Br. J. Pharmac. (1971), **43**, 295–301.

A new method of destroying adrenergic nerves in adult animals using guanethidine

G. BURNSTOCK, BARBARA EVANS, B. J. GANNON,
J. W. HEATH AND VICKI JAMES

Department of Zoology, University of Melbourne, Parkville, 3052, Victoria, Australia

Summary

1. The structure of sympathetic neurones in the rat has been examined with histological, fluorescence histochemical and electron microscopical methods after chronic treatment for 6 weeks with guanethidine (25 or 30 mg/kg/day i.p.).

2. Less than 2% of the nerve cell bodies in the superior cervical ganglion remained at this time and in these cells the mitochondria were badly damaged. Few fluorescent adrenergic nerve fibres were found outside the central nervous system. This situation persisted even 4 months (the longest period studied) after cessation of treatment.

3. This procedure is proposed as a new method of producing sympathectomy. It has the advantage of being applicable to adult animals in a variety of experimental and pathological situations. It is uniquely advantageous for denervation of the male reproductive tract.

Introduction

Selective destruction of adrenergic nerves is a valuable tool in the analysis of the components of autonomic innervation of both visceral and cardiovascular systems and for studies of supersensitivity, quite apart from its value in clinical situations.

Several methods of producing degeneration of adrenergic nerves are currently available, each with advantages and disadvantages. Surgical methods are particularly useful for selective sympathectomy of specific organs (Cooper, 1966) but these are technically difficult in some cases, especially with organs where intramural adrenergic ganglion cells are present. A further disadvantage for some experiments is that the period of denervation is brief, since reinnervation by sympathetic nerves from neighbouring organs or vessels occurs soon after denervation is complete (Langley & Anderson, 1904).

Another method of producing sympathetic denervation in adult tissue, introduced in 1960, is immunosympathectomy, which involves injection of new-born animals with the antiserum of nerve growth factor (N.G.F.) (see Levi-Montalcini & Angeletti, 1966). This ingenious method has several disadvantages: it is not easy to obtain anti-N.G.F. of reliable and consistent quality; sympathectomy is not complete, since the degree of interference with the development of adrenergic nerves depends on the time of injection in young or prenatal animals relative to the normal sequential laying down of nerves in different organ systems (Iversen, Glowinski &

Axelrod, 1965); afferent as well as efferent nerves are affected; and it is not possible to produce sympathectomy by injecting adult animals.

A third method, introduced by Thoenen & Tranzer in 1968, involves treatment of animals with 6-hydroxydopamine. Degeneration of adrenergic nerves with this method is limited to the terminal varicose regions of the nerve and does not seriously damage the cell bodies, so that after several weeks new nerve processes grow out to reinnervate the system (Thoenen & Tranzer, 1968; Goldman & Jacobowitz, 1971). The drug affects various systems differentially; the heart and large vessels are affected first (or with the lowest doses), then in order, the more peripheral vessels, visceral organs, and lastly the genital system, which, even with sublethal doses, is never fully sympathectomized. More recently it has been shown that, if 6-hydroxydopamine is injected into new-born rodents, the sympathetic ganglia as well the terminal varicosities are destroyed (Angeletti & Levi-Montalcini, 1970; Jaim-Etcheverry & Zieher, 1971). However, even with this procedure, these authors showed that adrenergic denervation of the genitalia is not achieved.

A new and simple method of producing sympathectomy with guanethidine in adult animals is described in this work.

Methods

Twenty-five male and twenty-five female rats (Sprague-Dawley), weighing 150–250 g, were injected daily (i.p.) with guanethidine sulphate (Ismelin-CIBA). Doses of 25 or 30 mg/kg guanethidine were administered for periods of up to 6 weeks.

