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A CORRELATION BETWEEN THE EFFECTS OF ANTI-MITOTIC DRUGS ON MICROTUBULE ASSEMBLY *IN VITRO* AND THE INHIBITION OF AXONAL TRANSPORT IN NORADRENERGIC NEURONES

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SUMMARY

1. Podophyllotoxin, colchicine and griseofulvin inhibit the intra-axonal movement of noradrenaline storage vesicles in cat hypogastric nerve/inferior mesenteric ganglion preparations maintained *in vitro*, cause the disappearance of axonal microtubules and inhibit the assembly of microtubules from tubulin *in vitro*. The order of potency for the three effects is podophyllotoxin > colchicine > griseofulvin.

2. Lumicolchicine is without effect on the three parameters and does not interfere with the binding of tritiated colchicine to tubulin.

3. Podophyllotoxin causes a more rapid loss of microtubules from axons than the same concentration of colchicine.

4. The experiments provide strong evidence that microtubules are components of the system responsible for the intra-axonal migration of noradrenaline storage vesicles.

INTRODUCTION

Noradrenaline storage vesicles are formed in the cell bodies of postganglionic sympathetic neurones and subsequently migrate along the axons to the terminal varicose plexus at about 2–5 mm/hr (see Dahlström, 1971; Banks & Mayor, 1972). Antimitotic drugs, such as colchicine, which depolymerize axonal microtubules, also inhibit the intra-axonal movement of the storage vesicles (Dahlström, 1968; Banks, Mayor, Mitchell & Tomlinson, 1971*a*; Banks, Mayor & Tomlinson, 1971*b*). Observations of this sort have led to the idea that microtubules are components of a system governing the intra-axonal migration of subcellular organelles. This hypothesis would have to be rejected if microtubules could be removed by drug treatment without affecting vesicle transport but, so far, the destruction of microtubules has always been attended by an

inhibition of transport. However, the ability to destroy microtubules may not be the only action of some antimitotic drugs. For example, colchicine causes ribosomes to clump, alters the arrangement of nuclear, endoplasmic and myelin membranes (Bunge & Bunge, 1968; Karlsson, Hansson & Sjöstrand, 1971; Peterson & Murray, 1966; Walsh, 1973), and inhibits the uptake of nucleosides by cultured cells (Mizel & Wilson, 1972). Thus whether antimitotic drugs inhibit movement by depolymerizing microtubules or by some other action, unconnected with microtubules, remains to be decided.

The experiments described in this paper show that the antimitotic drugs podophyllotoxin (see Zweig & Chignell, 1973), colchicine, and griseofulvin (see Paget & Walpole, 1958), have the same relative order of potency in three tests, namely (i) inhibition of axonal transport, (ii) depolymerization of axonal microtubules and (iii) inhibition of microtubule assembly *in vitro*. Furthermore, they show that lumicolchicine, which is claimed to be much less active than colchicine in dispersing microtubules but more potent as an inhibitor of nucleoside uptake (Mizel & Wilson, 1972; Wilson, Bamburg, Mizel, Grisham & Creswell, 1974), is of lower potency than colchicine in the three tests.

METHODS

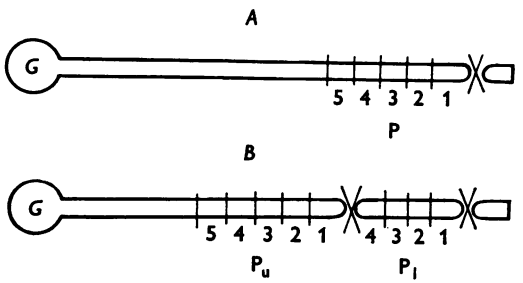
Ganglion/nerve preparations. The hypogastric nerves of adult cats were constricted with fine silk ligatures about 2 cm distal to the inferior mesenteric ganglion. The ganglia and attached hypogastric nerves were removed from the animal and incubated at 37° C in a test-tube in oxygenated Eagle' (M.E.M.) tissue culture medium (Wellcome Research Laboratories, Beckenham, Kent, U.K.) as described by Banks *et al.* (1971*a*). In some experiments, after incubating for 24 hr, a second ligature was placed on the hypogastric nerves about 0·5 cm proximal to the first ligature and incubation was continued for a further 24 hr.

Noradrenaline estimation. At the end of the incubation period the preparation was placed on a dry card and segmented as shown in Text-fig. 1. Noradrenaline in the segments was measured by the method of Häggendal (1963) described by Banks *et al.* (1971*a*). Noradrenaline accumulation was always confined to segments P_1 to P_3.

