Neuroscience, 1976. Vol. 1, pp. 413–421. Pergamon Press. Printed in Great Britain.

THE RECOVERY OF ACETYLCHOLINESTERASE ACTIVITY IN THE SUPERIOR CERVICAL GANGLION OF THE RAT FOLLOWING ITS INHIBITION BY DIISOPROPYLPHOSPHOROFLUORIDATE: A BIOCHEMICAL AND CYTOCHEMICAL STUDY

P. SOMOGYI[1] and I. W. CHUBB
University Department of Pharmacology, South Parks Road, Oxford OX1 3QT

Abstract—Injection of diisopropylphosphorofluoridate into rats results in a marked inhibition of the acetylcholinesterase activity of the superior cervical ganglia and associated nerve trunks. We have followed the recovery of the activity of the enzyme in these two tissues. We have studied the recovery biochemically, analysing also the contribution of the various molecular forms of acetylcholinesterase, and cytochemically.

Shortly after injection of the poison there is a rapid recovery of the acetylcholinesterase activity in the ganglia. This phase continues until about 16 h post-injection, and then it stops. The activity remains at this level for a further 80 h then there is another, but slower, accumulation of enzyme activity. Ganglia which had been decentralized prior to administration of diisopropylphosphorofluoridate show an identical pattern of recovery although the absolute amount of enzyme at each stage is lower than in normal tissues. In contrast, the acetylcholinesterase activity of the preganglionic nerve trunk does not begin to recover from the poison until at least 48 h after its injection. It reaches normal levels by 200 h. Analysis of the multiple molecular forms of acetylcholinesterase show: a) that there are four major soluble forms and, chiefly, one membrane-bound form of the enzyme in the ganglion, b) there is one soluble and one membrane-bound form in the nerve trunk, c) that there is no preferential loss of any of the observed forms in response to decentralization of the ganglia and, d) that there is no preferential recovery of any form after diisopropylphosphorofluoridate poisoning. The cytochemistry reveals that the initial recovery phase is due to synthesis of the enzyme by the ganglion cells since under our incubation conditions these are the only structures which contain the enzyme for the first 48 h after injection. It is noteworthy that acetylcholinesterase activity could not be demonstrated in the synaptic region until at least 48 h after injection of the poison at which time it could also be detected in presynaptic axons; this indicates that the synaptic enzyme is derived from the preganglionic nerve.

The results are discussed in terms of what proportion of normal ganglionic levels of acetylcholinesterase activity is derived from the presynaptic cholinergic nerves and how much from the postganglionic, but adrenergic, cells. It is suggested that appreciably less of the acetylcholinesterase is derived from the nerve trunk than is lost upon decentralization of the ganglia; it appears that the nerve might regulate the amount of enzyme that the postganglionic cells can synthesise and store.

IN AN EARLIER study (CHUBB & SMITH, 1975b), the possibility that the acetylcholinesterase (AChE) of the bovine splanchnic nerve might be secreted from the nerve terminals within the adrenal medulla was examined by administering membrane-depolarizing agents to a perfused ox adrenal gland: it was found that an isoenzyme of AChE was secreted into these perfusates. The interpretation of these experiments was complicated, however, by the observation that the bovine chromaffin cells, which can be considered as post-ganglionic cells (COUPLAND, 1965), also contain appreciable AChE activity (SOMOGYI, CHUBB & SMITH, 1975). Thus, both the major secreting elements of the adrenal medulla, the preganglionic splanchnic nerve terminals and the post-ganglionic chromaffin cells, contain AChE; from which is the enzyme secreted?

To obtain more information on any relation which might exist between the AChE in preganglionic nerve fibres with that in the postsynaptic cells, we have studied the effects of diisopropylphosphorofluoridate (DFP) poisoning on the AChE activity of the superior cervical ganglion of the rat. This particular tissue was chosen because, as in the bovine adrenal medulla, preganglionic nerve fibres innervate postganglionic cells many of which are rich in AChE (ERÄNKÖ & HÄRKÖNEN, 1964; KÁSA & CSERNOVSKY, 1967). Furthermore, the greatest proportion of the postganglionic cells appear to be adrenergic (KLINGMAN, 1970; BURNSTOCK, EVANS, GANNON, HEATH & JAMES, 1971);

[1] Present address: 1st Department of Anatomy, Semmelweis University Medical School, Tüzoltó ut. 58, 1450 Budapest IX, Hungary.

Abbreviations: AChE, acetylcholinesterase; DFP, diisopropylphosphorofluoridate.

and the simple nerve supply to the ganglion makes it amenable to decentralization. The recovery of the enzyme activity after administration of DFP has been followed using both biochemical and cytochemical techniques.

EXPERIMENTAL PROCEDURES

Male rats of the Wistar strain weighing 200–250 g were injected (s.c.) with 1.9 mg/kg of DFP and 6 mg/kg atropine sulphate dissolved in 1% (v/v) ethyl alcohol in 0.9% saline. Control animals were treated either with atropine sulphate in the solvent, the solvent alone or were untreated. DFP was obtained from Boots Ltd., Nottingham, England.

Biochemical analyses

At different times after injection, the animals were killed by a blow on the head followed by exsanguination and their superior cervical ganglia, together with measured lengths of the preganglionic nerve trunks, were dissected free. The tissues were first desheathed then homogenized in cold 0.3 M sucrose using a small, hand-driven, all glass homogenizer. This homogenate was stored frozen until used.

AChE activity was measured in aliquots of the homogenates according to the modification (CHUBB & SMITH, 1975*a*) of the method of ELLMAN, COURTNEY, ANDRES & FEATHERSTONE (1961). Analysis of any multiple molecular forms of AChE was carried out using polyacrylamide gel electrophoresis as described previously (CHUBB & SMITH, 1975*a*). To inhibit specifically the activity due to AChE, low concentrations of 1:5-bis(4-allyldimethylammonium phenyl)-pentan-3-one dibromide (BW 284C51) were used; in the assay, 2×10^{-5} M and for the gels 10^{-4} M were the final concentrations chosen. Choline acetyltransferase activity was estimated using the method of FONNUM (1975).

Cytochemical studies

Control and DFP treated rats were anaesthetized with ether and perfused through the heart, first with Tyrode solution for 2 min and then for 30 min with a fixative solution (at room temperature) consisting of 2% (w/v) formaldehyde and 2% (w/v) glutaraldehyde in 0.1 M sodium cacodylate at a pH of 7.4. After fixation both ganglia, complete with small lengths of pre- and postganglionic nerve trunks, were dissected out and cut into small blocks. Parts of the ganglia where the nerve trunks entered or emerged were studied separately. Following a further 2–3 h immersion in the same fixative at room temperature, the blocks were incubated for AChE activity using the acetylthiocholine method (KOELLE & FRIEDENWALD, 1949; KÁSA & CSILLIK, 1966) as described earlier (SOMOGYI *et al.*, 1975). To ensure that the activity observed was due to AChE and not to the non-specific cholinesterase (E.C. 3.1.1.8) which also exists in this ganglion (KLINGMAN & KLINGMAN, 1969) ethopropazine hydrochloride [10-(2-diethyl-amino-*n*-propyl)phenothiazine hydrochloride, 10^{-4} M final concentration] was routinely included in the incubation medium. The rest of the procedure was identical to that used earlier with adrenal medullae (SOMOGYI *et al.*, 1975).

To ensure equal treatment for all specimens, thick sections (40–60 μm) of ganglia from either treated or untreated animals were incubated at room temperature in separate compartments of the same dish and for the same period (*30 min*). Control sections were incubated in the full medium but either a) without substrate, or b) in the presence of 10^{-5} M eserine sulphate, or c) in the presence of both 10^{-4} M BW 284C51 and 10^{-4} M ethopropazine hydrochloride.

Surgical procedures

To study the influence of the nerve supply to the ganglion on the recovery of the AChE activity after DFP poisoning, the left superior cervical ganglia of some rats under chloral hydrate anaesthesia (30 mg/kg, i.p.) were decentralized by removing 2–3 mm of the preganglionic nerve trunk. Three days after decentralization the animals were injected with DFP and atropine, and then at different times the ganglia were removed and analyzed as described above.

Statistical analysis

The differences in AChE content of normal and decentralized ganglia at different times after DFP treatment were statistically analyzed using the method of paired comparisons. That is, each value from a decentralized ganglion was compared with its own contralateral control and the data thus obtained pooled for analysis.

RESULTS

Expression of results

Treatment of rats with either the vehicle alone or with 6 mg/kg atropine sulphate had no significant effect on the AChE content of the ganglia; control values therefore refer to pooled values from all these animals. The results of the recovery experiments are expressed as a percentage of the AChE activity found in these control tissues. This normalization of the data was necessary because of large discrepancies in the AChE levels of ganglia from two groups of rats studied 9 months apart. While the means of these two groups were significantly different (means $\pm$ S.E.M.: (i) 31.5 ± 1.1 mU, $n = 20$; (ii) 42.2 ± 0.7 mU, $n = 60$; 1 unit is defined as the hydrolysis of 1 μmol of substrate per min at 30°C and pH 7.0), the percentage of AChE activity which had recovered at any time was not, irrespective of which group was analyzed. The percentages of the recovered activity from both batches were pooled, and it is this combined data which is presented. The reasons why the AChE content of the ganglia should be so different remain unexplained.

