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FURTHER EVIDENCE FOR THE INVOLVEMENT OF MICROTUBULES IN THE INTRA-AXONAL MOVEMENT OF NORADRENALINE STORAGE GRANULES

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SUMMARY

1. Constricted cat hypogastric nerve/inferior mesenteric ganglion preparations maintained *in vitro* for 48 hr have been used to study the effects of different concentrations of colchicine on axonal microtubules and on the proximo-distal movement of catecholamine containing dense-cored vesicles in non-myelinated axons.

2. In low concentrations (0·03–0·3 μg/ml.), colchicine had no effect on the number of microtubules per axon and did not diminish the amount of noradrenaline accumulating proximal to the constriction.

3. Higher concentrations of colchicine (1·0–10 μg/ml.) produced a dramatic reduction in the number of microtubules per axon. This was associated with marked reductions in the number of dense-cored vesicles and the amount of noradrenaline accumulating above the constriction.

4. There was some morphological evidence for a structural relationship between dense-cored vesicles and microtubules.

5. These results further support the view that axonal microtubules are involved in the relatively fast proximo-distal transport of noradrenaline containing dense-cored vesicles in non-myelinated sympathetic axons.

INTRODUCTION

The proximo-distal movement of noradrenaline storage vesicles within the axons of post-ganglionic sympathetic nerves can be inhibited by colchicine and vinblastine *in vivo* (Dahlström, 1970, 1971; Hökfelt & Dahlström, 1971), and *in vitro* (Banks, Mayor, Mitchell & Tomlinson, 1971). Since these drugs are known to interact with the protein subunits of cytoplasmic microtubules (Schmitt, 1969; Borisy & Taylor, 1967) there has been much speculation that axonal microtubules are intimately concerned with the process of rapid axonal transport.

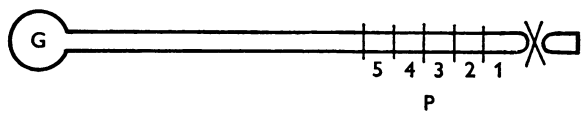
Recently it has been shown that a dose of colchicine which profoundly reduced the amount of noradrenaline transported along cat hypogastric nerves *in vitro* also caused an almost complete loss of axonal microtubules (Banks *et al.* 1971).

Further ultrastructural evidence for the involvement of microtubules in the axonal transport of vesicles has been obtained from the central nerve of the lamprey where a close association between microtubules and presumptive synaptic vesicles has been observed (Smith, Järlfors & Beránek, 1970; Smith, 1971).

The purposes of the present investigation were, first, to ascertain whether the effects of colchicine on noradrenaline transport and the abundance of microtubules held over a wide range of colchicine concentrations and, secondly, to search for morphological evidence of a close association between dense-cored vesicles containing noradrenaline and axonal microtubules.

METHODS

Ganglion nerve preparation. Constricted hypogastric nerve/inferior mesenteric ganglion preparations from cats of both sexes weighing 1·0–2·5 kg were used. In some instances the colonic nerves were also ligated 1–1·5 cm distal to the inferior mesenteric ganglion and included in the preparation. The ganglion/ligated nerve preparation was suspended in a test tube and bathed in oxygenated Eagle's Minimal Essential Medium, without added calf serum but with antibiotics, for 48 hr (see Banks *et al.* 1971 for further details). Eagle's Medium (MEM) was obtained from Wellcome Research Laboratories, Beckenham, Kent.



Text-fig. 1. Diagram indicating the segmentation of the constricted nerves for chemical analysis and electron-microscopy. G, inferior mesenteric ganglion. Segments 1–5 proximal, P, to site of constriction X.

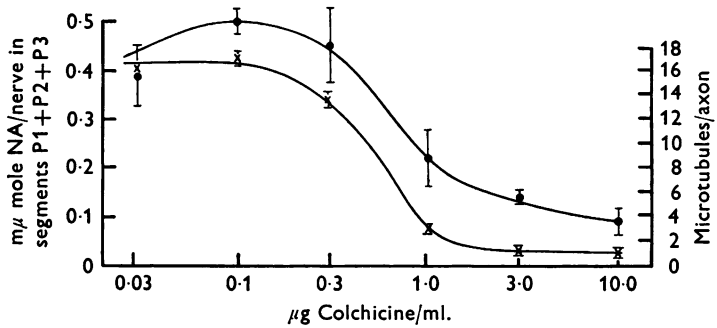
Segmentation of nerve. After 48 hr incubation, the nerves were placed on a dry post-card and the region of the nerve proximal to the site of constriction was cut into small segments 0·8 mm long (see Text-fig. 1).