Electron microscopy. Lengths of nerve taken from midway between ganglion and ligature were prepared for electron microscopy. The tissue was fixed in 4% glutaraldehyde in 0·1 M phosphate buffer (pH 7·35) at room temperature for $2\frac{1}{2}$ hr. After washing for 24–48 hr in 0·1 M phosphate buffer containing 10% sucrose the material was post-fixed at 4° C in 2% OsO_4 in Millonig's phosphate buffer, dehydrated in graded ethanols, passed through epoxy propane and embedded in Araldite (CIBA Ltd.). Ultra-thin sections were stained with 1% uranyl acetate in H_2O/ethanol (50:50, v/v) followed by lead citrate (Reynolds, 1963). Transverse sections were examined in an A.E.I. Corinth electron microscope. The numbers of microtubules in non-myelinated axons were counted on electron micrographs taken from random fields.

Microtubule formation in vitro. The method used was that developed for brain tubulin by Shelanski, Gaskin & Cantor (1973).

About 15 g bovine splenic nerve was placed on ice at the slaughter house and subsequently desheathed and cut into small segments in the laboratory before being homogenized in a 10 mM phosphate buffer (pH 6·7) containing 10 mM $MgCl_2$ (1:1, w/v) using an M.S.E. overhead homogenizer. The homogenate was centrifuged at 100,000 *g* (average) for 15 min and the resulting supernatant was recentrifuged at 100,000 *g* (average) for 30 min. Two ml. samples of the final supernatant were incubated at 37° C for 1 hr with 2·5 ml. 8 M glycerol, 0·5 ml. 10 mM phosphate (pH 6·7) containing 50 mM EGTA and 5 mM GTP and 0·1 ml. dimethylsulphoxide with or without drugs. At the end of the incubation, the mixtures were centrifuged at 10,000 *g* (average) for 30 min and the resulting pellets were dissolved in 2 ml. Mg^{2+}-free phosphate buffer (pH 6·7) and their protein content measured by the method of Lowry, Rosebrough, Farr & Randall (1951). The protein content of the pellets was used as a measure of microtubule assembly. Some pellets were fixed in buffered glutaraldehyde, examined in the electron microscope and shown to contain structures similar to microtubules seen in axons.



Text-fig. 1. Diagrams indicating the segmentation of the constricted nerves for noradrenaline analysis. *G*, inferior mesenteric ganglion. ×, sites of constriction. *A*, segments 1–5 proximal to a single constriction. *B*, segments $P_{L_{1-4}}$: above lower constriction applied when preparations excised from animal. Segments $P_{U_{1-5}}$: above second, or upper, constriction applied after initial 24 hr incubation.

^{3}H-colchicine binding to tubulin. This was estimated by the method of Weisenberg, Borisy & Taylor (1968).

Chopped, desheathed nerves were homogenized in 10 mM phosphate buffer (pH 6·7) containing 1 mM GTP and 10 mM-$MgCl_2$ (1:1, w/v) and centrifuged at 100,000 *g* (average) for 15 min. The supernatant was recentrifuged at 100,000 *g* (average) for 30 min and then diluted ten times in the phosphate, GTP, Mg^{2+}-buffer; to 0·5 ml. diluted supernatant were added 0·1 ml. 10^{-3} M colchicine, 0·1 ml. dimethylsulphoxide (with or without drugs) and 0·05 ml. buffer. Finally, 0·25 ml. ^{3}H-colchicine (5×10^{-6} M; 2 μCi/n-mole) was added and the mixture was incubated at 37° C for 30 min. The incubation was ended by placing the tubes on ice for 1 min, then the mixture was poured on to a DEAE filter paper and allowed to drain through. The filter disk was subsequently washed five times with 2 ml. buffer and transferred to a scintillation vial to which was added 5 ml. NE220 scintillator. The radioactivity bound to the filter disk was measured using a Packard Tricarb Liquid Scintillation Spectrometer. Radioactivity present in the filtrate and washings was also measured and recoveries averaged 105% ± 0·03 S.E.M. of mean (11). Control experiments showed that free [^{3}H]colchicine did not bind to the filter and that radioactivity retained by the filter could be ascribed to tubulin-bound colchicine.

Lumicolchicines (mol. wt. 399). These were prepared by irradiating colchicine (0·5 μg/ml. in dimethylsulphoxide/water 9:1, v/v) with ultraviolet light; the conversion was followed spectrophotometrically as described by Wilson *et al.* (1974).

Drugs. Colchicine (mol. wt. 399) was obtained from BDH Ltd, Poole, Dorset, U.K., griseofulvin (mol. wt. 343) from ICI Pharmaceuticals Ltd, Macclesfield, Cheshire, U.K., and podophyllotoxin (mol. wt. 414) from Aldrich Chemical Co. Ltd, Wembley, Middx., U.K. [^{3}H]colchicine was obtained from the Radiochemical Centre, Amersham, Bucks., U.K.

