Br. J. Pharmac. (1969), **37**, 10–18.

The effects of iproniazid and reserpine on the accumulation of granular vesicles and noradrenaline in constricted adrenergic nerves

P. BANKS, K. KAPELLER* AND D. MAYOR

Departments of Biochemistry and Human Biology and Anatomy, University of Sheffield

1. The accumulation of granular vesicles proximal to a constriction applied to hypogastric nerves has been investigated with the electron microscope in cats treated with reserpine and/or iproniazid.

2. The ultrastructural changes have been correlated with the accumulation of noradrenaline at the same site in similarly treated animals.

3. The findings give further support to the view that granular vesicles constitute a major storage site for intraneuronal noradrenaline.

It is well known that the noradrenaline (NA) content of various tissues increases after inhibition of monoamine oxidase and decreases after treatment with reserpine (see Iversen, 1967). Furthermore, the accumulation of NA above a constriction applied to adrenergic nerves can be enhanced by monoamine oxidase inhibitors and decreased by the administration of reserpine (Dahlström, 1965, 1967; Dahlström & Waldeck, 1968; Kapeller & Mayor, 1967).

Electron microscopic studies have shown that the monoamine oxidase inhibitor iproniazid increases, and reserpine decreases, the number of granular vesicles in presumptive adrenergic nerve terminals (see Pellegrino De Iraldi & De Robertis, 1963, 1964; Clementi, 1965; Devine & Laverty, 1965; Devine & Simpson, 1966; Hökfelt, 1966; Tranzer & Thoenen, 1968).

After ligation of post-ganglionic adrenergic nerves, both granular vesicles and NA accumulate proximal to the constriction (Banks, Mangnall & Mayor, 1969; Kapeller & Mayor, 1967; Mayor & Kapeller, 1967). In the present experiments the effects of iproniazid and reserpine on the accumulation of granular vesicles have been studied electron microscopically and the findings correlated with the quantitative changes in the NA content of constricted adrenergic nerves.

Methods

Under nembutal anaesthesia (30 mg/kg followed by an additional 15 mg regardless of weight), the hypogastric nerves of adult cats of either sex (weight 1·5–4 kg) were constricted tightly at one point about 1 cm distal to the inferior mesenteric

* On leave of absence from Anatomicky Ustav, Komenski University, Bratislava, Czechoslovakia.

ganglion with a fine silk ligature which was left *in situ* (see Kapeller & Mayor, 1967, 1969a, for further operative details). The nerves were excised at intervals up to 48 hr after operation for electron microscopy and noradrenaline estimation.

Drug treatment

Iproniazid (Iproniazid phosphate ; " Marsilid ", Roche Products Ltd.) 50 mg/kg in sterile 0·9 w/v sodium chloride solution in water was given intraperitoneally immediately after operation and then at intervals of 10–16 hr. The last dose was given 16 hr before death.

Reserpine (" Serpasil ", Ciba). A single intraperitoneal injection of 5 mg/kg was given 16 hr before death irrespective of the survival time after operation.

Iproniazid and reserpine. Iproniazid was given as indicated above and reserpine 5 mg/kg was given with the last dose of iproniazid.

Electron microscopy

For electron microscopy the nerves were fixed by immersion in 1% osmium tetroxide in 0·1 M veronal acetate buffer at pH 7·4 for 1·5–2 hr. While in the fixative the nerves were divided into segments 0·7–1 mm long, as illustrated in Fig. 1. Each segment was processed separately and embedded in Araldite. Ultra-thin silver grey sections were stained on the grid with lead citrate (Renyolds, 1963) and examined in a Philips EM200 electron microscope.

Noradrenaline estimation

Noradrenaline present in corresponding segments (each was 0·8 mm long) from both hypogastric nerves from two cats (four pooled segments) was determined by the fluorimetric method of Häggendal (1963) as described by Banks, Mangnall & Mayor (1969).