Noradrenaline present in corresponding segments from both hypogastric nerves was measured using a fluorimetric method (see Häggendal, 1963; Banks *et al.* 1971).

Electron microscopy. Segments P 1 to P 3 proximal to the constriction (see Text-fig. 1) and pieces of the nerve situated about halfway between the ganglion and segment P 3 were prepared for electron microscopy. The tissue was fixed in either osmium tetroxide alone or in glutaraldehyde followed by osmium tetroxide. Micrographs, at a final magnification of 51,375 ×, from random fields of transverse sections of the nerve were used for the quantitative estimation of the number of microtubules in non-myelinated axons. The analysis of the numbers of granular vesicles per unit axon profile area in segment P 1 was made from micrographs of random fields at a final magnification of 18,200 ×.

RESULTS

Previous work (Banks *et al.* 1971) has shown that when constricted hypogastric nerve/inferior mesenteric ganglion preparations were incubated *in vitro* there was a linear increase in the amount of noradrenaline accumulating proximal to the constriction over a period of at least 48 hr. After a 48 hr period of incubation in the absence of colchicine segments P1+P2+P3 (Text-fig.1) contained 0·34 ± 0·02 (7) mμ-mole noradrenaline per nerve and 16·5 ± 0·5 (91) microtubular profiles per axon were seen in transverse sections of the nerve taken from a point midway between the ganglion and the site of constriction.



Text-fig. 2. The effect of colchicine upon the accumulation of noradrenaline (NA) and the abundance of axonal microtubules in constricted post-ganglionic sympathetic nerves. Nerve/ganglion preparations were incubated at 37° C for 48 hr in Eagle's Medium in the presence of varying concentrations of colchicine as described in the text. ● mμ mole noradrenaline per nerve in segments P1+P2+P3. × Microtubules per axon. The noradrenaline present in segments P1+P2+P3 was the mean of at least three and not more than six experiments at each concentration of colchicine. Between 80 and 110 axons were examined at each colchicine concentration to obtain the number of microtubules per axon.

The bars represent the S.E. of the mean.

Incubating the preparations for 48 hr with colchicine at concentrations of 0·03, 0·1 and 0·3 μg/ml. did not diminish the amount of noradrenaline accumulating against the ligature (Text-fig. 2). Indeed rather more noradrenaline accumulated than in the previous control experiments (Banks *et al.* 1971). When compared with these control experiments colchicine at 0·03 and 0·1 μg/ml. had no significant effect on the number of microtubular profiles seen in transverse sections of axons, while at 0·3 μg/ml. it caused a 9% reduction in the number of microtubules/axon. After a 48 hr period of incubation with colchicine at 1·0, 3·0 or 10·0 μg/ml. there was a very profound reduction in both the amount of noradrenaline accumulating

and in the number of microtubular profiles seen (Text-fig. 2). The concentration of colchicine giving half-maximal reduction in the amount of noradrenaline accumulating over 48 hr was about 0·7 μg/ml. ($1{\cdot}75 \times 10^{-6}$ M) whilst that causing a half-maximal reduction in microtubule numbers was 0·6 μg/ml. ($1{\cdot}5 \times 10^{-6}$ M).

After 48 hr incubation *in vitro* in the absence of colchicine the marked variation in the organelle content and in the degree of axonal swelling found in segment P1 was similar to that seen in comparable experiments on hypogastric nerve ligated *in vivo* (see Kapeller & Mayor, 1969; Banks, Mangnall & Mayor, 1969). In longitudinal sections and in serial transverse sections considerable variation was seen along relatively short lengths of individual axons. These features and the wide variation in the number of dense-cored vesicles in individual axons after incubation in both the absence and the presence of colchicine necessitated a quantitative assessment of the abundance of granular vesicles in segment P1.

