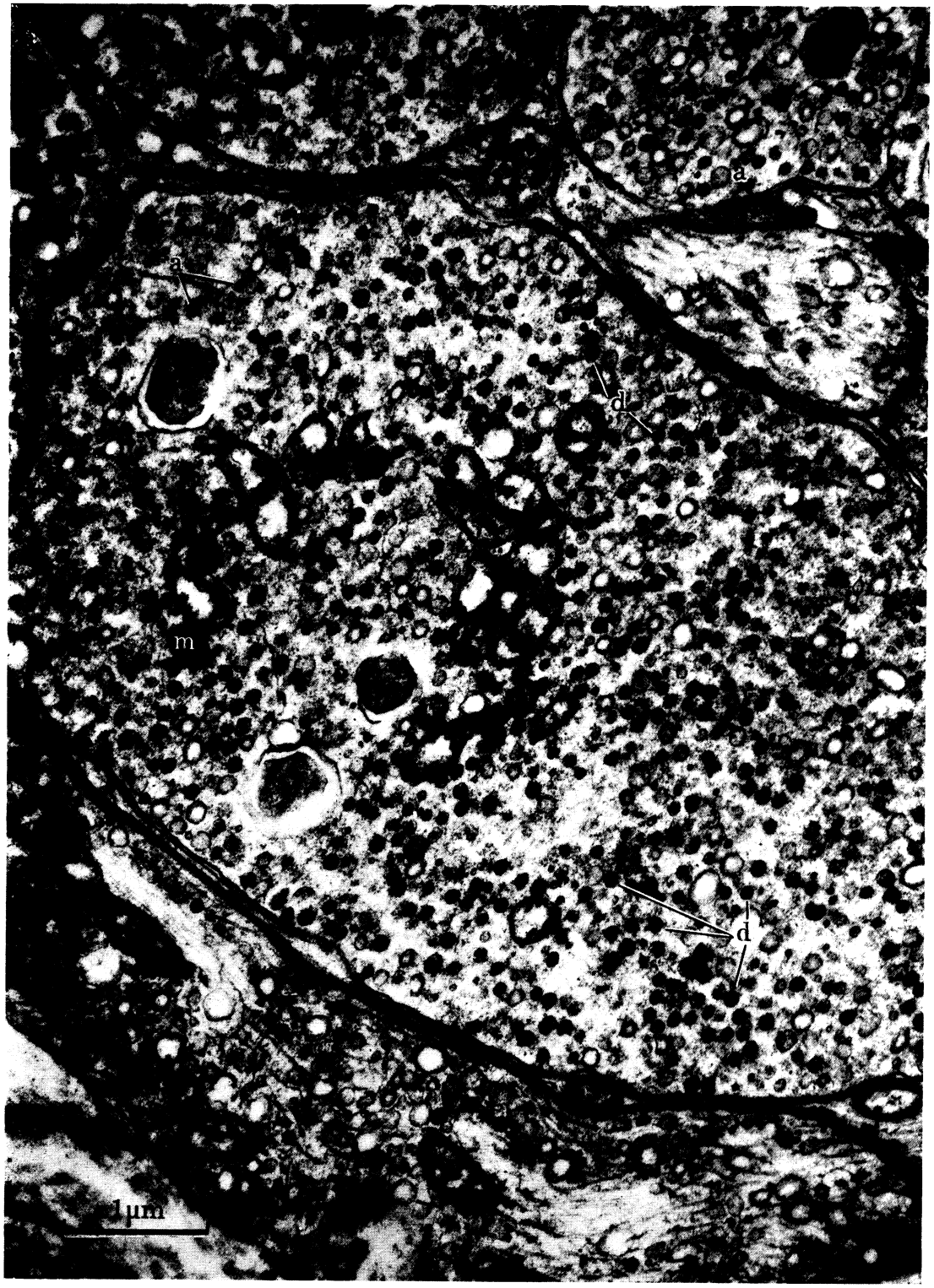

FIGURE 2. Electron micrograph of axons in cat hypogastric nerve 24 h after ligating the nerve trunk about 1 cm below the ganglion. The section was made within 0.8 mm of the ligature on the proximal or ganglionic side. Note the accumulation of dense-cored vesicles (*d*) mitochondria (*m*) and agranular vesicles (*a*). (The electron micrograph was made by Mr D. R. Tomlinson in the Human Biology and Anatomy Department at Sheffield.)