Results

Electron microscopy

The ultrastructural features of normal non-myelinated axons in cat hypogastric nerves and the changes produced by constriction have been described in detail elsewhere (Kapeller & Mayor, 1967, 1969a, 1969b). The only detectable effects of the two drugs used was on the population of granular vesicles in the constricted axons, so the present account is confined to a consideration of these structures.

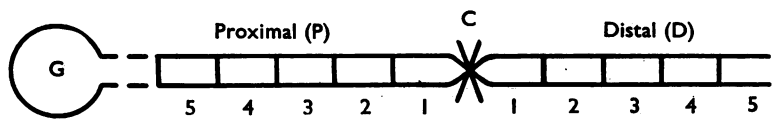

FIG. 1. Diagram to show the segments of the nerves proximal and distal to the constriction (C) used for electron microscopy and biochemical analysis. G represents the inferior mesenteric ganglion. The length of each segment was 0·8 mm for biochemical analysis and between 0·7 and 1·0 mm for electron microscopy.

After operation alone there was a rapid accumulation of granular vesicles on the proximal side of the constriction. Initially, this accumulation was located immediately adjacent to the site of constriction, but as time progressed it extended proximally along the nerve. However, at all times the greatest concentration of granular vesicles was found within the first 1 mm of nerve above the constriction. The number of granular vesicles in individual axonal profiles in regions of the nerve corresponding to segments P1 and P2 (see Fig. 1) was very variable. Some axons contained no or very few granular vesicles, while others contained large numbers.

Although typical granular vesicles were occasionally seen distal to the constriction, they did not accumulate in this part of the nerve at any time in the present experiments.

Effect of iproniazid. After treatment with iproniazid granular vesicles were more numerous than after operation alone and more axonal profiles contained these vesicles (Plate 1). Axons exhibiting densely packed granular vesicles were more common after treatment with iproniazid compared with operation alone.

Effect of reserpine. Only a few structures resembling granular vesicles remained after treatment with reserpine (Plate 2). The majority of the axonal profiles contained no granular vesicles. Agranular vesicles were, however, numerous.

Effect of iproniazid and reserpine. When the two drugs were given together granular vesicles accumulated above the constriction. They were more numerous than after reserpine alone but less common than after iproniazid. Taking into account the variation between adjacent axonal profiles, the appearances were frequently indistinguishable from those seen after operation alone.

Distal to the constriction the two drugs, either singly or in combination, had no detectable effect on the axons or their contents.

The majority of the granular vesicles accumulating above the constriction were between 600 and 900 Å in diameter ; most commonly they were slightly irregular in outline. The morphology of their electron dense core was variable. In some it almost completely filled the vesicle while in others it was in the form of a small osmiophilic dot. In the latter variety, the rest of the vesicle was filled with material of moderate electron density similar to that found in so-called " agranular " vesicles. There was no obvious relationship between the overall size of the granular vesicle and the morphology of its contents. After treatment with reserpine, the few remaining granular vesicles occupied the lower end of the size range and in general they were almost completely filled by an electron dense inclusion.

Noradrenaline content

Normal cat hypogastric nerves contain little detectable NA. In a previous investigation (Banks *et al.*, 1969) the NA content was found to be $0{\cdot}01 \pm 0{\cdot}004$ (S.D.) mμ-moles per single 0·8 mm segment of nerve.

PLATES 1 AND 2. Electron micrographs taken from the middle of segment P1 above the constriction. For comparison they are reproduced at the same final magnification. The tissue was fixed in osmium tetroxide and sections were stained with lead citrate. Plate 1: 24 hr after operation and 16 hr after iproniazid. The moderately swollen non-myelinated axons (A) contain large numbers of granular vesicles (g). These vesicles show considerable variation in their morphology. Plate 2: 24 hr after operation and 16 hr after reserpine. Granular vesicles (g) are very rare. The swollen non-myelinated axons (A) contain mainly agranular vesicles (a).