The fluorescence histochemical method for localizing monoamines was used in accordance with the Falck-Hillarp technique (see Falck & Owman, 1965). The tissue was frozen in liquid propane cooled with liquid nitrogen and then freeze-dried at $-38°$ C and 10^{-3} mmHg (1 mmHg$\equiv$1·333 mbar), using P_2O_5 as moisture trap, for 24 hours. The tissue was then allowed to return to room temperature over 7 h and heated to 35° C before incubation in a sealed vessel at 80° C for 1 h with paraformaldehyde (70% relative humidity). After vacuum embedding in paraffin wax, sections (10 μm) were mounted on heated glass slides with paraffin oil. Stretch preparations of irides were prepared according to Falck (1962). The following wide range of sympathetic ganglia and adrenergically innervated tissues was examined; superior cervical ganglion, inferior mesenteric ganglion, hypogastric ganglion, anterior cerebral artery, iris, submaxillary gland, atrial appendages, ventricles, anterior mesenteric artery branches, ileal wall, uterus, vas deferens, seminal vesicle, coagulating gland, epididymis, prostate gland, tail artery, hypothalamus. Tissues were examined in a Leitz Ortholux fluorescence microscope equipped with a Leitz Orthomat microscope camera. Exciting light from an Osram HBO 200 high pressure mercury lamp was passed through a 5 mm BG12 excitation filter to a lightfield substage condenser. Zeiss 10× or 16× fluorite objectives and a 530 mm barrier filter were fitted to the optical train.

The superior cervical and hypogastric ganglia were selected for detailed study by electron microscopy. The tissue, cut into 1 mm cubes, was fixed for 1 h in 1% osmium tetroxide buffered at pH 7·4 with 0·2 M phosphate buffer (following an initial 10 min fixation of the whole ganglion), postfixed in phosphate buffered 3% glutaraldehyde for 1 h, followed by 45 min in phosphate buffered 1% osmium tetroxide. The fixed material was then block stained in a 2% aqueous solution of