Recovery of acetylcholinesterase activity after administration of diisopropylphosphorofluoridate

Total acetylcholinesterase. The recovery of AChE activity after a single dose of DFP follows a complex curve which can be broken down into at least two parts (Fig. 1). The first is an initial rapid recovery during which the AChE content of the ganglion increases by about 1.5% of the initial stores per hour. This rate of increase continues up to 16 h, by which time the ganglion contains 43% of its original amount of AChE, and then stops. Over the next 22 h the level remains fairly constant and then, from about 96 h after the injection, it increases again. The rate of increase during the initial part of this second phase is much slower than that during the first, about 0.2%

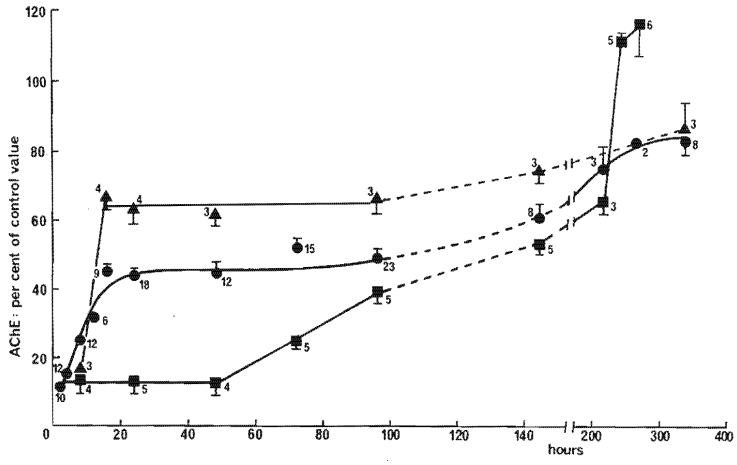


FIG. 1. The recovery from DFP poisoning of the AChE in the superior cervical ganglion and preganglionic nerve trunk of rats. DFP was injected (s.c.) at time zero. The numbers associated with each point are the number of values from which the data were compiled. The bars represent one standard error of the mean. ● total AChE activity and, ▲, soluble AChE activity in the ganglia; ■ total AChE activity in the preganglionic nerve trunk.

of the stores per hour. At around 300 h after injection of DFP, the recovery curve again levels off so that normal AChE values are not achieved until 600 h after DFP treatment.

The time course of recovery of AChE activity in the preganglionic trunk is also illustrated in Fig. 1. This dose of DFP reduces the AChE content of the nerve trunk to about 12% of normal levels. The enzyme activity stays at this level until 48 h but between this time and 72 h it begins to rise. By 144 h approximately 50% of the original amount of AChE has returned and, as a result of a rapid increase after 216 h, the control level (0.102 ± 0.005 mU/mm, $n = 15$) is achieved by 240 h. The levels attained at this time are generally above the control level but are not statistically significantly different.

Soluble acetylcholinesterase. High-speed centrifugation of the 0.3 M sucrose homogenates of superior cervical ganglia reveals that an appreciable proportion of the AChE cannot be sedimented, even after centrifugation for 90 min at 100,000 g_{av}. This activity, which amounts to 39.1% of the total AChE, will be referred to as soluble enzyme (Table 1). The definition of the term soluble as used in this context, and justification of its use, has previously been discussed (CHUBB & SMITH, 1975*a*).

The recovery of this soluble AChE activity after DFP treatment was also studied. As shown in Fig. 1, the initial rapid recovery found when total AChE activity was estimated is also found for the recovery of AChE in the high speed supernatant. After this phase is complete, i.e. up to 16 h, the recoveries of the total and soluble activities differ. The accumulation of the activity of the soluble enzyme is much slower than that found for the total AChE; there is no clearly defined second recovery phase but just a very slow steady increase. Also, the recovery of the soluble enzyme appears to lag slightly behind the recovery of the membrane-bound activity. Thus, 2 h post-DFP administration there is measurable AChE activity but only a small proportion of this appears as soluble enzyme. This proportion increases so that between 16 and 144 h it is above that found in untreated tissues and then, as the total activity increases, it falls to its normal proportion of about 40% of the total activity.

Preganglionic denervation. The influence of the preganglionic nerve on the recovery of AChE and its contribution to the enzyme concentration in the ganglion has also been examined. Decentralization of the ganglion for 3 days results in a nearly complete (98%) loss of choline acetyltransferase activity from the

TABLE 1. TOTAL AND SOLUBLE ACETYLCHOLINESTERASE, AND CHOLINE ACETYLTRANSFERASE ACTIVITIES, IN NORMAL AND DECENTRALIZED SUPERIOR CERVICAL GANGLIA

	Acetylcholinesterase (mU/ganglion)			Choline acetyltransferase nmol product/h/ganglion
	Total activity	Soluble	% Soluble	Total activity
Normal ganglion	42.2 ± 0.7	16.5 ± 1.0	39.1	17.7 ± 2.8
Decentralized ganglion	25.9 ± 1.7	11.6 ± 1.0	43.1	1.3 ± 0.8

The ganglia were decentralized 72 h prior to assay.

FIG. 3. Polyacrylamide gel electrophoresis demonstrating the soluble multiple molecular forms of AChE in superior cervical ganglia and preganglionic nerve trunks. The samples were applied to the top of the gel; migration was towards the bottom which was the anode. The bands are precipitates of copper thiocholine sulphate caused by the action of AChE in the gel on its substrate, acetylthiocholine. Gel (a), the soluble molecular forms of AChE in the ganglion. The sample run on this gel was a complete homogenate, the dense staining material which has not entered the gel can be shown, by extracting with Triton X-100, to consist primarily of the one membrane-bound form of AChE. Those forms which enter the gel are not altered, in number or relative content, if a high-speed supernatant fraction is applied to the gel. Gel (b) is a similar fraction from the preganglionic nerve trunk. To obtain a sample with sufficient AChE activity for it to be visible on the gel, the preganglionic nerve trunk was ligated for 24 h and the accumulated enzyme activity analyzed in a 2 mm segment on the proximal (central) side of the tie.

FIG. 4. Polyacrylamide gel electrophoresis of the soluble molecular forms of AChE in (a) a normal ganglion and (b) a ganglion which had been decentralized 72 h prior to analysis. The two samples, each containing the same amount of tissue, were treated as described in the legend to Fig. 3.

All micrographs show the localization of AChE in superior cervical ganglia of the rat. The times given in each case indicate the time in h between the injection of DFP and death. The control animals were injected with atropine in 1% (v/v) ethyl alcohol (see Experimental Procedures).

FIG. 5. Control. AChE reaction end-product in the endoplasmic reticulum of a ganglion cell. In the right upper corner of the micrograph a large dendrite is emerging from the soma. Smooth tubules of endoplasmic reticulum which show AChE activity are aligned parallel to the axis of the dendrite. Some reaction end-product can be seen between the plasma membranes of a satellite cell and the neuron (arrow) × 12,000.

FIG. 6. Control. Detail of the neuropil. Several nerve endings can be seen with reaction end-product on their surface (curved arrow) and one of them contains a tubule with enzyme activity associated (straight arrow). Preterminal axons (p) are also surrounded by reaction product. Synapse (s), dendrites (d). × 23,500.

FIG. 7. Control. Detail of a ganglion cell soma. Smooth, AChE containing, subsurface cisternae (thin arrow) run parallel to the plasma membrane. There is also some reaction end-product between the plasma membranes of the ganglion and satellite cells (thick arrow). × 28,000.

FIG. 8. Control. Preganglionic axons. The reaction end-product can be seen around the axons and, in one of them, in a tubule × 46,000.

FIG. 9. Two hours after DFP. Faint reaction due to AChE activity can be seen in cisternae of the endoplasmic reticulum in a ganglion cell body. × 8,400.

FIG. 10. Eight hours after DFP. A survey picture of ganglion cells and their proximal dendrites (d). The distribution of AChE corresponds to that of the control (see comparison Fig. 5). × 8,300.

FIG. 11. Eight hours after DFP. A nerve ending synapsing (arrow) on a dendritic shaft. Although AChE is present in the rough and smooth endoplasmic reticulum of the dendrite, there is no reaction end-product around the nerve terminal or in the synaptic cleft. × 20,000.

FIG. 12. Seventeen hours after DFP. A tubule of smooth endoplasmic reticulum (SER) with AChE activity in a post-ganglionic nerve fibre. A large dense-cored vesicle (arrow). × 60,000.

FIG. 13. Forty-eight hours after DFP. AChE is present within the ganglion cell but there is still no appreciable activity in the nerve terminals (t) or preterminal axons (a). × 24,000.

FIG. 14. Five hundred and four hours after DFP. Synaptic bouton on a small diameter dendrite (d). The AChE reaction end-product is present in the synaptic cleft (s) and around the nerve terminal. × 68,500.

FIG. 15. Five hundred and four hours after DFP. The Golgi region of a ganglion cell. In this cell the cisternae contain AChE activity. × 41,500.

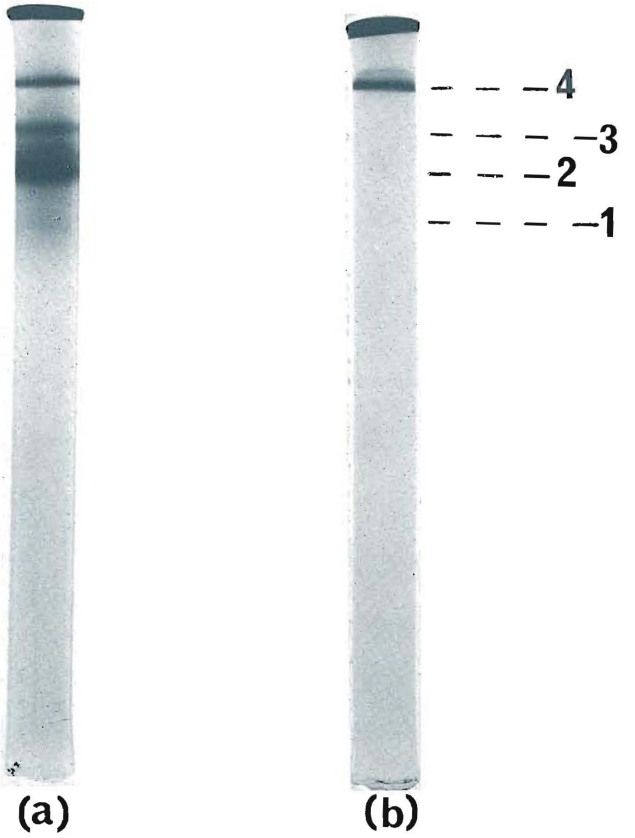


FIG. 3.