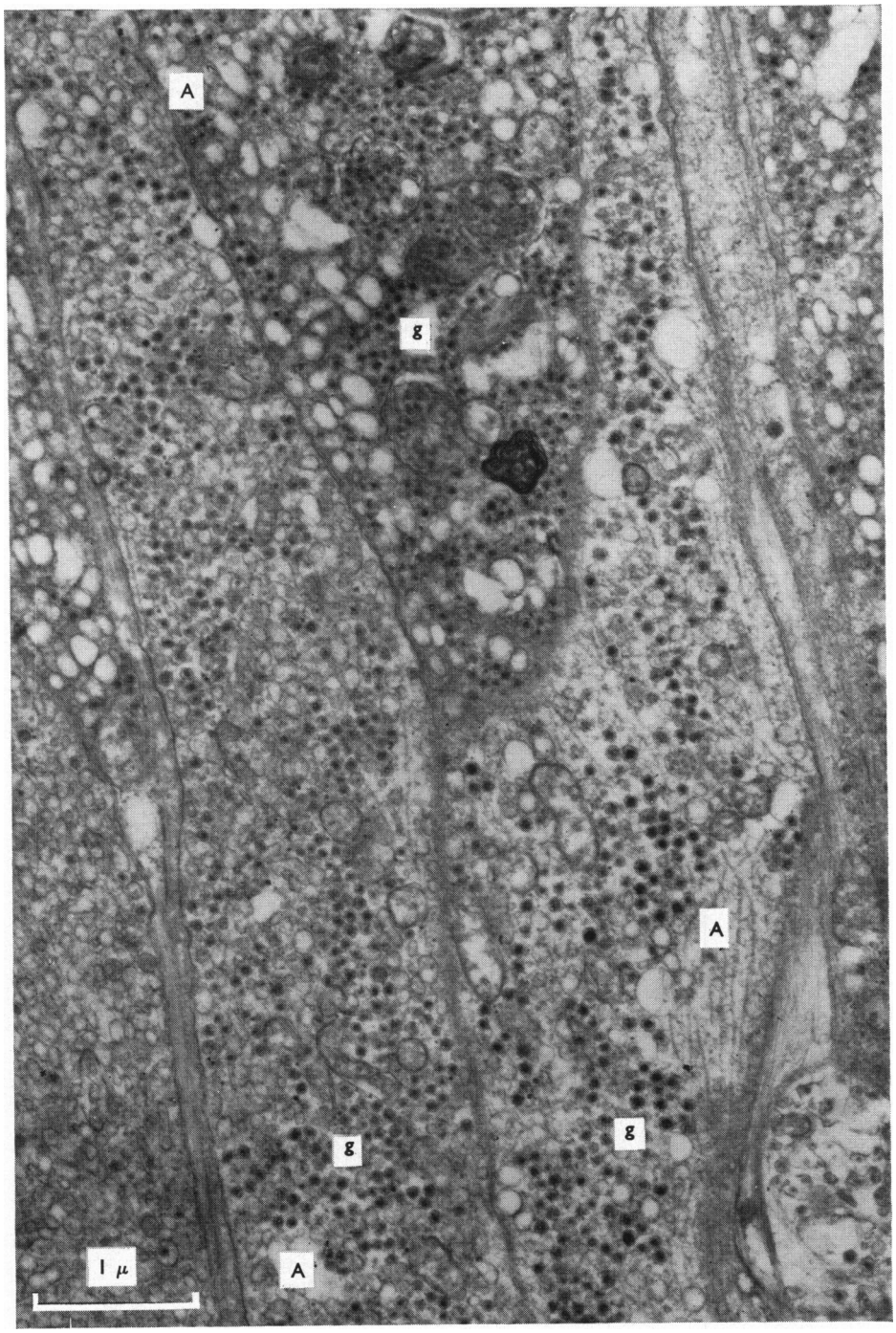

PLATE 1. For legend see facing page.