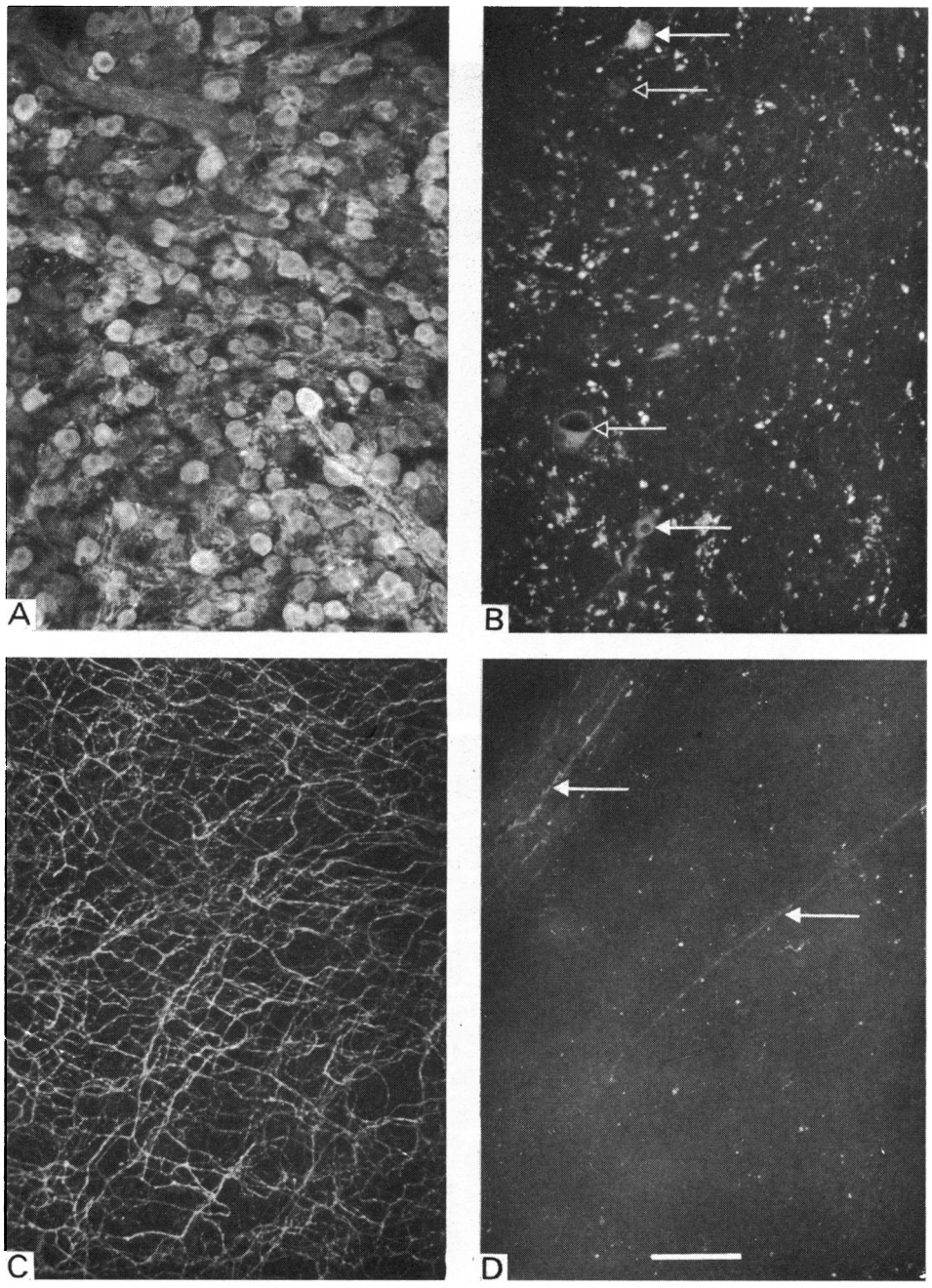

FIG. 1. Fluorescence histochemical demonstration of the effects of chronic guanethidine treatment ((25 mg/kg)/day, i.p.) on rat superior cervical ganglia and irides. A. Superior cervical ganglion from untreated animal. B. Superior cervical ganglion of rat after treatment for 6 weeks. Note the extreme reduction in numbers of ganglion cells. There are two cells showing large vacuoles (open arrows) and two cells in which vacuoles are not seen (solid arrows). The background particulate fluorescence represents orange autofluorescent material. C. Iris from untreated animal. D. Iris from animal killed 2 weeks after cessation of treatment for 6 weeks. Note that a few fibres (arrows) are seen in some areas of these preparations. Calibration bar for A-D = 100 μm.