RESULTS

Effects of drugs on noradrenaline accumulation and microtubule numbers

Nerve ganglion preparations were incubated for 24 hr at 37° C, with or without drugs, and subsequently the amount of noradrenaline accumulating within the 2·5 mm nerve immediately proximal to the ligatures was estimated. In each experiment a length of nerve trunk taken from midway between the ganglion and ligature was used to assess the abundance of microtubules within the non-myelinated axons. Table 1 shows that the potency of the drugs for inhibiting noradrenaline accumulation and for depolymerizing axonal microtubules was in the order podophyllotoxin > colchicine ≫ griseofulvin. A mixture of β and γ lumicolchicines (1·0 μg/ml.) was without effect on both parameters whereas at a concentration of 1·0 μg/ml. colchicine caused a 53 % reduction in noradrenaline accumulation and 68 % reduction in microtubule numbers.

Pl. 1, 2 and 3 show transverse sections of nerve taken from preparations treated with podophyllotoxin (0·5 μg/ml.), lumicolchicine (1 μg/ml.) and griseofulvin (100 μg/ml.) respectively. Concentrations of podophyllotoxin which reduced noradrenaline accumulation to undetectable levels completely prevented the accumulation of dense-cored, noradrenaline storage vesicles in the axons of segment P_1 (see Text-fig. 1); furthermore, the axonal swelling which accompanied the accumulation of organelles in control nerves was absent.

Effect of colchicine and podophyllotoxin on the noradrenaline content of storage vesicles

Nerve ganglion preparations were incubated in the absence of drugs for 24 hr at 37° C before a second ligature was placed on the nerve trunks about 0·5 cm proximal to the first ligature. Then, in all except the control experiments, either colchicine (final concentration 10 μg/ml.) or podophyllotoxin (final concentration 5 μg/ml.) was added to the bathing medium and the incubation was continued for a further 24 hr. In control experiments, the amounts of noradrenaline above the upper and lower constrictions did not differ significantly after the full 48 hr period of incubation (Table 2). Neither podophyllotoxin (5 μg/ml.) nor colchicine

TABLE 1. Effects of drugs on noradrenaline accumulation and microtubule abundance

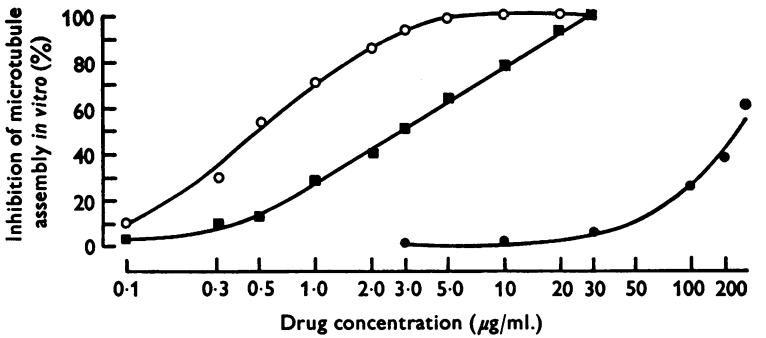
	Control	Podophyllotoxin			Colchicine		Lumicolchicine	Griseofulvin
Drug (μg/ml.) ...	—	0·1	0·5	5·0	0·5	1·0	1·0	100
Noradrenaline n-mole/nerve per 24 hr	0·17	0·13	0·02	>0·01	0·11	0·08	0·17	0·06
± S.E. of mean	± 0·01	± 0·04	± 0·0		± 0·0	± 0·01	± 0·04	± 0·01
(n) = no. of expts.	(5)	(3)	(3)	(3)	(2)	(2)	(5)	(3)
Microtubules/axon	24·2	9·7	0·4	0	18·2	7·8	26·9	12·1
± S.E. of mean	± 0·4	± 0·2	± 0·0		± 0·1	± 0·3	± 0·8	± 0·6
(n) = no. of axons counted	(78)	(78)	(60)	(45)	(77)	(75)	(102)	(63)

Nerve ganglion preparations were incubated at 37° C for 24 hr in the presence or absence of drugs. The figures for microtubule abundance refer to a single experiment and the figures in parentheses gives the number of axons counted. In all experiments, except one, in which microtubule numbers were counted, the noradrenaline accumulation was close to the mean value shown in the Table. The exception was the experiment with 0·1 μg/ml. podophyllotoxin in which noradrenaline accumulation was 0·06 n-mole/nerve/24 hr.

TABLE 2. The effects of colchicine and podophyllotoxin on stores of noradrenaline accumulated proximal to a constriction before the addition of drugs

Drug	Noradrenaline accumulating (n-mole/nerve ± S.E. of mean) Segments $P_{U_1}+P_{U_2}+P_{U_3}$, upper	Segments $P_{L_1}+P_{L_2}+P_{L_3}$, lower
Control	0·16 ± 0·02 (4)*	0·18 ± 0·02 (4)*
Colchicine (10 μg/ml.)	0·06 ± 0·01 (2)*	0·18 ± 0·03 (2)*
Podophyllotoxin (5 μg/ml.)	< 0·01 ± 0 (2)	0·17 ± 0·03 (2)

Ganglion/ligated hypogastric nerve preparations were incubated in test-tubes for 24 hr under normal conditions. A second ligature was then applied about 0·5 cm proximal to the first and the conditions of incubation were modified as indicated in the Table. The incubation was then continued for a further 24 hr at 37° C. Segments $P_{L_1}+P_{L_2}+P_{L_3}$ were immediately proximal to the lower, or first, constriction and Segments $P_{U_1}+P_{U_2}+P_{U_3}$ were immediately proximal to the upper, or second, constriction (see Text-fig. 1). Figures in parentheses indicate the number of observations. The results marked with an asterisk are taken from Banks *et al.* (1971*a*).