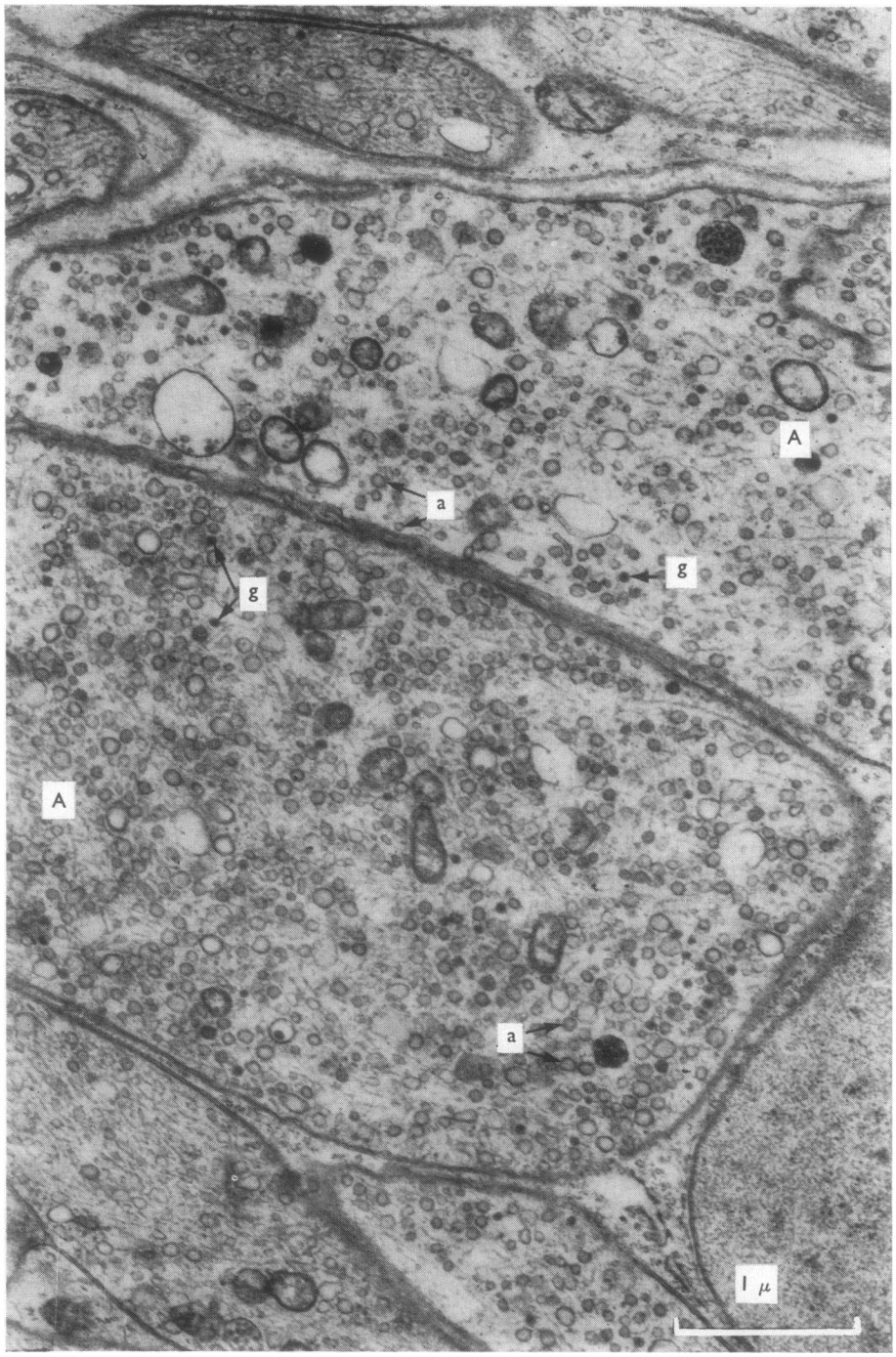

PLATE 2. For legend see page 12.