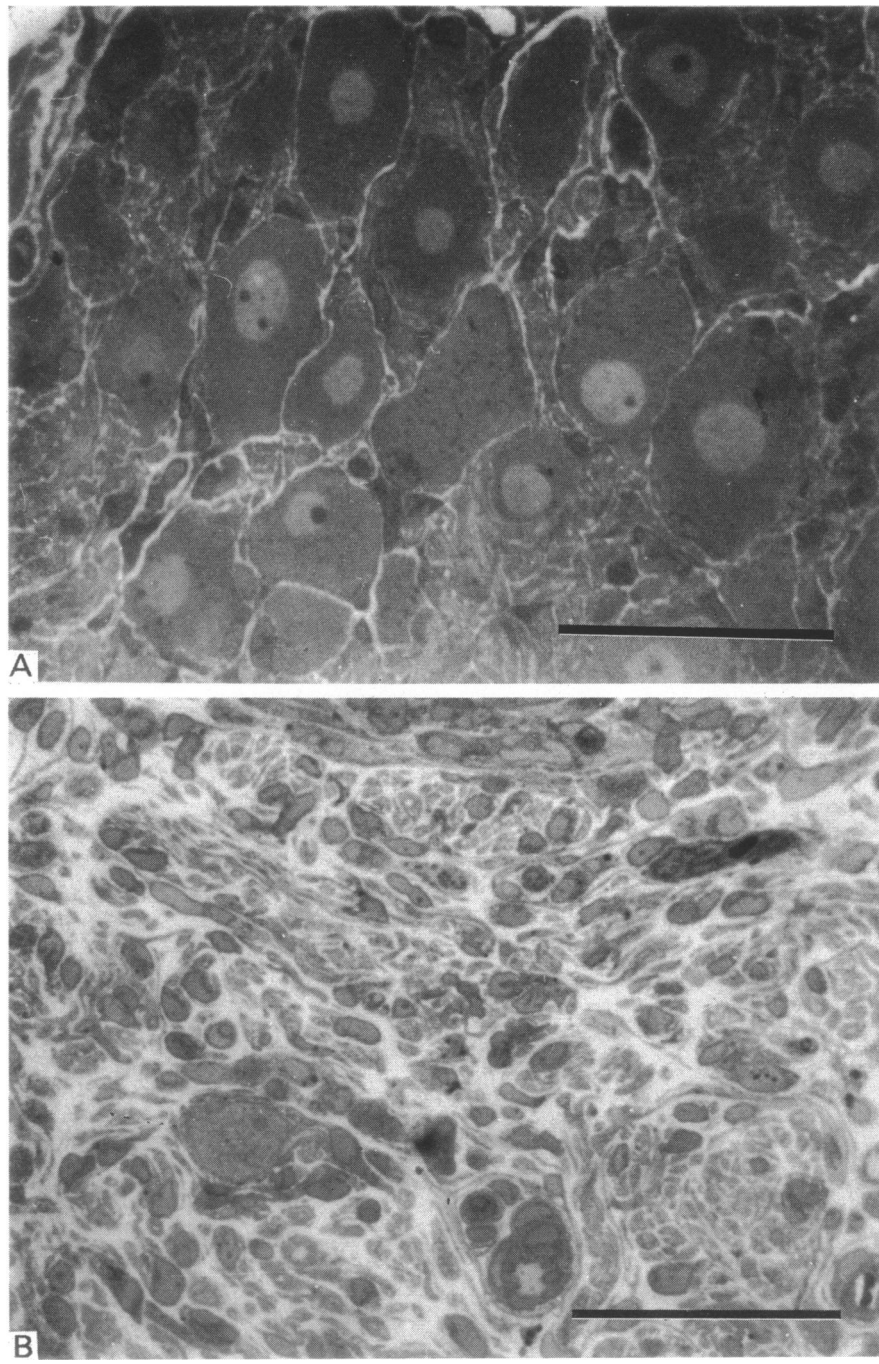

FIG. 2. Toluidine blue staining of Araldite sections of rat superior cervical ganglion. A. Control section. B. Section from ganglion of rat treated with guanethidine ((25 mg/kg)/day, i.p. for 6 weeks). Note that the single ganglion cell that remains shows signs of vacuolation. Calibration bars = 50 μm.