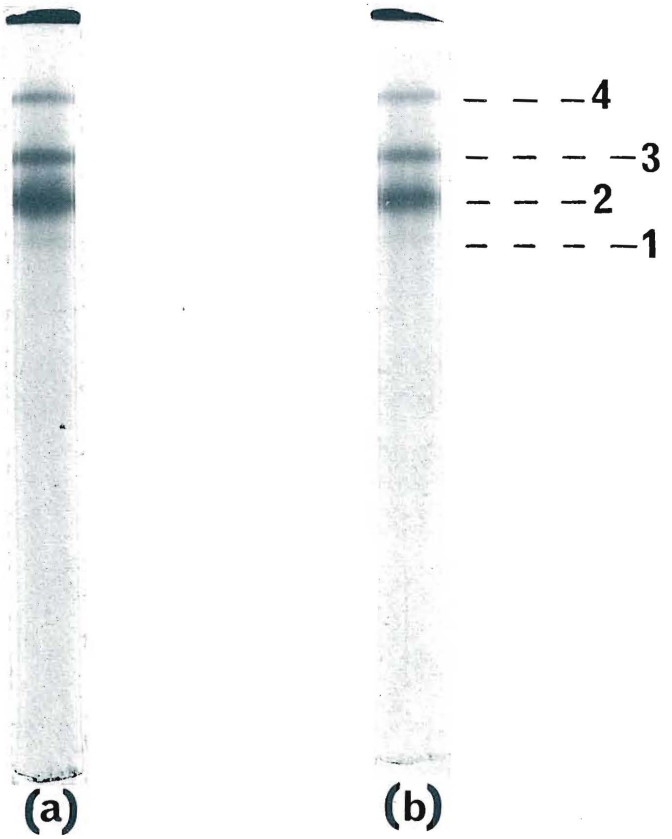
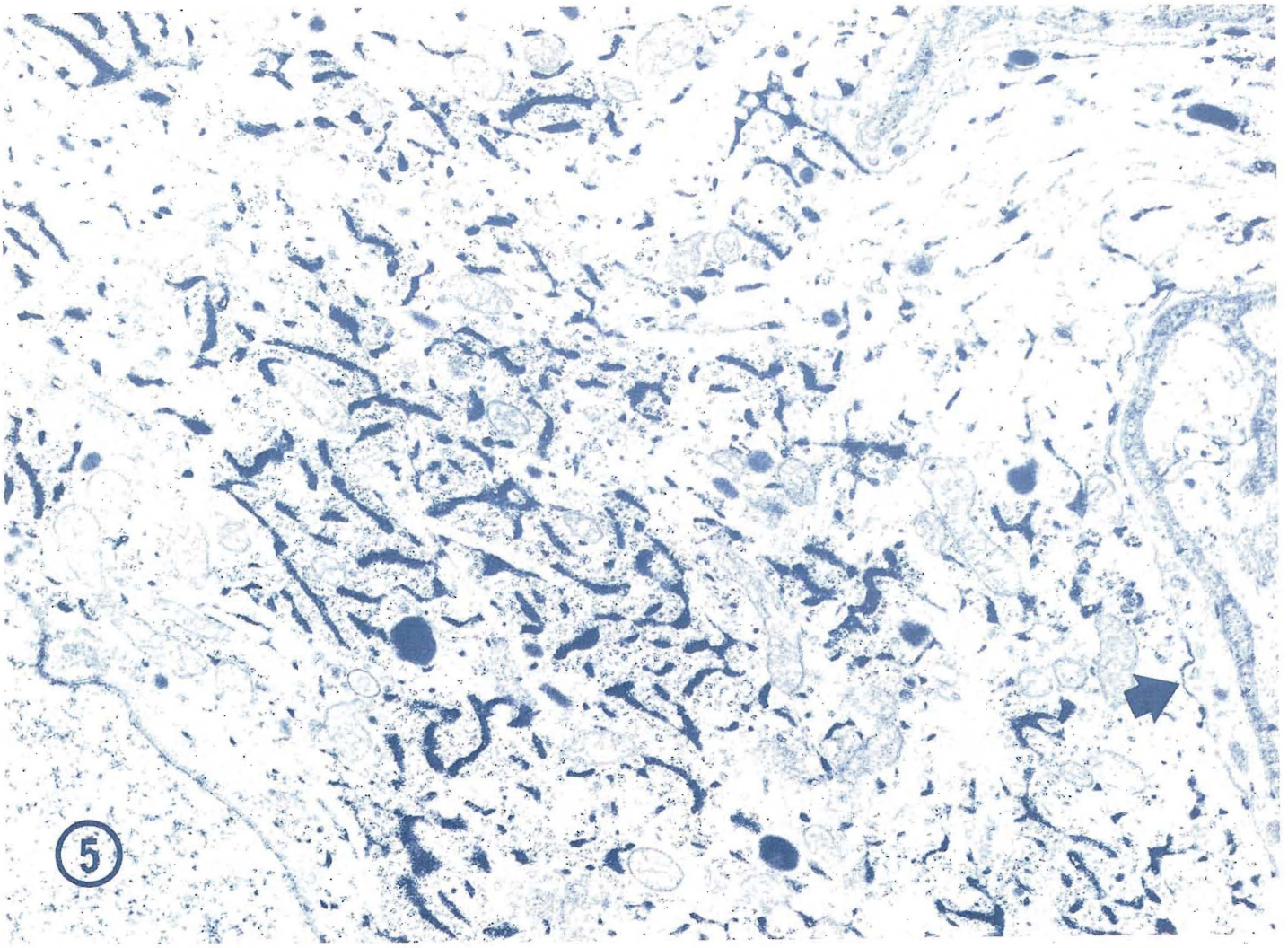
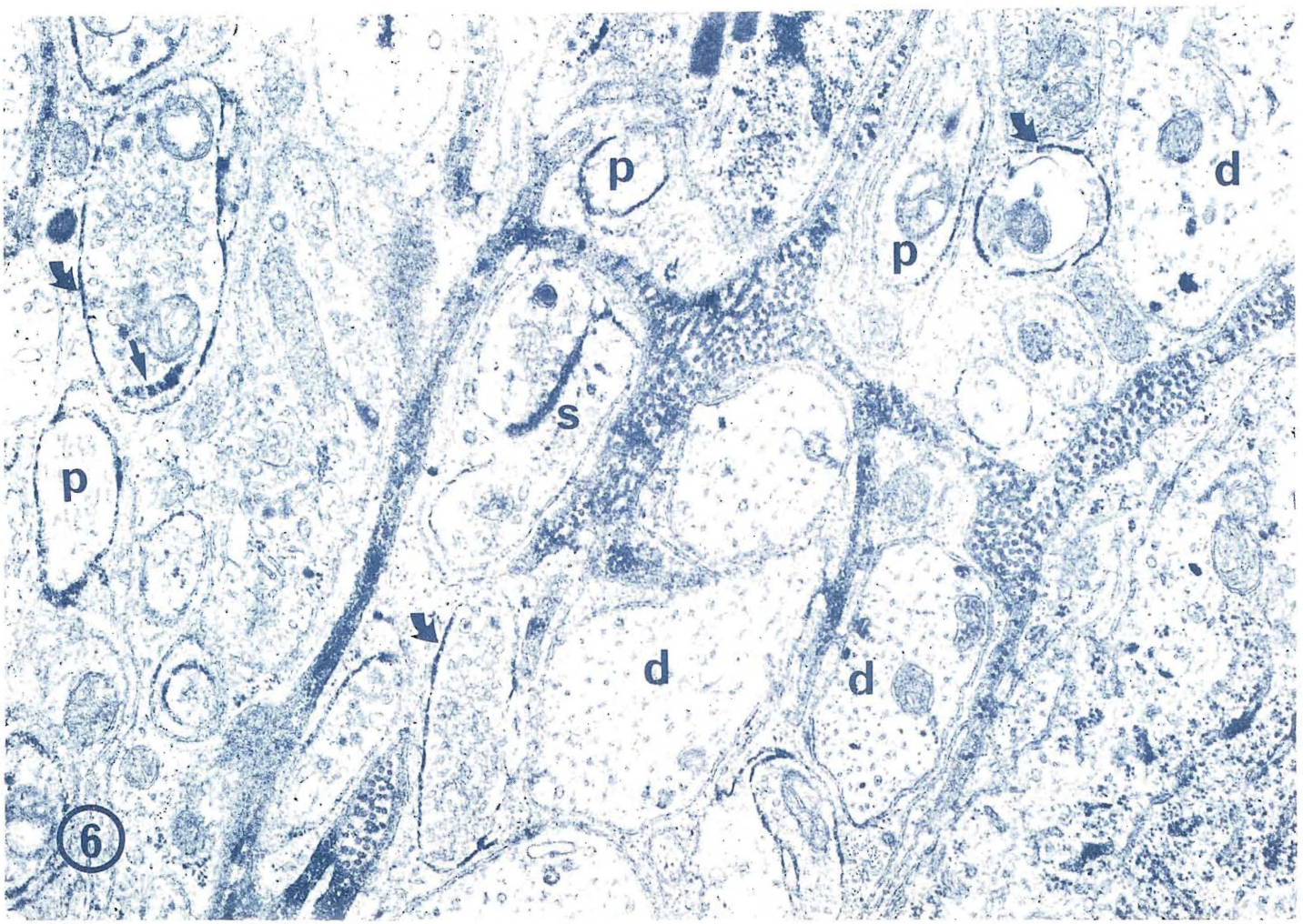