Text-fig. 2. The effects of podophyllotoxin, colchicine and griseofulvin on the assembly of microtubules *in vitro*. High-speed supernatants from homogenates of bovine splenic nerves were induced to form microtubules by incubation at 37° C in 4 M glycerol containing EGTA, GTP and Mg^{2+} as described under Methods. Tubules were collected by centrifugation and quantified by measuring their protein content. ●, griseofulvin; ○, podophyllotoxin; ■, colchicine.

(10 μg/ml.) had any effect upon the amount of noradrenaline above the lower constriction but both greatly reduced the amount above the upper ligature.

Effect of drugs on the assembly of microtubules in vitro

Samples of high-speed supernatant prepared from homogenates of bovine splenic nerve were incubated, with or without drugs, in 4 M

glycerol, 5 mM EGTA and 0·5 mM GTP at 37° C for 1 hr to induce the formation of microtubules as described under Methods (Pl. 4). Podophyllotoxin, colchicine and griseofulvin caused a dose dependent inhibition of tubule assembly as shown in Text-fig. 2; 50 % inhibition was effected by 0·5 μg/ml. podophyllotoxin, 3·0 μg/ml. colchicine and 180 μg/ml. griseofulvin. On account of its low solubility griseofulvin could not be tested above a concentration of 200 μg/ml. Incubation with lumicolchicines (10 μg/ml.), inhibited assembly by about 14 % whereas colchicine at the same concentration gave an inhibition of around 92 % (Table 3).

Effect of lumicolchicine and podophyllotoxin on the binding of [^3H]colchicine to tubulin

The binding of [^{3}H]colchicine to tubulin present in high-speed supernatants of bovine splenic nerve was measured in the presence and absence of drugs as described under Methods. With a colchicine concentration 50 μg/ml., lumicolchicine (50 μg/ml.) did not alter the binding of label significantly whereas podophyllotoxin (50 μg/ml.) inhibited binding by about 70 % (Table 4).

TABLE 3. Effects of colchicine and lumicolchicine on the assembly of microtubules *in vitro*

	Control	Colchicine (10 μg/ml.)	Lumicolchicine (10 μg/ml.)
Tubules formed (μg protein)	4220 ± 75·7 (4)	350 ± 59·7 (4)	3610 ± 77·2 (4)
Inhibition of tubule assembly (%)	—	92	14

Samples of high-speed supernatants from homogenates of bovine splenic nerves were incubated with or without drugs, in 4 M glycerol together with GTP, Mg^{2+} and EGTA for 1 hr at 37° C before microtubules were collected by centrifugation. Figures in parentheses indicate the number of observations.

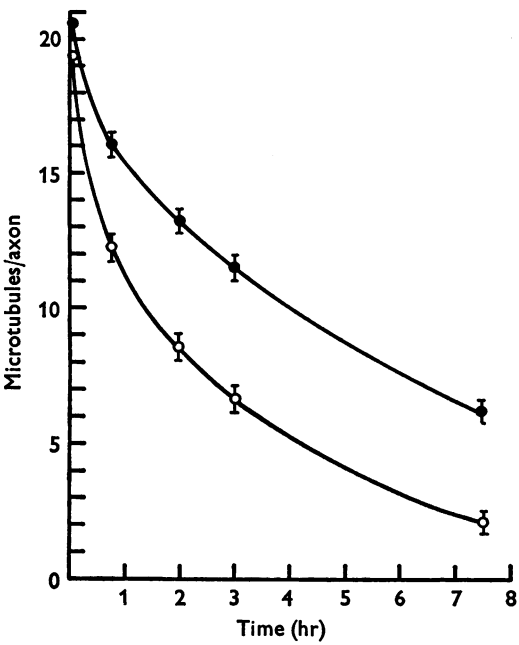
TABLE 4. Effects of lumicolchicine and podophyllotoxin on the binding of [^{3}H]colchicine to tubulin

	Control	Lumicolchicine (50 μg/ml.)	Podophyllotoxin (50 μg/ml.)
D.p.m. bound to filter ± S.E. of mean	20,503 ± 3,059 (4)	19,446 ± 2,380 (4)	5353 ± 616 (4)
Inhibition of binding (%)	—	5	74

Samples of high-speed supernatants from homogenates of bovine splenic nerves were incubated with [^{3}H]colchicine (50 μg/ml.) in the presence or absence of other drugs as described under Methods. [^{3}H]colchicine retained by DEAE cellulose filter paper was assumed to be bound to tubulin (see Weisenberg *et al.* 1968). Figures in parentheses indicate the number of observations.