After 48 hr incubation in Eagle's Medium control nerves from the previous experiments (Banks *et al.* 1971) contained $31{\cdot}69 \pm 3{\cdot}44$ (S.E.) dense-cored vesicles per unit axonal area. In the present experiments when colchicine was added to Eagle's Medium in concentrations of 0·03 and 3·0 μg/ml. the number of dense-cored vesicles seen per unit axon profile area was $44{\cdot}60 \pm 5{\cdot}96$ (S.E.) and $16{\cdot}54 \pm 1{\cdot}29$ (S.E.) respectively (Table 1).

TABLE 1. Effects of colchicine on the numbers of dense-cored vesicle (DCV) profiles per unit axon profile area in P1 segments of constricted cat hypogastric nerves, 48 hr *in vitro*

Drug	DCV profiles/unit axon profile area
Colchicine 0·03 μg/ml.	$44{\cdot}60 \pm 5{\cdot}96$ (24)
	$-P < 0{\cdot}10$
No drug	$31{\cdot}69 \pm 3{\cdot}44$ (34)
	$-P < 0{\cdot}001$
Colchicine 3·0 μg/ml.	$16{\cdot}54 \pm 1{\cdot}29$ (22)

In view of the large accumulation of organelles and the marked distortion of the axons immediately adjacent to the constriction, the microtubules were frequently difficult to identify with certainty and there was no obvious relationship between these structures and granular vesicles. At the proximal end of segment P1 and further from the constriction where the axons were of more normal dimensions, microtubules were arranged approximately parallel to each other along the axons and groups of dense-cored vesicles or single vesicles were seen to be closely associated with the microtubules. Occasionally it appeared as though the vesicles were lined up along the microtubules (Pls. 1 and 2).

DISCUSSION

The view that noradrenaline storage vesicles can be equated with dense-cored vesicles receives confirmation from the finding that the reduction in the accumulation of noradrenaline caused by colchicine is paralleled by the reduction in the number of dense-cored vesicles accumulating above the ligature (see also Banks, Kapeller & Mayor, 1969).

The present experiments demonstrate that the inhibitory action of colchicine on the proximo-distal movement of noradrenaline correlates well with the ability of this drug to cause the disruption and disappearance of axonal microtubules. These observations, coupled with the demonstration that colchicine does not deplete dense-cored vesicles of their stored noradrenaline (Banks *et al.* 1971), support the view that the integrity of axonal microtubules is a prerequisite for the fast intra-axonal movement of noradrenaline storage vesicles. Interest thus focuses on the nature of the functional relationship between axonal microtubules and the relatively rapid, 1–2 mm/hr, proximo-distal movement of dense-cored vesicles.

It is possible that the microtubules facilitate the movement of dense-cored vesicles by maintaining the characteristic cylindrical form of the axons and thus favouring the interaction of vesicles with other axonal organelles. However, in our experiments the removal of microtubules by treatment with colchicine did not lead to any obvious change in the overall axonal morphology. Furthermore, the disappearance of microtubules does not prevent the movement of mitochondria towards the ligature as judged subjectively from electron micrographs taken from segment P1 immediately above the ligature.

It would appear, therefore, that some more specific relationship exists between dense-cored vesicles and microtubules than merely the maintenance of axonal morphology. In this context, the fact that dense-cored vesicles are sometimes found to be aligned with microtubules is noteworthy and is reminiscent of, although less dramatic than, the association between presumptive synaptic vesicles and microtubules demonstrated by Smith *et al.* (1970) in the lamprey nervous system.

However, although there is undoubtedly a relationship between microtubules and dense-cored vesicles, the evidence at present for some definite physico-chemical contact between them is sparse and the details of an interaction, if any, between these two organelles are still obscure (see Davison, 1970*a*, *b*).