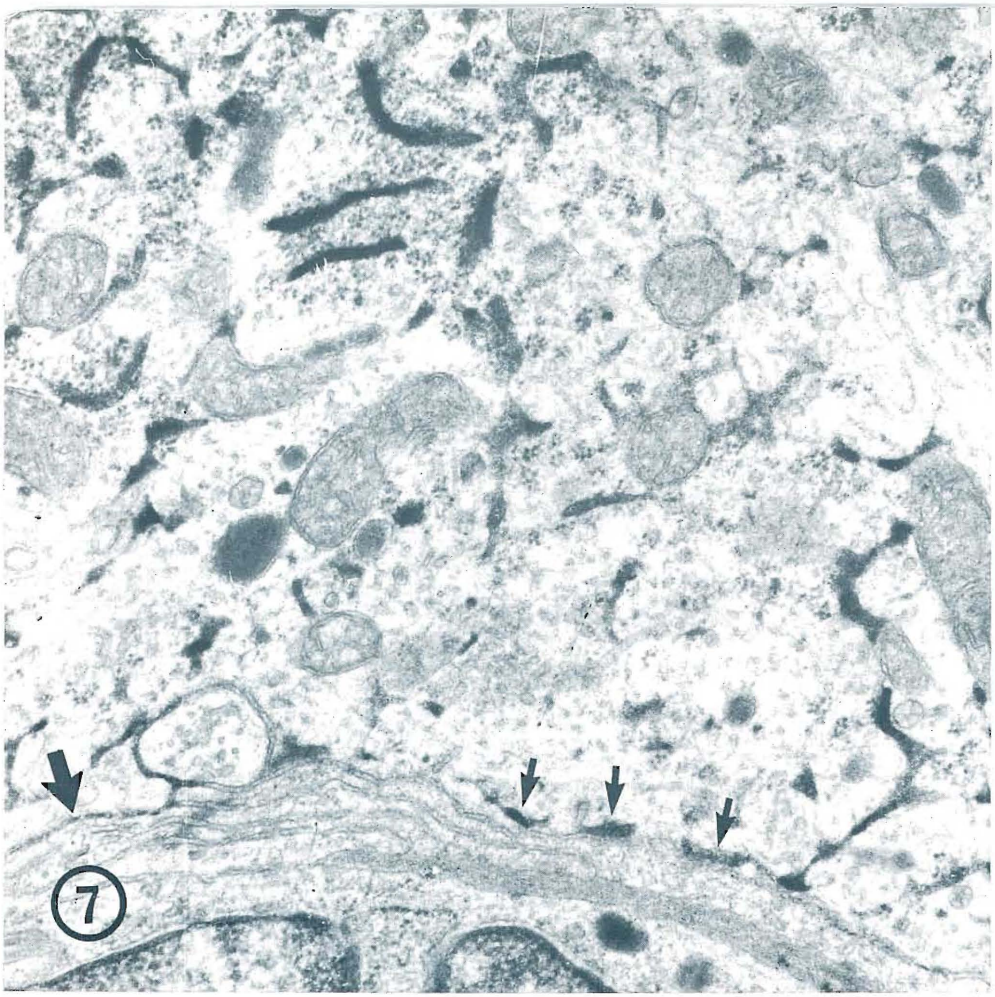
FIG. 4.