Effect of operation alone. After operation alone, the amount of NA proximal to the constriction increased steadily. At first it was located mainly in segment P1 (see Fig. 1) but at longer times after operation the more proximally placed segments contained more NA than normal. However, at all stages the greatest concentration of NA was in segments P1 and P2 (Fig. 2) corresponding to the site of accumulation of the granular vesicles.

Segment D1 below the constriction occasionally contained up to 2–3 times the normal complement of NA by 48 hr after operation. Most commonly, however, no NA was detectable distal to the constriction.

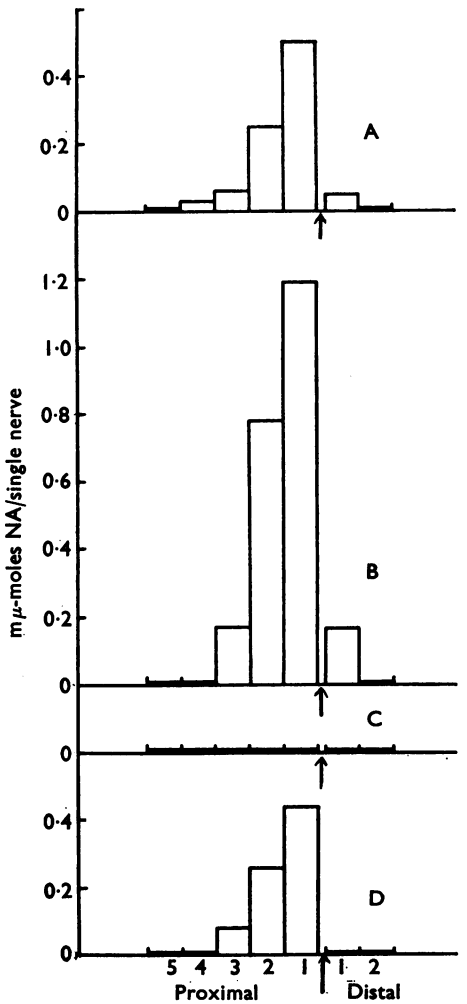

FIG. 2. Histograms showing the distribution of noradrenaline in constricted hypogastric nerves of cat 48 hr after operation. The site of constriction is indicated by the vertical arrows, the segmentation is as indicated in Fig. 1. The NA content of a single 0·8 mm segment of normal nerve was $0{\cdot}01 \pm 0{\cdot}004$ (S.D.) mμ-moles. Each histogram represents the mean values obtained from two experiments involving a total of four cats (eight hypogastric nerves). A, Operation only; B, operation plus iproniazid; C, operation plus reserpine; D, operation plus iproniazid and reserpine.

Effect of iproniazid. Although the basic pattern was similar to that seen after operation alone, considerably more NA accumulated above the constriction after treatment with this monoamine oxidase inhibitor. By 48 hr after operation (Fig. 2) the total NA content of segments P1 + P2 + P3 was three times greater than after operation alone.

Effect of reserpine. From a previous study (Banks *et al.*, 1969) it was known that there was a linear increase in the amount of NA accumulating in the first three segments proximal to the constriction during the initial 4 days after operation. The reserpine was therefore given at a time when a certain amount of NA had already accumulated above the constriction. In the case of the experiment illustrated in Fig. 2, this drug was given 32 hr after operation when about 0·66 mμ-moles of NA should have accumulated in each nerve (see Banks *et al.*, 1969). Sixteen hours after reserpine treatment (48 hr after operation) no NA could be detected in any segment of the nerve.

Effect of iproniazid plus reserpine. Forty-eight hours after operation the pattern of the accumulation of NA was similar to that seen after operation alone. There was less NA in segments P1 to P3 compared with operation plus iproniazid but very much more than after treatment with reserpine.