The rate of loss of microtubules from hypogastric nerves incubated in the presence of colchicine and podophyllotoxin

Nerve ganglion preparations were incubated at 37° C for 4 hr in oxygenated Eagle's medium before a length of hypogastric nerve was removed for electron microscopy, and the preparation was transferred to medium containing colchicine (10 μg/ml.) or podophyllotoxin (10 μg/ml.). Small lengths of nerve were removed from the preparation at intervals over the subsequent 7½ hr. Text-fig. 3 shows that podophyllotoxin caused a more rapid and complete loss of tubules than colchicine.

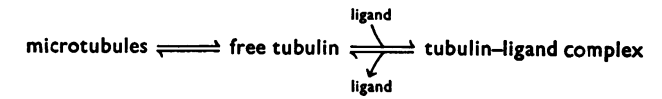


Text-fig. 3. The rates of loss of axonal microtubules in the presence of podophyllotoxin and colchicine. Nerve/ganglion preparations were incubated for 4 hr at 37° C before a length of nerve was taken for electron microscopy. The preparation was then transferred to Eagle's medium containing the drug, and sections of nerve were removed for examination at intervals over the ensuing 7½ hr. ●, colchicine (10 μg/ml.); ○, podophyllotoxin (10 μg/ml.).

DISCUSSION

The view that microtubules are necessary for the intra-axonal migration of noradrenaline storage vesicles is based on several pieces of evidence. First, vesicle movement occurs in lengths of axon lacking functional contact with either cell body or nerve ending and must, therefore, be

generated locally within the axon or within the axon–Schwann cell complex (Dahlström, 1967; Mayor & Kapeller, 1967; Banks, Mangnall & Mayor, 1969). Second, microtubules, similar to those that are aligned parallel to the long axis of axons, are known to be concerned with the intracellular movement of particles of various kinds, most notably with that of chromosomes during mitosis (see reviews by Olmsted & Borisy, 1973; Bardele, 1973). Third, antimitotic drugs such as colchicine and vinblastine, which disperse the array of microtubules forming the mitotic spindle and arrest the movement of chromosomes, inhibit the migration of noradrenaline storage vesicles and destroy axonal microtubules when in contact with the nerve trunk alone (Dahlström, 1968; Banks *et al.* 1971*a*). Furthermore, the concentration of colchicine causing half-maximal inhibition of vesicle movement is similar to that giving half-maximal disaggregation of microtubules (Banks *et al.* 1971*b*). Fourth, colchicine and vinblastine bind to tubulin, the protein subunit of microtubules; both ligands are believed to cause microtubules to break-down by establishing an equilibrium:



which is very much in favour of the tubulin–ligand complex (for reviews see Wilson *et al.* 1974; Olmsted & Borisy, 1973). The two ligands bind at different sites and the resulting ligand–protein complexes differ in their properties; the colchicine–tubulin complex is readily soluble whereas the vinblastine–tubulin complex forms insoluble aggregates of distinctive appearance.

The fourth strand of evidence is of great importance because it shows that colchicine and vinblastine exert a direct effect on microtubules and it has led to the view that the effects of the drugs on vesicle transport can be ascribed to the accompanying depolymerization of microtubules. However, the alternative view that both drugs have two actions, one resulting in the inhibition of transport and the other leading to the break-down of microtubules must be considered. This paper provides evidence which helps to distinguish between these hypotheses.

Both podophyllotoxin, which binds to tubulin at the same site as colchicine, and griseofulvin, which may not (see Wilson *et al.* 1974), inhibit vesicle transport and remove axonal microtubules. In both respects podophyllotoxin is more potent, and griseofulvin is less potent, than colchicine. All three drugs inhibit the assembly of microtubules *in vitro* and again their efficacy is in the order podophyllotoxin > colchicine $\gg$ griseofulvin. The finding that griseofulvin depolymerizes cellular microtubules and prevents tubule assembly *in vitro* conflicts with the

report of Grisham, Wilson & Bensch (1973); this discrepancy probably arises from differences in the concentrations used. In our studies 0·25 mM griseofulvin diminished transport, tubule stability and tubule assembly whilst at 0·05 mM no effects were observed: the maximum concentration of griseofulvin used by Grisham *et al.* was 0·06 mM.