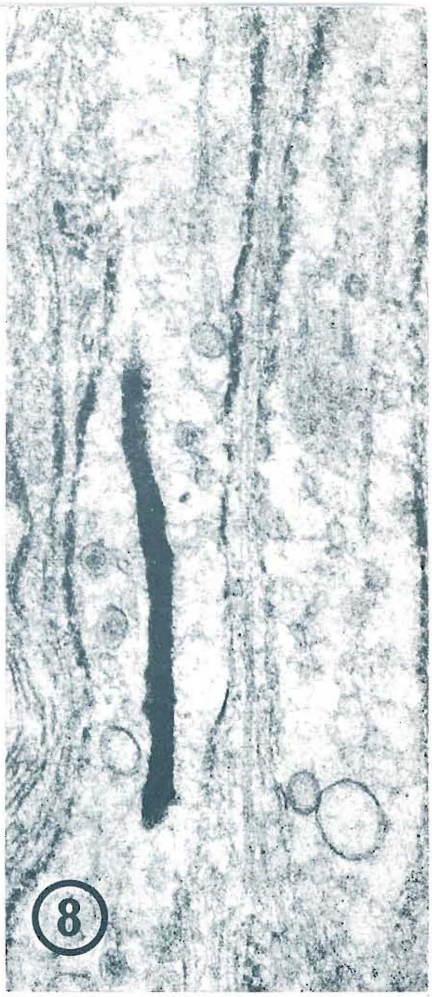
5



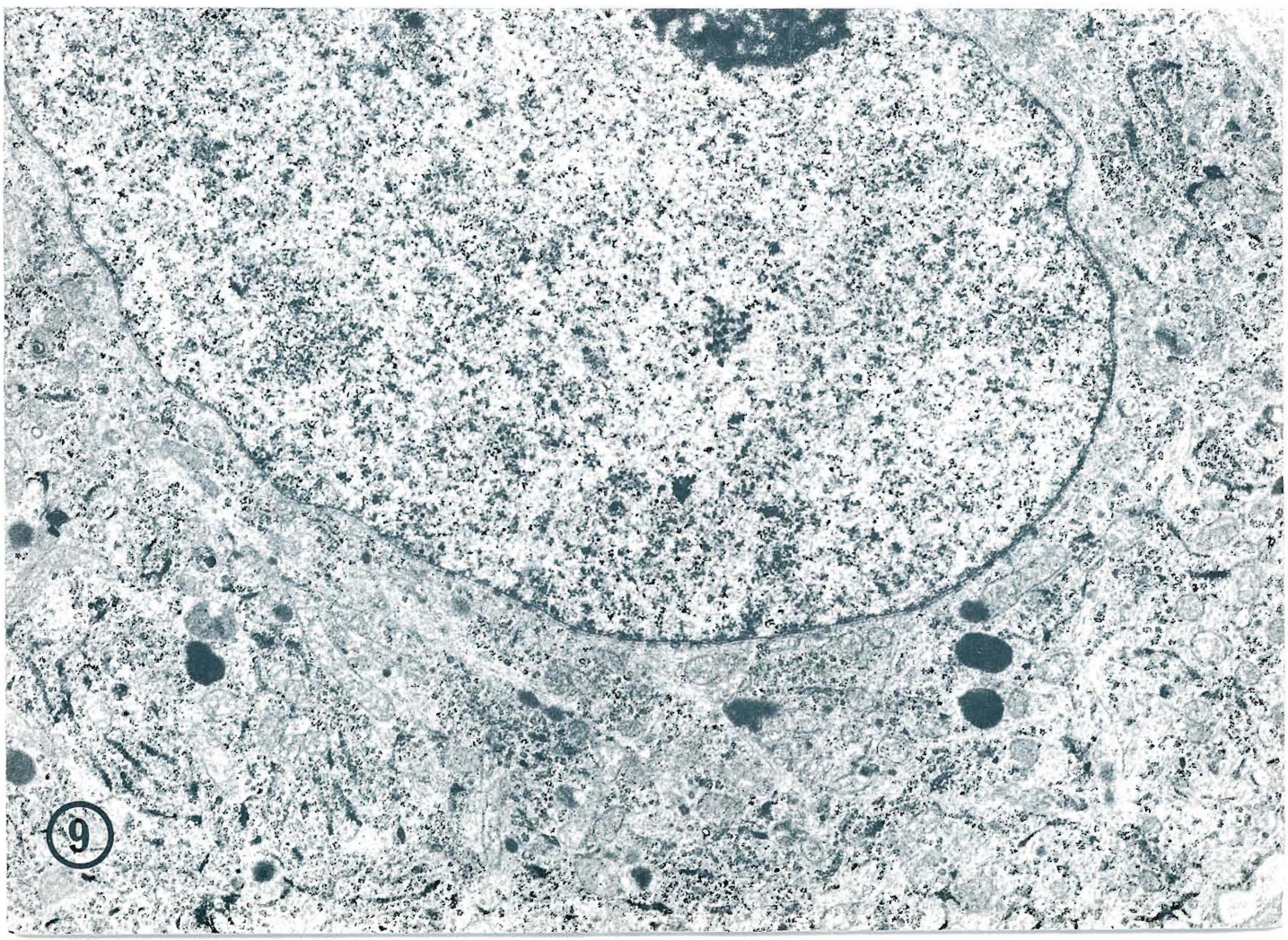
6



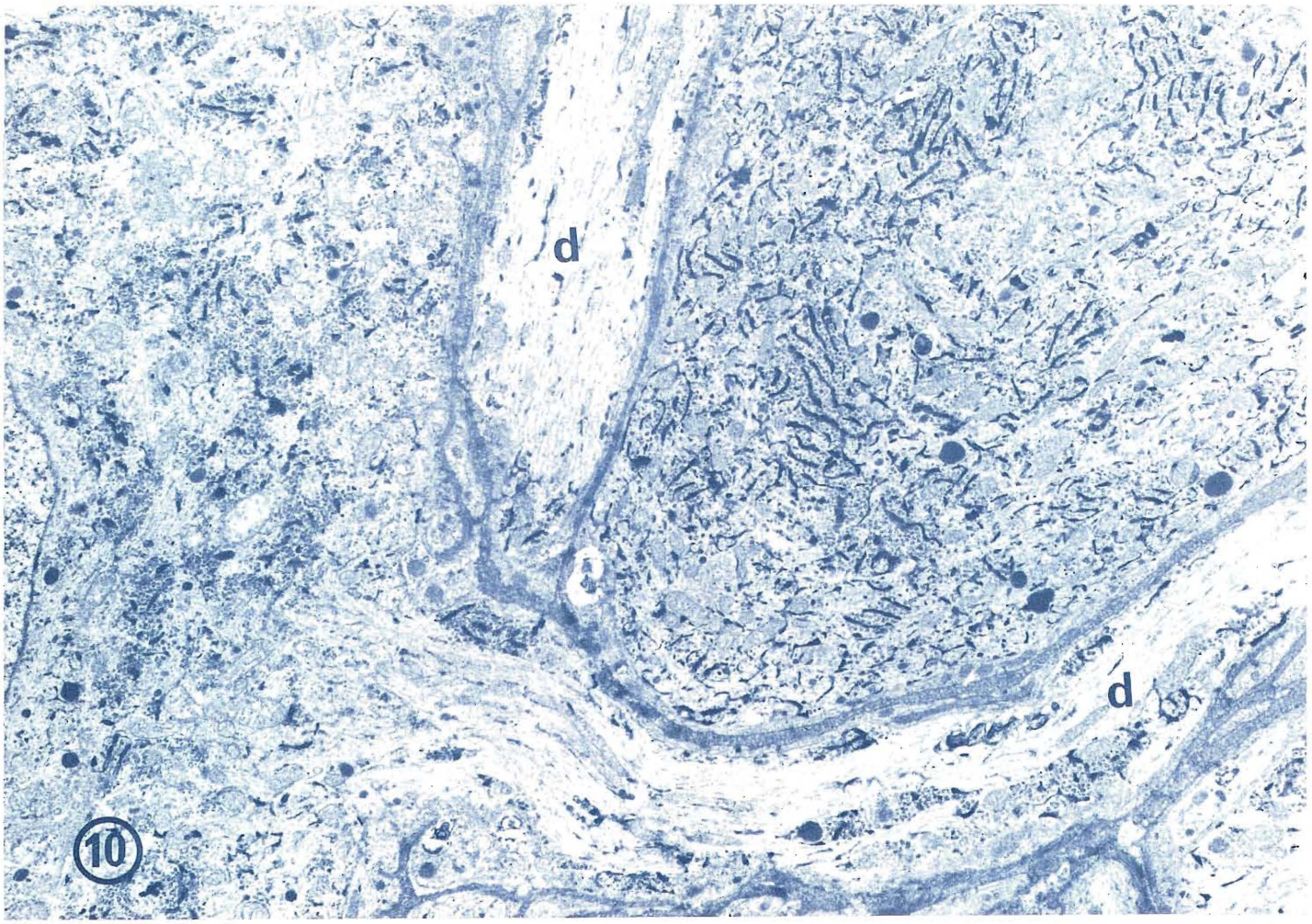
7

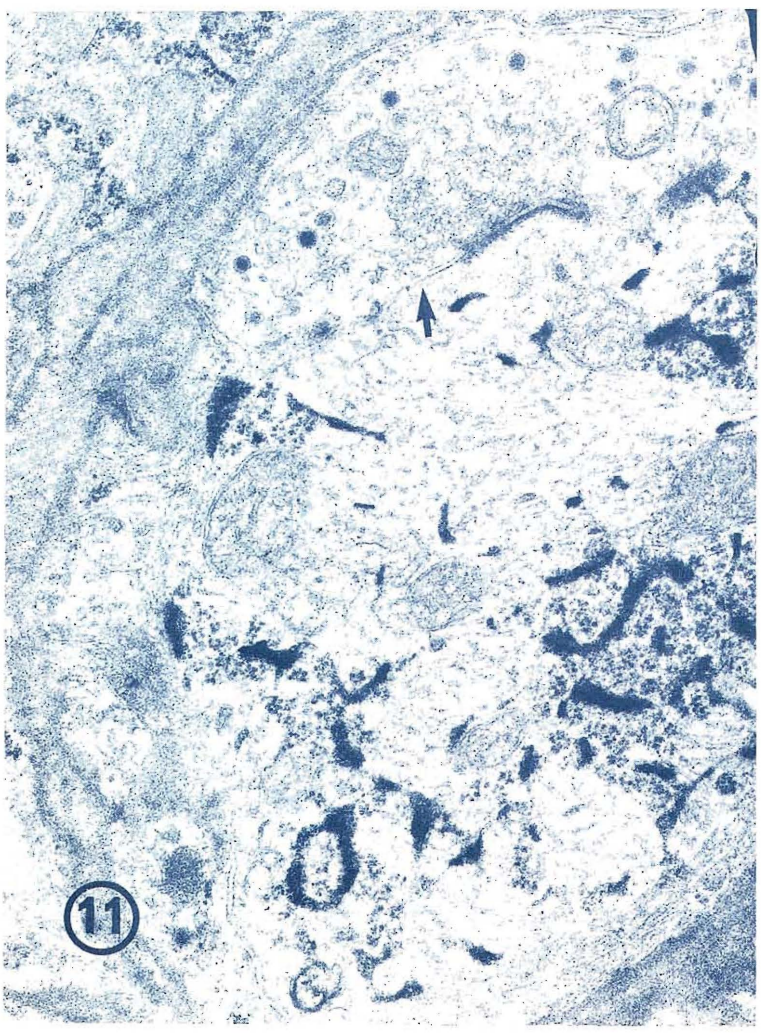


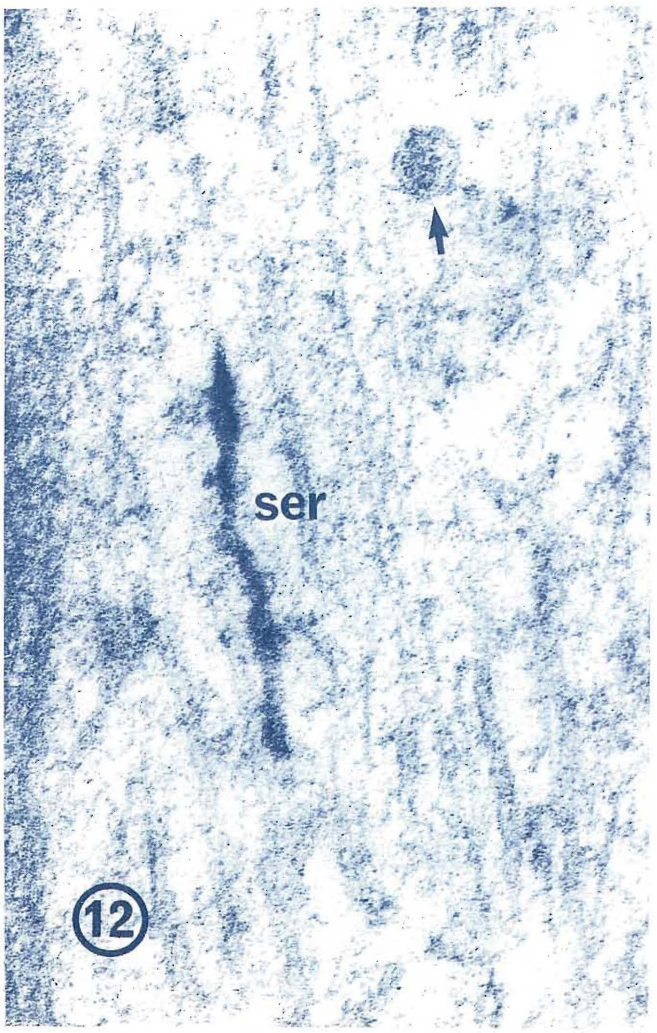
8

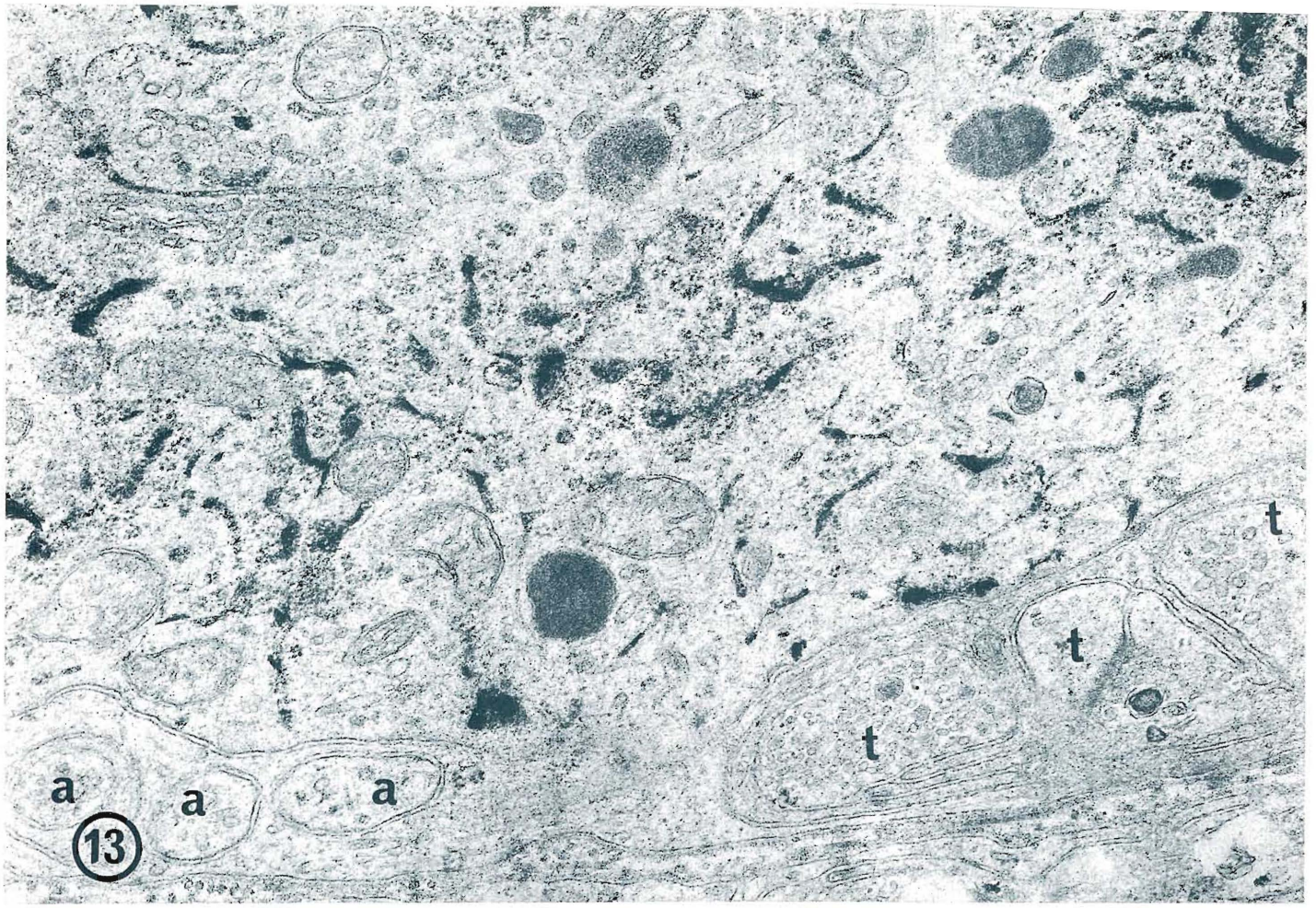


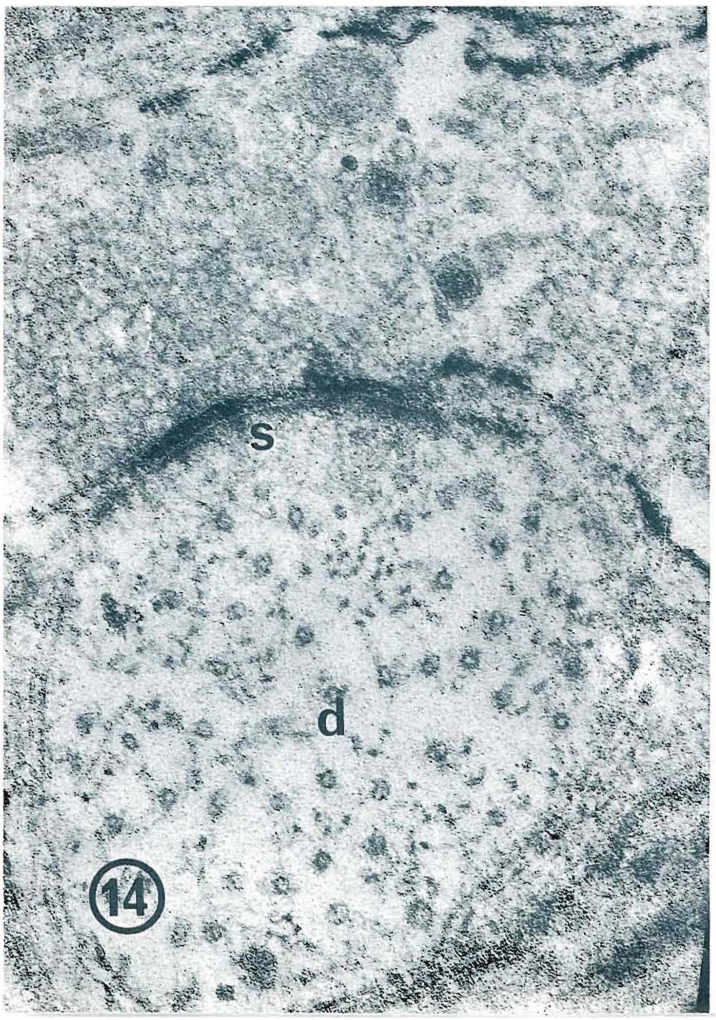
9

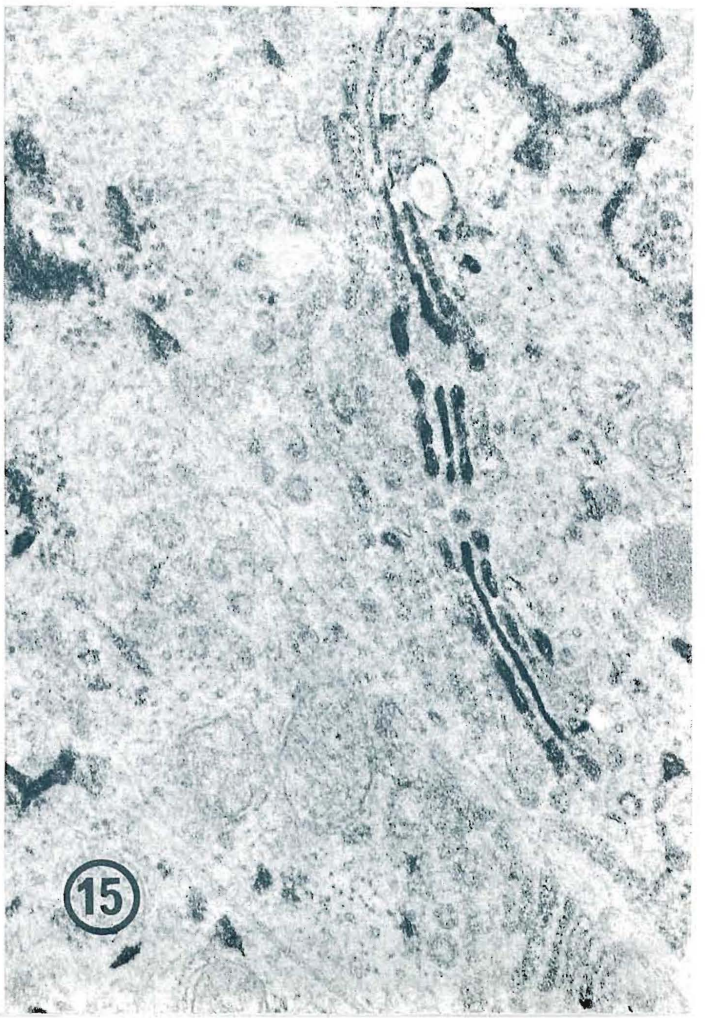

d
d
10


11


ser
12


a
a
a
t
t
t
13


s
d
14


15

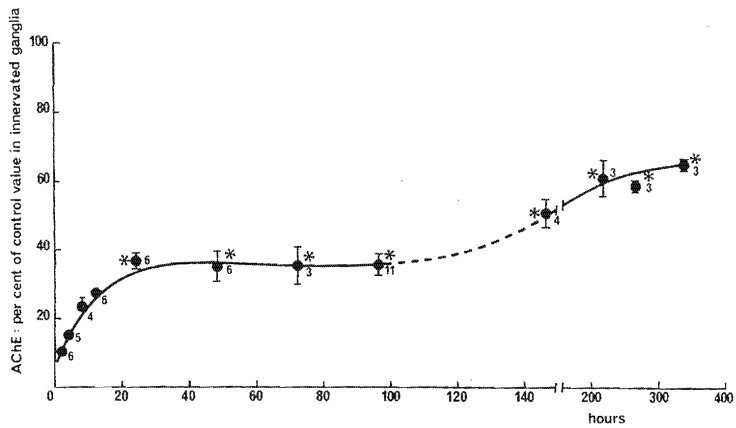


FIG. 2. The recovery from DFP poisoning of the total AChE activity in decentralized superior cervical ganglia. The ganglia were decentralized 72 h prior to DFP administration which was injected at time zero. The numbers associated with each point show the numbers of ganglia used. The bars represent one standard error of the mean. The points marked with an asterisk are those which differ significantly, at the $P < 0.05$ level, from the corresponding control value.

ganglion and a 40% decrease in the AChE concentration (Table 1). The decrease in total AChE activity is accompanied by an equivalent loss of soluble enzyme; the proportion which is soluble in a decentralized ganglion is still 40%.

The results illustrated in Fig. 2 show the recovery of AChE in the decentralized ganglia after treatment with DFP. There is a rapid recovery phase, up to 16 h, the rate of which does not differ significantly from that found in normal tissues. Again there is a plateau which is followed by a second, slower, recovery phase just as was found in the control ganglia. The decentralized ganglion reaches its own control level, i.e. the level to which the enzyme falls after decentralization, much sooner then the innervated tissue reaches its normal level (200 h against 600 h). The recovery of the soluble AChE activity occurs in a similar fashion to that found in the normal ganglion.

Multiple forms of AChE in the ganglia and the preganglionic nerve trunk