Discussion

These experiments have further demonstrated the close relationship between the accumulation of granular vesicles, seen with the electron microscope, and NA determined fluorimetrically in constricted adrenergic nerves (Banks *et al.*, 1969). They also support the view that the granular vesicles constitute the principal intra-axonal storage site for bound NA.

After treating the animals with the monoamine oxidase inhibitor iproniazid, the amount of NA accumulating proximal to the ligature during the first 48 hr was three times greater than after operation alone. This indicates that some, perhaps a substantial percentage, of the NA synthesized in these neurones is usually (in the absence of iproniazid) oxidized by the monoamine oxidase and thus prevented from contributing to the net store of NA above the constriction. It would appear, therefore, that after inhibition of monoamine oxidase the potential of these neurones to synthesize and accumulate NA can be observed more completely than after operation alone.

The significance of the apparent wastage of NA via the monoamine oxidase pathway is not clear. Furthermore, if the extra NA accumulating after iproniazid is stored in granular vesicles, as is suggested by their numerical increase after treatment with the drug, the further question arises as to why the full storage capacity for NA is not utilized in the absence of iproniazid. It is possible that the monoamine oxidase activity keeps the concentration of unbound, extra-granular, NA in the system below the level required effectively to inhibit tyrosine hydroxylase (see Iversen, 1967) and in consequence permits the continued synthesis of NA. It is conceivable, therefore, that some advantage may accrue to adrenergic neurones by ensuring that the synthetic pathway for NA remains continually active and able to respond rapidly to changes in the demands for the transmitter.

Reserpine, in the doses used in these experiments, depleted the nerve of the NA which had already accumulated at the time of its administration and prevented the

further accumulation of the transmitter. This was associated with an almost complete disappearance of the granular vesicles from the region above the constriction. Agranular vesicles were numerous in reserpine-treated neurones, but it has not been possible to decide whether these are more numerous than in control neurones, consequently it is not clear whether some of the agranular vesicles present after treatment with reserpine are the " ghosts " of granules that have been depleted of their osmiophilic cores. The depleting action of reserpine was reduced by the simultaneous administration of iproniazid, a finding supporting other investigations (see Pellegrino De Iraldi & De Robertis, 1963 ; Clementi, 1965 ; Dahlström, 1967). The present findings are consistent with the view that reserpine acts by diverting the amines from the storage sites in the granular vesicles to the monoamine oxidase carried by the mitochondria (see Iversen, 1967). However, they shed no further light on the mechanism by which reserpine causes such a diversion. The functional significance of the granular vesicles which remain after treatment with reserpine is uncertain. They may be resistant to the drug, or have been recharged with NA following depletion ; alternatively they may not be related to NA storage but have some other functional significance. Thus, although it is clear that NA is associated with granular vesicles which exhibit considerable variation in their morphology, it is by no means certain that all the granular vesicles accumulating in constricted non-myelinated axons are carriers of noradrenaline (Pellegrino De Iraldi & De Robertis, 1968 ; Mayor, 1968).

We thank Professors W. Bartley and R. Barer for their help in preparing this paper and Mr. B. Christian and Mr. R. Hardy and his staff for technical assistance. We acknowledge facilities made available through grants from M.R.C. and S.R.C. We are also grateful to the Wellcome Trust and to the Smith, Kline & French Foundation for grants in aid of this work. The drugs were generously supplied by Roche Products Ltd. and Ciba. K. K. acknowledges a grant from the British Council.

REFERENCES

BANKS, P., MANGNALL, D. & MAYOR, D. (1969). The redistribution of cytochrome-oxidase, noradrenaline and adenosine triphosphate in adrenergic nerves constricted at two points. *J. Physiol., Lond.*, **200**, 745–762.