Although colchicine, vinblastine and griseofulvin bind to different sites on tubulin, they share the common property of breaking down microtubules; consequently their ability to inhibit vesicle transport could be explained if the movement requires an intact system of microtubules. If there is no involvement of microtubules, it is difficult to account for the fact that these different drugs have similar effects on vesicle movement in addition to, and quite distinct from, their actions on microtubules. Wilson *et al.* (1974), have drawn attention to the value of using lumicolchicines as diagnostic tools for microtubule dependent processes; in some instances these drugs are more potent inhibitors than colchicine but as they do not bind to tubulin they should not exert their effects by depolymerizing microtubules. Experiments described in this paper show that a mixture of lumicolchicines was without effect on vesicle transport, axonal microtubules or microtubule assembly, and failed to interfere with colchicine binding to tubulin. These data suggest that the action of colchicine on vesicle movement cannot be attributed to its lumicolchicine-like activity but must be ascribed to its ability to bind to tubulin and thus to depolymerize microtubules. In a recent paper Price (1974) has reported that, in contrast to colchicine, lumicolchicines failed to inhibit fast axonal transport in the visual system of rabbits.

During the course of the present experiments we noted that, after 24 hr in the presence of podophyllotoxin (10 μg/ml.), microtubules were almost absent from axons and noradrenaline accumulation above the single constriction was scarcely detectable. This contrasted with earlier experiments (Banks *et al.* 1971*a*) which showed a significant, but much diminished, accumulation of noradrenaline after 48 hr in the presence of colchicine (10 μg/ml.), although microtubules were almost absent. The earlier experiments could indicate that vesicle transport does not require intact microtubules. However, as the rate of loss of microtubules in the presence of colchicine (10 μg/ml.) was slower and much less complete than in the presence of podophyllotoxin (10 μg/ml.), it is probable that movement of vesicles would continue for longer in colchicine treated preparations than in those treated with podophyllotoxin if it depended upon the presence of microtubules.

In conclusion, the results described in this paper are consistent with the view that microtubules are necessary components of the system responsible for the intra-axonal migration of noradrenaline storage vesicles.

Note added in proof.

Since this paper was submitted for publication we have seen the paper by J. C. Paulson & W. C. McClure (1974), Microtubules and axonal transport (*Brain Res.* **73**, 333–377), which describes the effects of several antimitotic drugs and lumicolchicine on axonal transport in the rat optic system.

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EXPLANATION OF PLATES

PLATE 1

Electron micrograph of a transverse section of hypogastric nerve from midway between ganglion and ligature after incubation for 24 hr in Eagle's medium containing podophyllotoxin (0·5 μg/ml.). The axons (*A*) appear to be normal except for the absence of microtubules (compare Pl. 2). Neurofilaments (*N*), mitochondria (*M*) and Schwann cells (*S*) have their normal appearance. (Bar = 1 μm.)

PLATE 2

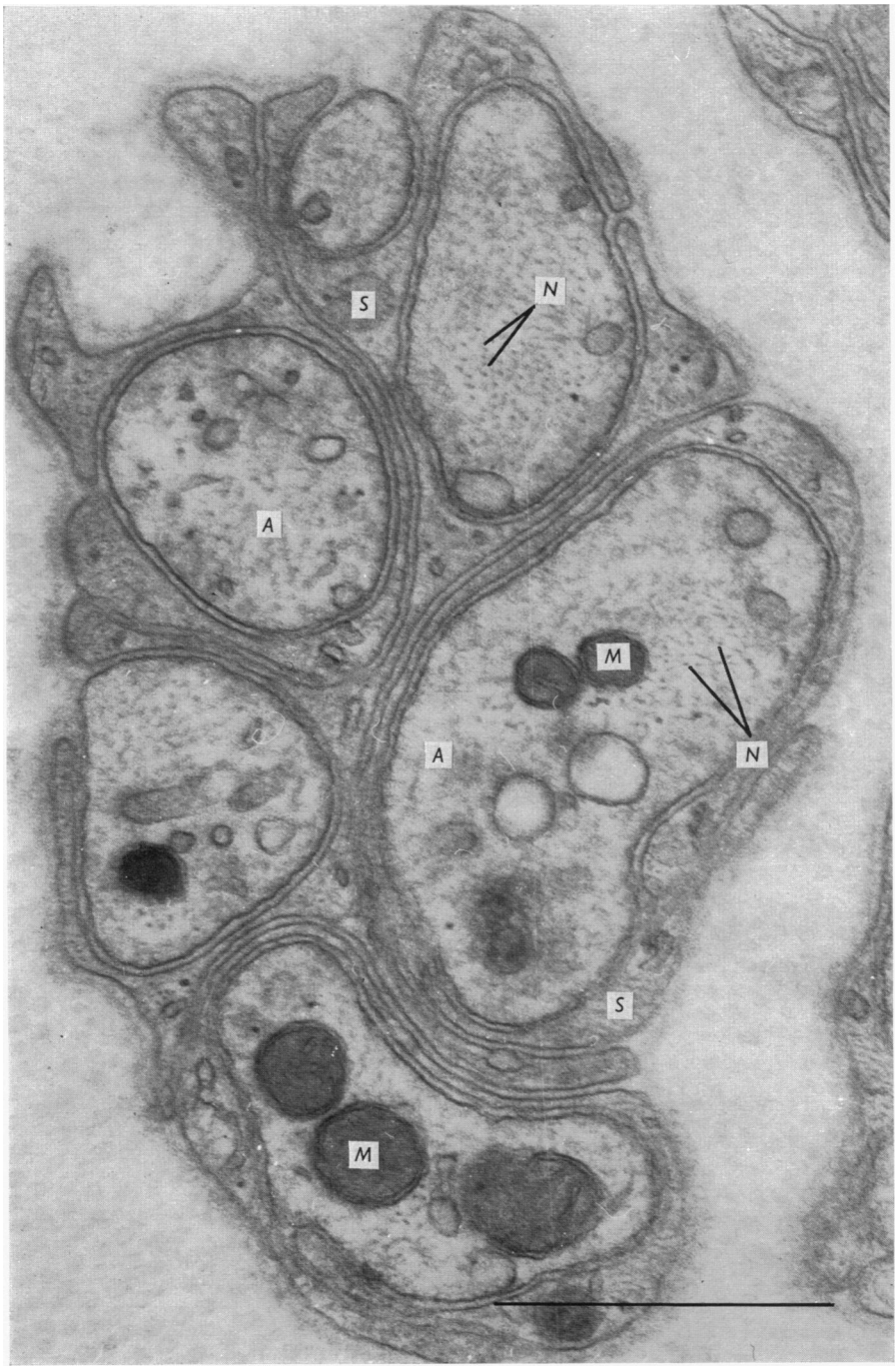
Electron micrograph of a transverse section of hypogastric nerve from midway between ganglion and ligature after incubation for 24 hr in Eagle's medium containing lumicolchicine (1 μg/ml.). The axons and Schwann cells are indistinguishable from those in control preparations (see Pl. 3, Banks & Mayor, 1972). Microtubules (*Mt*) and neurofilaments (*N*) within the axons are clearly shown. (Bar = 1 μm.)