Electrophoretic analysis of the AChE of the ganglia shows that the enzyme exists in several molecular forms (Fig. 3). Using the incubation conditions described previously (CHUBB & SMITH, 1975*a*), the high-speed supernatant fraction of the homogenates can be shown to contain 4 major forms of AChE, all of which are absent if 10^{-4} M BW284C51 is included in the incubation medium. An additional form of AChE is revealed if the homogenates are first extracted with 0.5% Triton X-100 and then subjected to electrophoresis. This membrane-bound form of AChE has a slower electrophoretic mobility than the slowest of the soluble forms (cf. the bovine adrenal medulla; CHUBB & SMITH, 1975*a*). Thus, a ganglion contains four major soluble forms of AChE, numbered 1–4 in order of decreasing electrophoretic mobility, and a major membrane-bound form, AChE-5. The molecular forms of AChE in the nerve trunk are considerably fewer than those found in the ganglion (Fig. 3). There is a single soluble species, equivalent in electrophoretic mobility to AChE-4, and another which is membrane-bound and similar to AChE-5.

The influence of decentralization on the various soluble forms of AChE present in the ganglion is shown in Fig. 4. The tissues for the two gels shown in this figure were taken from the one animal; a ganglion denervated 72 h prior to its removal and its contralateral control. The same wet weight of tissue was applied to the gels and so the apparent difference in the activity on each gel is due to the 40% decrease in soluble AChE activity which occurs in response to denervation. As shown, however, this decrease is not due to the disappearance of any one form they all seem to be affected approximately equally.

Analysis of the soluble molecular forms of AChE present in the ganglia at different times after DFP treatment shows little indication of a preferential recovery of a particular form; as soon as enzyme activity can be measured (2 h post-DFP) all the forms can be seen on gels. There is too little activity in the preganglionic nerve trunk to study the earlier recovery of AChE in these axons. When activity can be measured (72–144 h), the pattern is identical to that found in untreated tissues.