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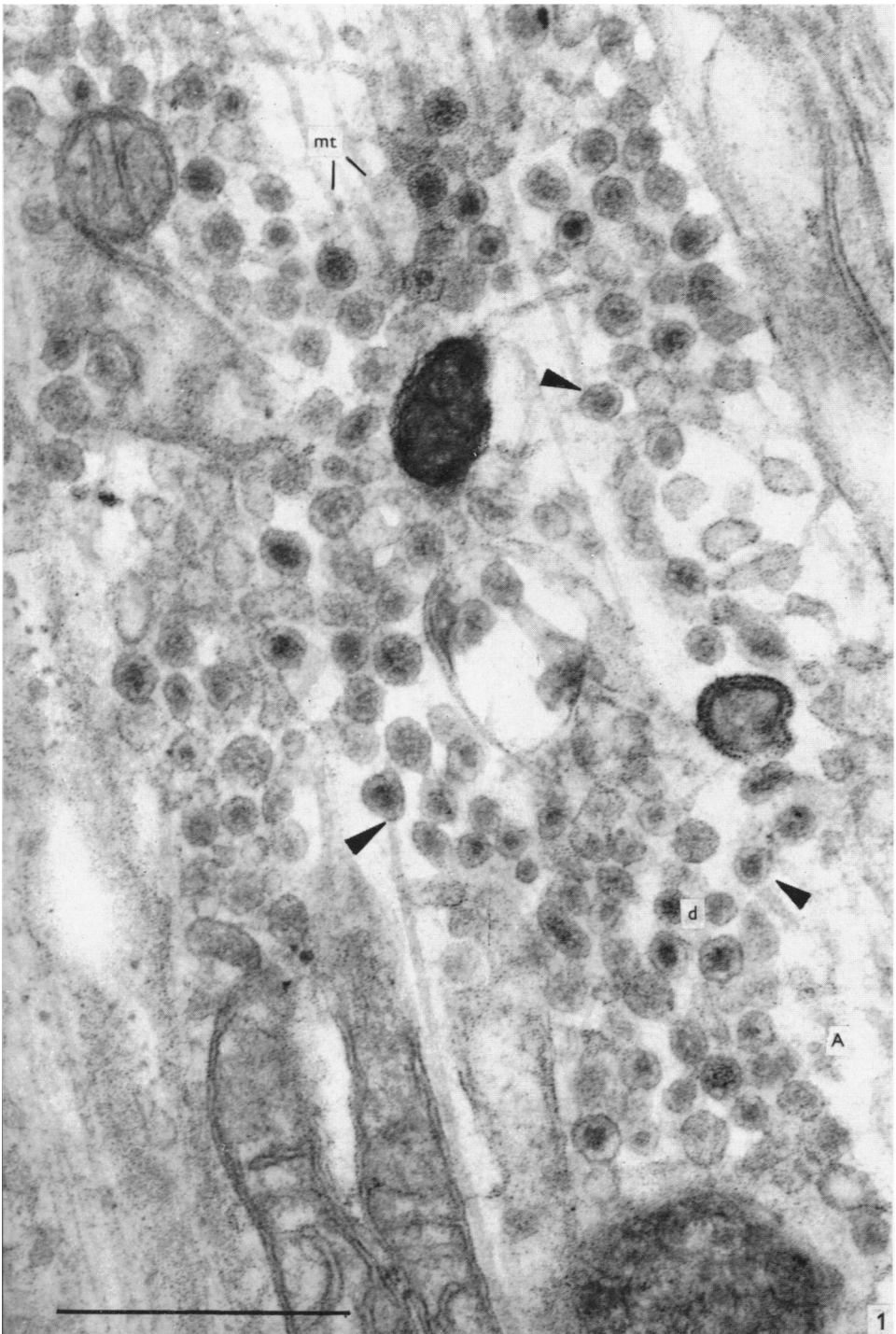