PLATE 3

Electron micrograph of a transverse section of hypogastric nerve from midway between ganglion and ligature after incubation for 24 hr in Eagle's medium containing griseofulvin (100 μg/ml.). The axons (*A*) in this preparation have a much reduced population of microtubules (*Mt*) (compare Pl. 2). (Bar = 1 μm.)

PLATE 4

Electron micrograph of microtubules formed by incubating an extract of bovine splenic nerve in 4 M glycerol containing EGTA, Mg^{2+} and GTP. *A*, tubules cut in transverse section; tubule subunits can be seen in some profiles. Whilst many profiles conform to microtubule dimensions some are larger (*) or have bizarre forms (↑). (Bar = 1 μm.) *B*, tubules shown in longitudinal profile.



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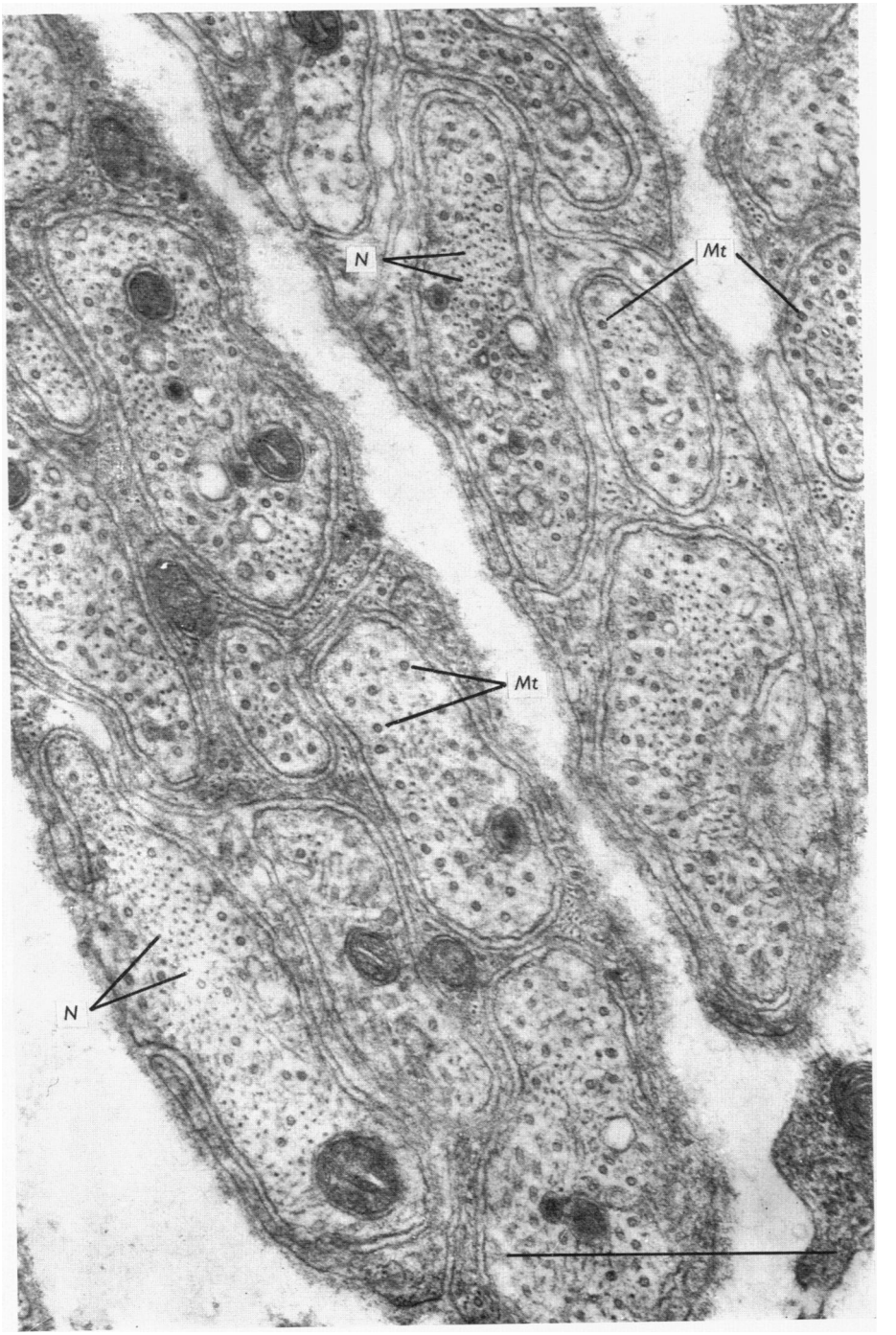
(*Facing p.* 294)