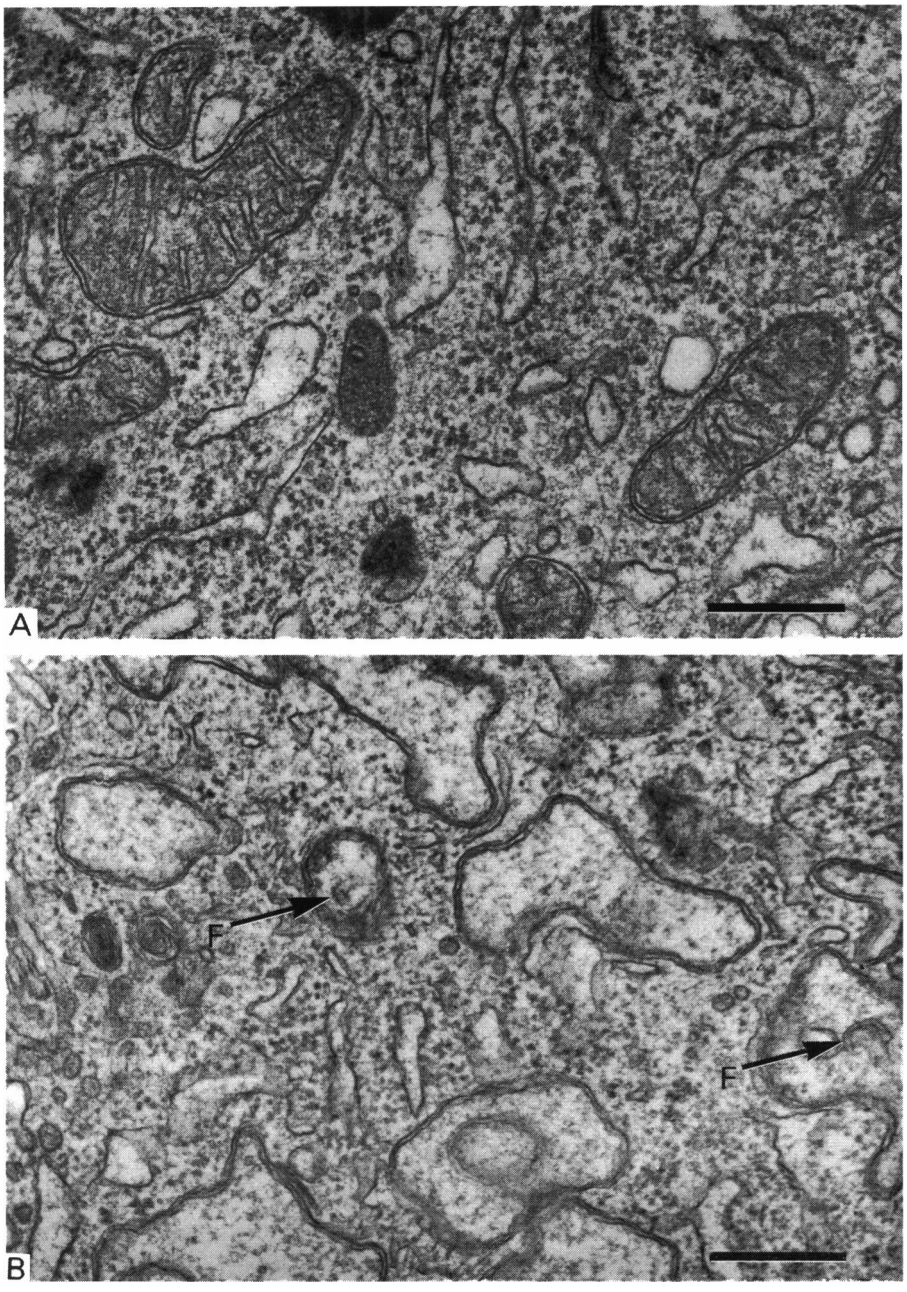

FIG. 3. Electron micrographs of ganglion cell bodies from rat superior cervical ganglion. A. Control. Note that the cristae of the mitochondria are prominent and that a dense matrix occupies the remainder of the organelle. B. Typical region of a ganglion cell body from an animal treated with guanethidine ((30 mg kg)/day, i.p. for 2 weeks). Note that the mitochondria are swollen and their outlines irregular. Only fragments of cristae (F) remain and the matrix is dispersed. Calibration bars = 0·5 μm.

uranyl acetate for 30 minutes. The tissue was briefly washed in distilled water and rapidly dehydrated in acetone. It was infiltrated in a 50/50 solution of acetone and Araldite, and finally embedded in Araldite. Thin sections were cut with a Huxley-Cambridge ultramicrotome. The sections were stained with saturated aqueous uranyl acetate followed by lead citrate (Reynolds, 1963) and subsequently examined with a Hitachi Hu 11B electron microscope.

Thick sections (about 0·5 μm) of Araldite embedded material were stained with Toluidine blue (0·5 g Toluidine blue, 0·5 g borax, distilled water to 100 ml) and examined by light microscopy.

Results

Fluorescence histochemistry

Less than 2% of the ganglion cells remained in the superior cervical ganglion after treatment for 6 weeks with guanethidine (25 mg/kg) (Fig. 1B cf. 1A) and some of these cells showed abnormal vacuolation (Fig. 1B). Few fluorescent nerve fibres were visible outside of the CNS at this time in the wide range of tissues examined (see **Methods**). This effect persisted even 4 months after the cessation of treatment (the longest period studied) apart from occasional adrenergic fibres which reappeared in the iris (Fig. 1D, cf. 1C) and superior cervical ganglion. SIF cells (see Van Orden, Shaffer, Burke & Lodoen, 1970) in the ganglia and paraganglia were apparently unaffected by the treatment.

Histology

Toluidine blue staining of Araldite sections also revealed that less than 2% of the neurone cell bodies in the superior cervical ganglion remained after 6 weeks treatment (Fig. 2B cf. 2A).

Electron microscopy

Analysis of neurones in the superior cervical ganglia after treatment for 6 weeks showed that most of the cell bodies had completely degenerated and those few that remained were severely damaged. Mitochondria appeared to be the main site of neurone damage (see also Jensen-Holm & Juul, 1970). Figure 3B shows the typical appearance of damaged mitochondria which are characterized by swelling, loss or severe damage of cristae and an irregular outline compared to control mitochondria (Fig. 3A). Every ganglion cell examined in the superior cervical ganglion after treatment for 2 weeks showed some degree of mitochondrial damage. In contrast, examination of the hypogastric ganglion after similar treatment showed that some ganglion cells were apparently unaffected although many cells contained damaged mitochondria.

Discussion

Chronic treatment with high doses of guanethidine described in this work results in widespread sympathectomy, which persists over long periods. This method has the advantage of being applicable to adult animals in a variety of experimental situations and pathological conditions. It is particularly valuable for studies of the reproductive organs, where it is not possible to obtain adrenergic denervation either by immuno-sympathectomy or by treatment with 6-hydroxydopamine. Even with chronic treatment of animals with low doses (5 mg/kg) of guanethidine over

long periods (18 weeks), long lasting damage to adrenergic nerves in the male reproductive tract is produced, although adrenergic nerves in other parts of the body are not seriously affected (Gannon, Iwayama, Burnstock, Gerkens & Mashford, 1971). While the changes have been demonstrated in rats, it is not yet known whether they occur to the same extent in other species.