Cytochemical localization of acetylcholinesterase in control and diisopropylphosphorofluoridate treated ganglia

Control ganglia. The electron dense end-product due to AChE activity could be seen in all ganglion cells (see also, GISIGER, VENKOV & GAUTRON, 1975). The precipitate was present in the tubules and cisternae of both rough and smooth endoplasmic reticulum (Figs. 5–7), in the nuclear envelope, subsurface cisternae (Fig. 7) and occasionally in saccules of the Golgi apparatus. Most of the cells were found to contain

reaction product throughout their endoplasmic reticulum, including the small dendritic branches, but occasionally we observed cells with a reaction in only some of the cisternae. In contrast to this abundant intracellular localization, AChE activity was only rarely observed on the plasma membrane of the ganglion cells. When reaction product was present between the plasma membranes of the neurons and the enveloping satellite cells, it was restricted to short distances in the extracellular space (Figs. 4, 5, 7).

Preganglionic axons entering the ganglion, as well as their preterminal regions containing synaptic vesicles, have reaction end-product all around their surface membrane (Figs. 6, 8). Nerve terminals were often entirely, but sometimes only partially surrounded by reaction product; the synaptic cleft was invariably active. Only very rarely was end-product ever seen within the cytoplasm of the nerve terminal although when it was it could be seen in tubules (Fig. 6). A feature of the axons within the ganglia is that both the pre- and the postganglionic nerves contain AChE activity in elements of smooth endoplasmic reticulum.

Two hours after diisopropylphosphorofluoridate administration. At this time some cells were totally devoid of activity while others showed a faint reaction. Those which did react had the end-product in the endoplasmic reticulum of the cell soma and the dendrites (Fig. 9). Observations made at such a short time after DFP administration are complicated by the fact that the inhibition of AChE activity by the poison is not complete; it is difficult to assess whether the observed activity is due to the presence of newly synthesized or residual enzyme. However, since there is no demonstrable activity associated with either the preganglionic nerve fibres or terminals, it seems most likely that the activity seen is largely due to newly synthesized enzyme. Thus, as soon as 2 h after DFP new enzyme has already arrived in the dendrites, in smooth endoplasmic reticulum (Fig. 9).

Eight hours after diisopropylphosphorofluoridate administration. There was no observable difference between the localization of AChE in the ganglion cells compared with that found in the controls (Fig. 10). All the cells contained reaction end-product and it could be seen in even the most distal regions of the dendrites. It was also possible to find occasional tubules of smooth endoplasmic reticulum containing AChE in the post-ganglionic axons.

In marked contrast to the ganglion cells, the preganglionic nerve fibres and their terminals showed no sign of having enzyme activity. There was no activity at synapses even though the postsynaptic cells (e.g. a dendrite, Fig. 11) contained appreciable amounts of AChE.

Seventeen hours after diisopropylphosphorofluoridate administration. The distribution of AChE in the ganglion cells still corresponded to that of the control tissues and to those examined 8 h after DFP. The end-product could be seen in endoplasmic reticulum throughout the cells and in the postganglionic axons (Fig. 12). In general, the nerve terminals and preganglionic axons were still devoid of AChE activity. A weak reaction was seen around a few terminals where it was restricted to small areas in the extracellular space, between the axolemma and the enveloping Schwann cell.

Twenty four and 48 h after diisopropylphosphorofluoridate administration. The distribution of the enzyme (Figs. 12, 13) is no different from that observed 17 h post-DFP. Again the nerve axons and terminals were almost totally devoid of activity although a few showed a positive reaction. Those which showed a reaction were possibly more numerous 48 h after DFP than they were after 17 h (Fig. 12).

Five hundred and four hours after diisopropylphosphorofluoridate administration. By this time, there was no discernible difference between treated and control ganglia. Both the pre- and the postganglionic elements appear to have regained their activity, and all the synaptic clefts contained AChE (Fig. 14). The Golgi regions of some cells also contained the reaction end-product (Fig. 15).

DISCUSSION

Localisation of acetylcholinesterase activity

The cytochemical experiments have revealed that the ganglion cells and the nerve trunks which innervate them are the two structures within the superior cervical ganglion which contain the bulk of the AChE present in the organ. Of these two it has been estimated, on the basis of results from studies of the effects of denervation, that the ganglion cells contain 55% of the total activity; the remainder disappears upon decentralization of the ganglion (KLINGMAN & KLINGMAN, 1969).

The cytochemical experiments also reveal that the localization of the AChE activity within the two structures is different. In the cells the enzyme is mainly associated with the rough endoplasmic reticulum and occasionally with the smooth tubules and the plasma membrane. This is in complete contrast to the axons in which the AChE is always found to be associated with the plasma membrane and with the infrequent elements of the *smooth* endoplasmic reticulum. Thus, it can be concluded that in this respect the ganglion appears to be composed of two separate parts; one, the cells, which contain 55% (60% in our experiments) of the total AChE activity and which can synthesize the enzyme and another, the nerve, which contains 40–45% but is unable to synthesize the enzyme.

How much of the acetylcholinesterase is presynaptic?

Using this information it is possible to predict the changes in the levels of the AChE activity of the ganglion following recovery from DFP poisoning. First, 55–60% of the activity should recover at a rate which corresponds to the rate of synthesis of the enzyme

in ganglionic nerve cell bodies; this is likely to be a rapid recovery phase (DAVIS & AGRANOFF, 1968; AUSTIN & JAMES, 1970; WILSON, NIEBERG, WALKER, LINKHART & FRY, 1973; CHIPPENDALE, COTMAN, KOZAR & LYNCH, 1974). This first phase would then be followed by another phase whose debut would depend on the rate at which enzyme synthesized in the cell bodies of the preganglionic neurons was transported down the axons to the nerve terminals within the ganglion. As shown in Fig. 1, however, this curve is not quite the one found in practice; the recovery of the enzyme activity stops at the end of the first phase when it reaches only 43% and not 55–60% of its original level. In view of this finding it would seem worthwhile to re-examine the premises on which the prediction was made.

Is the amount of an enzyme, or any other constituent of a cell, which remains after denervation an accurate reflection of the amount present in a normal, innervated cell? This has long been assumed to be true for cholinesterase, provided that sufficient time has been allowed for the nerves to degenerate (see e.g. SAWYER & HOLLINSHEAD, 1945; KOELLE, 1963) and there is no difficulty in the interpretation of this type of experiment if the measured constituent is unique to either of the two cells which comprise the synapse. Ambiguity does arise, however, when the constituent is present in both the pre- and the postsynaptic cells, as is the case for AChE in ganglia. In a situation such as this, how is it possible to know whether changes found as a result of denervation are due solely to degeneration of the nerve trunk and not to any influence that the nerve might exert on the constituents of the postsynaptic cell?

This problem can be approached by examining the effects of denervation on the soluble forms of AChE. It is known (Fig. 3) that there are four of these and that only one (AChE-4) is found in the preganglionic nerve trunk. It is also known (Table 1) that denervation of the ganglion reduces the amount of soluble AChE to 11.6 mU, a loss of 5 mU of soluble activity. We have estimated that AChE-4 comprises about 30% (or 5 mU) of the soluble activity present within a normal ganglion (I. W. CHUBB & D. G. WILLIAMS, unpublished). Thus, a loss of 5 mU of soluble activity could be accounted for by the complete loss of the one soluble form which is known to be associated with the nerve trunk. It was found, however, that AChE-4 does *not* disappear from the ganglion after decentralization; it does not even seem that there is a preferential loss of this molecular form of AChE over all the others. It seems likely, therefore, that the effect of decentralization is to reduce, more or less evenly, the concentrations of all the soluble forms of AChE in the ganglion.

Another approach to the problem of how much of the AChE in the ganglion is derived from the preganglionic axons and terminals can be made by considering the quantitative data of KLINGMAN and KLINGMAN (1969) and the cytochemistry reported here. KLINGMAN and KLINGMAN (1969) have estimated that 1 mg of nerve trunk contains 7.4 mU of AChE activity. In their experiments, the weight of the ganglion did not change from its original 1.5 mg after decentralization, but they found that 13 mU of AChE activity was lost. This observation makes it unlikely that the loss of AChE from the ganglion is due simply to the loss of nervous tissue unless, of course, it is markedly enriched in AChE activity. However, these quantitative estimates and the cytochemistry indicate that this too is unlikely. Knowing the amount of AChE per mg of nerve trunk, and assuming that about 1% of the weight of the ganglion is due to the weight of the preganglionic nerves within it (see BIRKS & MACINTOSH, 1957), it can be calculated that to account for the observed loss of AChE activity, then the AChE of the preganglionic nerve elements in the ganglion would need to be concentrated 120–150 fold relative to that in the nerve trunk before it enters the ganglion. The cytochemical experiments, although they cannot exclude the possibility, give no indication of an appreciable concentration of enzyme activity near the nerve terminals. There do not appear to be significant differences in the amount of end-product associated with axons, axon terminals and synapses when all are present in the one tissue section (e.g. Fig. 6).

It is dangerous to imply that cytochemistry is quantitative, but as used here it can be justified on the following grounds. The reaction end-product which appears first is at those locations where the enzyme activity is normally highest. Thus, by using different incubation times the relative enzyme concentrations in any one tissue section could be roughly determined. Also, when large amounts of end-product accumulate, it is found as crystals. It follows that if an incubation is prolonged to reveal all sites of AChE then those which have the highest concentrations ought to have crystalline end-product. Conversely, if the incubation time is restricted to prevent crystal growth, only areas of high activity will be revealed. The results described (Fig. 6) are therefore best interpreted as indicating that the reactive areas all have similar concentrations of AChE.

All this evidence makes it likely that one effect of decentralization is to influence the amount of acetylcholinesterase within the postsynaptic cells. Is there support for this suggestion?

Does the presynaptic nerve influence the level of acetylcholinesterase activity in postsynaptic neurons?