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β-hydroxylase are all constituents of dense-cored vesicles and the data are consistent with the view that the dense-cored vesicles move in a proximo-distal direction within the axons of postganglionic sympathetic neurons. In view of the fact that chromogranin A and dopamine β-hydroxylase are released during the secretory response of the adrenal medulla together with the catecholamines, it is of interest that two groups of workers have now found these two proteins to be released from perfused spleens following stimulation of the splenic nerve (De Potter *et al.* 1969; Geffen, Livett & Rush 1969*b*).

Conclusion

Most of the noradrenaline present in postganglionic neurons is stored in dense-cored vesicles that have a molar ratio of catecholamine to ATP of about 4:1 and that also carry the enzyme dopamine β-hydroxylase and the characteristic protein of chromaffin granules—chromogranin A.

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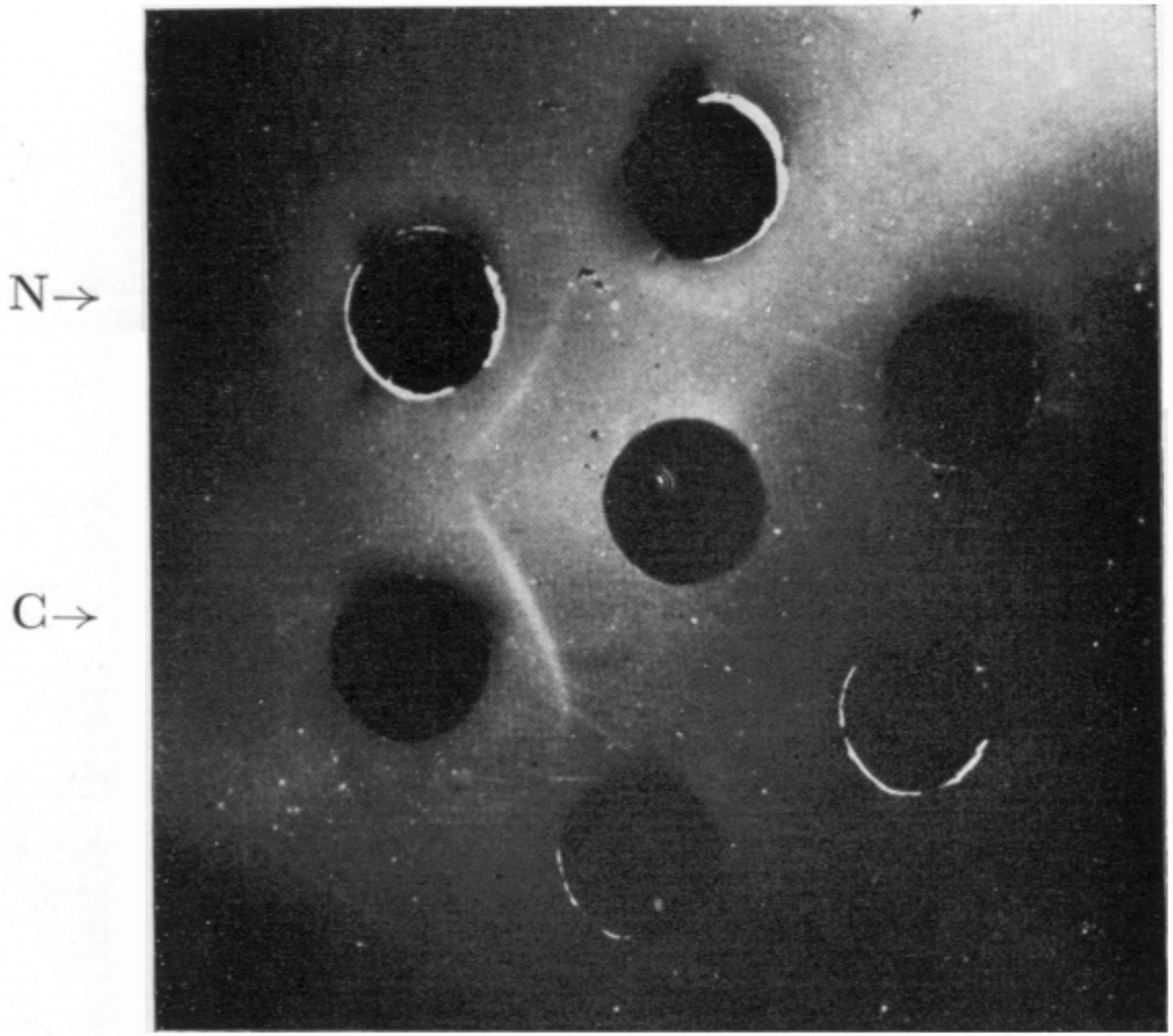