Some ganglion cells in the superior cervical ganglion remain after treatment for 6 weeks with high doses of guanethidine. The possibility that these neurones are cholinergic seems unlikely since similar numbers of cells are seen to remain with both fluorescence histochemical and histological methods; thus, the remaining ganglion cells are apparently adrenergic. Furthermore, the few cells that do remain appear severely damaged (see Fig. 1B). Cholinergic ganglion cells have not been clearly demonstrated in the rat superior cervical ganglion by other workers (see Eränkö, 1967). In contrast, the ultrastructure of some cells in the hypogastric ganglion, which contains cholinergic as well as adrenergic nerve cell bodies, remains unchanged after treatment with guanethidine.

The sequential changes in mitochondrial damage and eventual total cell degeneration and phagocytotic elimination produced by guanethidine will be described in detail in a further communication.

This work was supported by grants from the Australian Research Grants Committee, the National Heart Foundation of Australia and the National Health and Medical Research Council.

REFERENCES

ANGELETTI, P. U. & LEVI-MONTALCINI, R. (1970). Sympathetic nerve cell destruction in newborn mammals by 6-hydroxydopamine. *Proc. natn Acad. Sci., U.S.A.*, **65**, 114–121.

COOPER, T. (1966). Surgical sympathectomy and adrenergic function. *Pharmac. Rev.*, **18**, 611–618.

ERÄNKÖ, O. (1967). Histochemistry of nervous tissues: catecholamines and cholinesterases. *Ann. Rev. Pharmac.*, **7**, 203–222.

FALCK, B. (1962). Observations on the possibilities of the cellular localization of monoamines by a fluorescence method. *Acta physiol. scand.*, **56**, suppl. 197, 1–25.

FALCK, B. & OWMAN, C. H. (1965). A detailed methodological description of the fluorescence method for the cellular demonstration of biogenic monoamines. *Acta Univ. Lund.*, Sect. II, No. 7, 1–24.

GANNON, B. J., IWAYAMA, T., BURNSTOCK, G., GERKENS, J. & MASHFORD, M. L. (1971). Prolonged effects of chronic guanethidine treatment on the sympathetic innervation of the genitalia of male rats. *Med. J. Aust.*, in the Press.

GOLDMAN, H. & JACOBOWITZ, D. (1971). Correlation of norepinephrine content with observations of adrenergic nerves after a single dose of 6-hydroxydopamine in the rat. *J. Pharmac. exp. Ther.*, **176**, 119–133.

IVERSEN, L. L., GLOWINSKI, J. & AXELROD, J. (1965). Reduced uptake of tritiated noradrenaline in tissues of immunosympathectomized animals. *Nature, Lond.*, **206**, 1222–1223.

JAIM-ETCHEVERRY, G. & ZIEHER, L. M. (1971). Permanent depletion of peripheral norepinephrine in rats treated at birth with 6-hydroxydopamine. *Eur. J. Pharmac.*, **13**, 272–276.

JENSEN-HOLM, J. & JUUL, P. (1970). Ultrastructure of rat sympathetic ganglia following guanethedine. *Acta pharmac. tox.*, **28**, suppl. 1, 56.

LANGLEY, J. N. & ANDERSON, H. K. (1904). On autogenetic regeneration in the nerves of the limbs. *J. Physiol., Lond.*, **31**, 418–428.

LEVI-MONTALCINI, R. & ANGELETTI, P. U. (1966). Immunosympathectomy. *Pharmac. Rev.*, **18**, 619–628.

REYNOLDS, 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.

THOENEN, H. & TRANZER, J. P. (1968). Chemical sympathectomy by selective destruction of adrenergic nerve endings with 6-hydroxydopamine. *Naunyn Schmiedebergs Arch. exp. Pharmak. Path.*, **261**, 271–288.

VAN ORDEN, L. S., SHAFFER, J. M., BURKE, J. P. & LODOEN, F. U. (1970). Differentiation of norepinephrine storage compartments in peripheral adrenergic nerves. *J. Pharmac. exp. Ther.*, **174**, 357–368.

(*Received June* 10, 1971)