Some sort of nervous influence on the AChE content of the ganglion cells seems the most plausible explanation for the way which the enzyme activity recovers in the initial stages after poisoning. When normally innervated ganglia reach the end of the early and rapid recovery phase, they contain approximately 40–50% of the AChE level found in untreated tissues. Cytochemistry reveals that the AChE at this early stage is confined to the postganglionic cells. There-

fore, if the nerve was without influence it might be expected that the same amount of AChE would be synthesized by ganglion cells irrespective of whether they were innervated. However, the denervated cells accumulate significantly less AChE than their innervated counterparts, although they contain a greater proportion of their ultimate level (50–53% compared with 43%). Thus, the nerves seem able to govern the total amount of AChE that ganglion cells can contain.

Effectively, we have used two means of removing the contribution of the preganglionic nerve to the AChE concentration of the whole ganglion. On the one hand this has been done surgically and on the other the nerves are still present but are devoid of AChE activity. The mere presence of the nerves is sufficient to increase the amount of AChE that the cells contain.

It is also noteworthy that the synapse is devoid of AChE activity until the preganglionic nerve axons recover their activity. This implies that the synaptic AChE is not derived from the innervated cell but is supplied from within the presynaptic axon.

The way in which the nerve might influence the AChE concentration of the ganglion is not clear. The presence or absence of the nerve could influence such factors as the amount of enzyme synthesized, the amount transported away from the ganglion (see Fig. 12) and the amount secreted from the cells (KREUTZBERG & TÓTH, 1974; CHUBB & SMITH, 1974, 1975b). In this respect it is worthwhile to note several interesting observations made on the AChE of the neuromuscular junction. The AChE content of muscles is known to decrease rapidly but incompletely in response to denervation (GUTH, 1968; CRONE & FREEMAN, 1972); after DFP poisoning the original level of activity is recovered only if the muscle is innervated (FILOGAMO & GABELLA, 1966; ROSE & GLOW, 1967); as much as 85% of the AChE is associated with the postsynaptic cell while less than 10% is associated with the axon (SALPETER 1967); non-innervated, cultured muscle cells are capable of rapid synthesis of AChE, much of which is soluble, but they seem unable to retain the activity since it is lost to the incubation medium (WILSON *et al.*, 1973) and nerve explants or extracts, which need not directly contact the muscle, are capable of preventing the denervation-induced loss of cholinesterase activity from muscle cells in culture (LENTZ, 1974). Clearly the nerve provides the muscle with an ability to synthesize and then to retain a defined amount of AChE. There are enough similarities between the muscles and the ganglia to ask whether a similar mechanism might be operating in both tissues.

There is as yet no way in which a definitive answer to this question can be given. We feel, however, that the evidence is sufficiently suggestive to propose that an interaction between pre- and postganglionic cells of a type similar to that between nerve and muscle should be considered possible. From the data presented in Fig. 2, it is unlikely that AChE is an inducible enzyme in the same sense as tyrosine hydroxylase (THOENEN, 1972). The initial hyperactivity of the innervated ganglion cells after inhibition of the AChE, which must be in marked contrast to the low activity of the denervated cells, does not significantly influence the rate at which the AChE is synthesized. It seems more likely, therefore, that the nerve bestows upon the cells an ability to synthesize and possibly more importantly, to retain, a fixed amount of AChE, just as it appears to do for muscles.

In the context of secretion of AChE from the adrenal medulla (see the introduction), this study enables us to draw one conclusion. Of the several AChE compartments which exist in the ganglion and adrenal medulla, the most rapidly turning-over is likely to be that in the ganglion, and hence most probably the chromaffin, cells. It thus becomes more probable that the molecular form which is secreted from the medulla is derived from the chromaffin cells and not from the innervating nerve.

Acknowledgements—This work was supported by grants from the Medical Research Council, The Wellcome Trust and the Smith, Kline and French Foundation. We thank Mrs. M. BURNETT for expert technical assistance. I.W.C. is a Royal Society Horace Le Marquand and Dudley Bigg Research Fellow.

REFERENCES

AUSTIN L. & JAMES K. A. C. (1970) Rates of regeneration of acetylcholinesterase in rat brain subcellular fractions following DFP inhibition. *J. Neurochem.* **17,** 705–707.

BIRKS R. I. & MACINTOSH F. C. (1957) Acetylcholine metabolism at nerve endings. *Br. med. Bull.* **13,** 157–161.

BURNSTOCK G., EVANS B., GANNON B. J., HEATH J. W. & JAMES V. (1971) A new method of destroying adrenergic nerves in adult animals using guanethidine. *Br. J. Pharmac.* **43,** 295–301.

CHIPPENDALE T. J., COTMAN C. W., KOZAR M. D. & LYNCH G. S. (1974) Analysis of acetylcholinesterase synthesis and transport in the rat hippocampus: recovery of acetylcholinesterase activity in the septum and hippocampus after administration of diisopropylfluorophosphate. *Brain Res.* **81,** 485–496.

CHUBB I. W. & SMITH A. D. (1974) Release of an isoenzyme of acetylcholinesterase from perfused adrenal gland. *J. Physiol., Lond.* **239,** 97–98P.

CHUBB I. W. & SMITH A. D. (1975*a*) Isoenzymes of soluble and membrane-bound acetylcholinesterase in bovine splanchnic nerve and adrenal medulla. *Proc. R. Soc. Lond. B.* **191,** 245–261.

CHUBB I. W. & SMITH A. D. (1975*b*) Release of acetylcholinesterase into the perfusate from the ox adrenal gland. *Proc. R. Soc. Lond. B.* **191,** 263–269.

COUPLAND R. E. (1965) In *The Natural History of the Chromaffin Cell.* pp. 1–279, Longmans, London.

CRONE H. D. & FREEMAN S. E. (1972) The acetylcholinesterase activity of the denervated rat diaphragm. *J. Neurochem.* **19,** 1207–1208.

DAVIS G. A. & AGRANOFF B. W. (1968) Metabolic behaviour of isoenzymes of acetylcholinesterase. *Nature, Lond.* **220,** 277–280.

ELLMAN G. L., COURTNEY K. K., ANDRES V. & FEATHERSTONE R. M. (1961) A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem. Pharmac.* **7,** 88–95.

ERÄNKÖ O. & HÄRKÖNEN M. (1964) Noradrenaline and acetylcholinesterase in sympathetic ganglion cells of the rat. *Acta. physiol. scand.* **61,** 299–300.

FILOGAMO G. & GABELLA G. (1966) Cholinesterase behaviour in denervated and re-innervated muscles. *Acta Anat.* **63,** 199–214.

FONNUM F. (1975) A rapid radiochemical method for the determination of choline acetyltransferase. *J. Neurochem.* **24,** 407–409.

GISIGER V., VENKOV L. & GAUTRON J. (1975) Acétylcholinesterase et biosynthèse des proteins du ganglion sympathique au repos et stimulé. *J. Neurochem.* **25,** 737–748.

GUTH L. (1968) "Trophic" influences of nerve on muscle. *Physiol. Rev.* **48,** 645–687.

KÁSA P. & CSERNOVSZKY E. (1967) Electron microscopic localization of acetylcholinesterase in the superior cervical ganglion of the rat. *Acta Histochem.* **28,** 274–285.

KÁSA P. & CSILLIK B. (1966) Electron microscopic localisation of cholinesterase by a copper-lead-thiocholine technique. *J. Neurochem.* **13,** 1345–1349.

KLINGMAN G. I. (1970) The distribution of acetylcholinesterase in sympathetic ganglia of immunosympathectomized rats. *J. Pharmac. exp. Ther.* **173,** 205–211.

KLINGMAN G. I. & KLINGMAN J. D. (1969) Cholinesterases of rat sympathetic ganglia after immunosympathectomy decentralization and axotomy. *J. Neurochem.* **16,** 261–268.

KOELLE G. B. (1963) Cytological distributions and physiological functions of cholinesterases. In: Cholinesterases and anticholinesterase agents. *Handb. exp. Pharmak. Suppl.* **15,** 187–298. (ed. KOELLE G. B.) Springer, Berlin.

KOELLE G. B. & FRIEDENWALD J. S. (1949) A histochemical method for localizing cholinesterase activity. *Proc. Soc. exp. Biol. Med.* **70,** 617–622.

KREUTZBERG G. W. & TOTH L. (1974) Dendritic secretion: a way for the neuron to communicate with the vasculature. *Naturwissenschaften* **61,** 37.

LENTZ T. L. (1974) Effect of brain extracts on cholinesterase activity of cultured skeletal muscle. *Expl. Neurol.* **45,** 520–526.

ROSE S. & GLOW P. H. (1967) Denervation effects on the presumed de novo synthesis of muscle cholinesterase and the effects of acetylcholine availability on retinal cholinesterase. *Expl. Neurol.* **18,** 267–275.

SALPETER M. M. (1967) Electron microscope radioautography as a quantitative tool in enzyme cytochemistry—I. The distribution of acetylcholinesterase at motor end plates of a vertebrate twitch muscle. *J. Cell Biol.* **32,** 379–389.

SAWYER C. H. & HOLLINSHEAD W. H. (1945) Cholinesterases in sympathetic fibres and ganglia. *J. Neurophysiol.* **8,** 137–153.

SOMOGYI P., CHUBB I. W. & SMITH A. D. (1975) A possible structural basis for the extracellular release of acetylcholinesterase. *Proc. R. Soc. Lond. B.* **191,** 271–283.

THOENEN H. (1972) Neuronally mediated enzyme induction in adrenergic neurons and adrenal chromaffin cells. *Biochem. Soc. Symp.* **36,** 3–15.

WILSON B. W., NIEBERG P. S., WALKER C. R., LINKHART T. A. & FRY D. M. (1973) Production and release of acetylcholinesterase by cultured chick embryo muscle. *Develop. Biol.* **33,** 285–299.

(*Accepted* 4 *June* 1976)