CLEMENTI, F. (1965). Modifications ultrastructurelles provoquées par quelques medicaments sur les terminaisons nerveuses adrenergiques et sur la medullaire surrenale. *Experientia*, **21**, 171–176.

DAHLSTRÖM, A. (1965). Observations on the accumulation of noradrenaline in the proximal and distal parts of peripheral adrenergic nerves after compression. *J. Anat.*, **99**, 677–689.

DAHLSTRÖM, A. (1967). The effect of reserpine and tetrabenzine on the accumulation of noradrenaline in the rat sciatic nerve after ligation. *Acta physiol. scand.*, **69**, 167–179.

DAHLSTRÖM, A. & WALDECK, B. (1968). The uptake and retention of ^{3}H noradrenaline in rat sciatic nerves after ligation. *J. Pharm. Pharmac.*, **20**, 673–677.

DEVINE, C. E. & LAVERTY, R. (1965). The effects of reserpine on the noradrenaline and dopamine contents of the rat vas deferens and on the fine structure of its nerve fibres. *Proc. Univ. of Otago med. Sch.*, **43**, 17–20.

DEVINE, C. E. & SIMPSON, F. O. (1966). The effect of noradrenaline-depleting drugs on the granular vesicles of the axons around rat intestinal blood vessels. *Proc. Univ. Otago med. Sch.*, **44**, 54–56.

HÄGGENDAL, J. (1963). An improved method for fluorimetric determination of small amounts of adrenaline and noradrenaline in plasma and tissues. *Acta physiol. scand.*, **59**, 242–254.

HÖKFELT, T. (1966). The effect of reserpine on the intraneuronal vesicles of the rat vas deferens. *Experientia*, **22**, 56.

IVERSEN, L. L. (1967). In *The Uptake and Storage of Noradrenaline in Sympathetic Nerves*. Cambridge: Cambridge University Press.

KAPELLER, K. & MAYOR, D. (1967). The accumulation of noradrenaline in constricted sympathetic nerves as studied by fluorescence and electron microscopy. *Proc. R. Soc. B*, **167**, 282–292.

KAPELLER, K. & MAYOR, D. (1969a). An electron microscopic study of the early changes proximal to a constriction in sympathetic nerves. *Proc. R. Soc. B*, **172**, 39–51.

KAPELLER, K. & MAYOR, D. (1969b). An electron microscopic study of the early changes distal to a constriction in sympathetic nerves. *Proc. R. Soc. B*, **172**, 53–63.

MAYOR, D. (1968). Granular vesicles and the electron microscopic localization of noradrenaline. *Proc. R. microscop. Soc.*, **3**, 94–95.

MAYOR, D. & KAPELLER, K. (1967). Fluorescence microscopy and electron microscopy of adrenergic nerves after construction at two points. *J. R. microscop. Soc.*, **87**, 277–294.

PELLEGRINO DE IRALDI, A. & DE ROBERTIS, E. (1963). Action of resperpine, iproniazid and pyrogallol on nerve endings of the pineal gland. *Int. J. Neuropharmac.*, **2**, 231–239.

PELLEGRINO DE IRALDI, A. & DE ROBERTIS, E. (1964). Ultrastructure and function of catecholamine containing systems. In *Proc. 2nd Int. Congr. Endocr. London*, Excepta Medica Internat. Congr. Series No. 83, 355–363.

PELLEGRINO DE IRALDI, A. & DE ROBERTIS, E. (1968). The neurotubular system of the axon and the origin of granulated and non-granulated vesicles in regenerating nerves. *Z. Zellforsch. mikrosk. Anat.*, **87**, 330–344.

RENYOLDS, E. S. (1963). The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. *J. cell Biol.*, **17**, 208–212.

TRANZER, J. P. & THOENEN, H. (1968). Various types of amine-storage vesicles in peripheral adrenergic nerve terminals. *Experientia*, **24**, 484–486.

(*Received January* 17, 1969)