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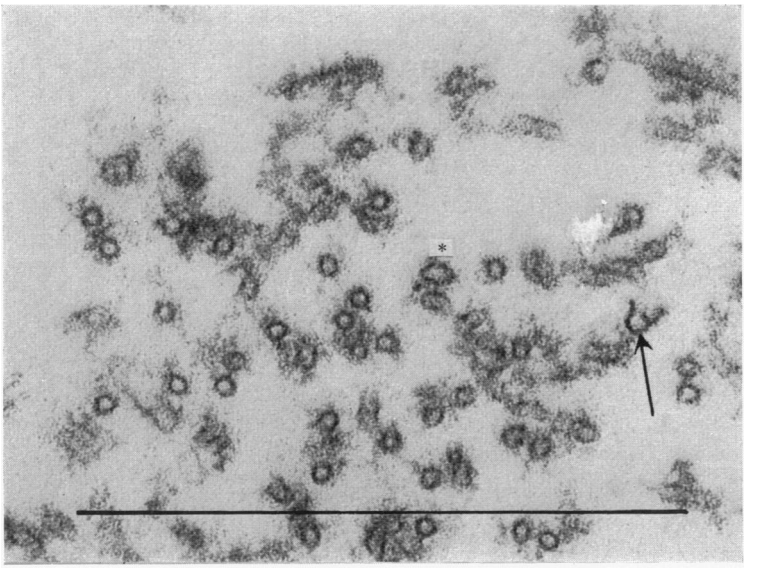
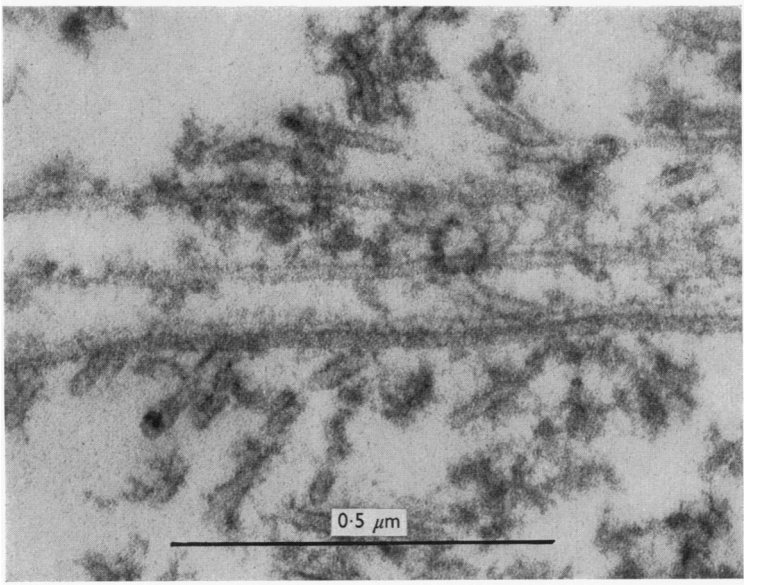
A
Mt
A
Mt



A



B