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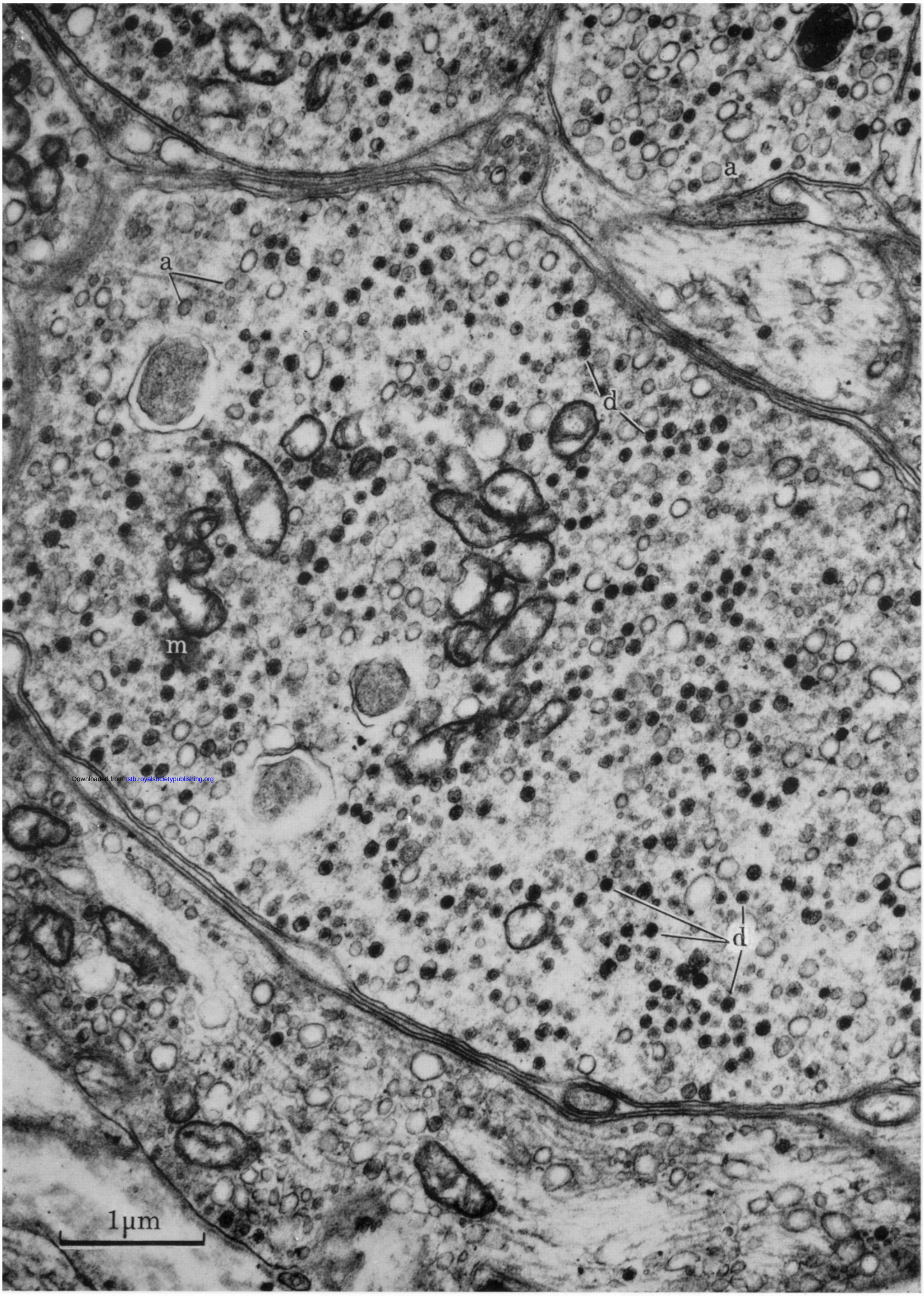

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