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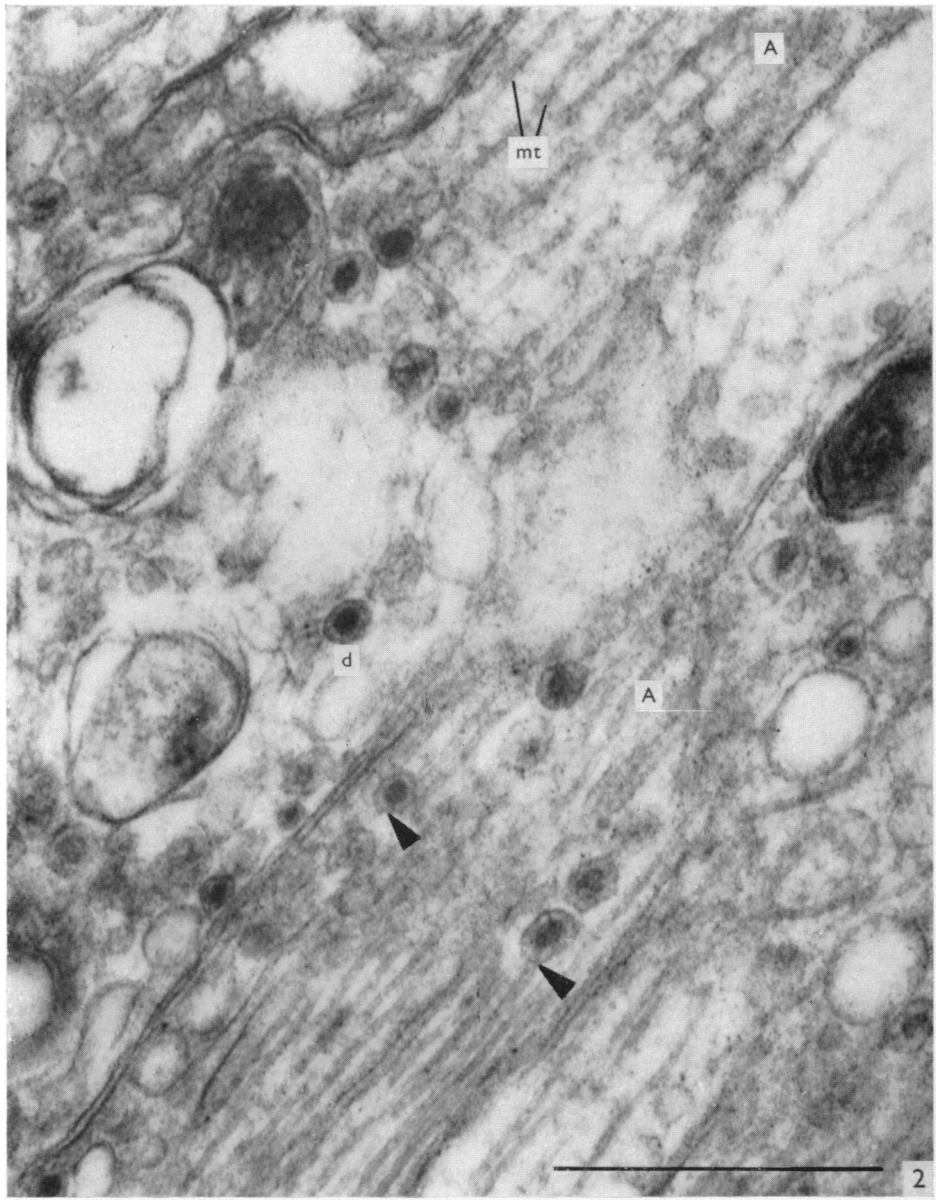
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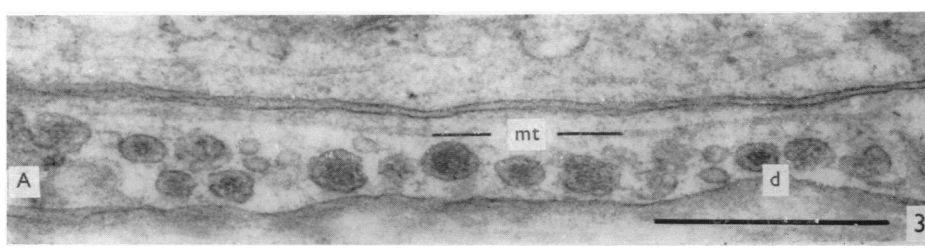
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EXPLANATION OF PLATES

The electron micrographs are from the proximal end of segment P 1 (see Text-fig. 1) after 48 hr incubation in Eagle's Medium only. The nerves were fixed in osmium tetroxide and the sections were stained with lead citrate. *A* indicates non-myelinated axon. Magnification markers represent 0·5 μ.

PLATE 1

Fig. 1. Illustrates the accumulation of dense-cored vesicles (*d*), orientated in some cases parallel to the axonal microtubules (*mt*), in a slightly swollen non-myelinated axon. Certain dense-cored vesicles (arrowed) are in close proximity to particular microtubules.

PLATE 2

Fig. 2. Illustrates instances of a morphological proximity (arrowed) between dense-cored vesicles (*d*) and axonal microtubules (*mt*) in longitudinal sections of non-myelinated axons of approximately normal diameter.

Fig. 3. Shows dense-cored vesicles (*d*) orientated parallel to microtubules (*mt*) in a narrow portion of a non-myelinated axon.