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Cholinesterase activity in the hypoglossal nucleus of the rat and the changes produced by axotomy: a light and electron microscopic study

B. A. FLUMERFELT* AND P. R. LEWIS

Departments of Anatomy and Physiology, University of Cambridge, and Department of Anatomy, University of Western Ontario, London, Canada

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INTRODUCTION

It is known that lower motor neurons whose axons innervate voluntary skeletal muscles have acetylcholine as their transmitter. Such neurons, for example those with their cell bodies in the ventral horns of the spinal cord and in certain cranial nuclei, are rich in acetylcholinesterase, the enzyme which hydrolyses the acetylcholine after its release at the myoneural junction. The hypoglossal nucleus is of special interest because it contains two groups of neurons with differing staining reactions for the cholinesterases. The majority of hypoglossal neurons stain only for acetylcholinesterase, but a small ventro-caudal cluster of cells, comprising about one sixth of the total, stain not only for acetylcholinesterase but also for another cholinesterase. This latter enzyme hydrolyses acetylthiocholine and is inhibited by ethopropazine, but its most characteristic property is rapid hydrolysis of butyrylthiocholine; BuChE would thus seem an appropriate abbreviation to distinguish it from true cholinesterase (AChE), the enzyme typically present in motor neurons (Lewis, Flumerfelt & Shute, 1971). The hypoglossal nucleus in the rat, therefore, provides an opportunity for comparing the distribution of these two cholinesterases in a single population of neurons.

The hypoglossal nucleus is also an excellent site at which to study chromatolytic changes following axotomy, in particular changes in cholinesterase activity. Several investigators, using the light microscope, have observed a marked fall in the intensity of cholinesterase staining of motor neurons following interruption of their axons (Schwarzacher, 1958; Filogamo & Candiollo, 1962; Söderholm, 1965; Navaratnam, Lewis & Shute, 1968; Lewis *et al.* 1971). The decrease in cholinesterase activity following axotomy has not been satisfactorily explained, however, and has yet to be investigated in detail with the electron microscope. It was therefore decided to study the relative responses of AChE and BuChE activity to axotomy by light and electron microscopy. It was hoped that such a study might throw light on the changes in enzyme activity following axotomy, and on the reason for the existence of BuChE as well as AChE in some neurons.

* Present address: Department of Anatomy, Health Sciences Centre, University of Western Ontario, London, Canada.

MATERIALS AND METHODS

Mature albino rats, mostly males weighing 300–400 g, were anaesthetized with chloral hydrate and the hypoglossal nerve (on the right side in the majority of experiments) transected before its bifurcation at a point deep to the posterior belly of the digastric muscle. In order to reduce the likelihood of rapid regeneration, the proximal stump was folded back on itself and in a few experiments a centimetre or more of the nerve trunk was removed completely. For the present study 23 animals were prepared for light microscopy; 10 covering the period up to one month after axotomy and 8 covering the further period up to and including 300 days. (Additional material from a study of topographical localization (Lewis *et al.* 1971) was also available.) For the electron microscopic study 9 animals were used to cover the period up to one month and 5 animals for longer periods up to just over a year. Again, some material from other studies was also available.

All animals for light microscopy were perfused under ether with 20% formalin-saline. The brains were dissected out and immersed in 10% neutral formalin at 4 °C for a further 4–6 hours and stored overnight in water at 4 °C. Serial frozen sections, 25–100 μm in thickness, were cut through the medulla oblongata and stained for cholinesterase activity by a modified thiocholine technique (Lewis, 1961). Incubation was carried out at room temperature in media containing 6 mM substrate, 9 mM copper sulphate, 16 mM glycine and 50 mM acetate buffer at a pH of 5·0. Acetylthiocholine iodide was employed as substrate to demonstrate AChE activity with 10^{-4} M ethopropazine hydrochloride added to inhibit BuChE activity. Butyrylthiocholine was used as substrate for the demonstration of BuChE activity. After incubation for 2–4 hours (AChE) or 4–7 hours (BuChE) the sections were well washed in water, treated with sodium sulphide and mounted in Canada balsam on glass slides. Four animals were kept as long as practicable, for periods of 12–22 months; every third section was stained for acid phosphatase, AChE or BuChE and complete cell counts were made for the hypoglossal nucleus on each side (Lewis *et al.* 1971).

Animals for electron microscopy were perfused with 5% glutaraldehyde in cacodylate buffer (pH 6·5). Following removal of the brain and a further period in fixative, thin slices of tissue were cut through the region of the hypoglossal nucleus and stained for cholinesterase activity (Lewis & Shute, 1966, 1969). Incubation was carried out at 4 °C in media containing 13 mM substrate (acetyl- or butyrylthiocholine), 18 mM copper sulphate, 33 mM glycine and 25 mM succinate buffer at a pH of 5·3 for 2–4 hours (AChE) or 4–6 hours (BuChE). After incubation the tissue slice was developed in sodium sulphide, trimmed, post-fixed in Dalton's chrome-osmium and embedded in Araldite. All the electron micrographs illustrated are from sections counterstained with lead citrate alone.

RESULTS

Cholinesterase distribution in the normal nucleus

In material prepared for the demonstration of AChE by light microscopy the hypoglossal nucleus was sharply delineated by the enzyme staining (Fig. 1*a*). The caudal part of the nucleus consisted of two well-defined groups of cells, one dorsal

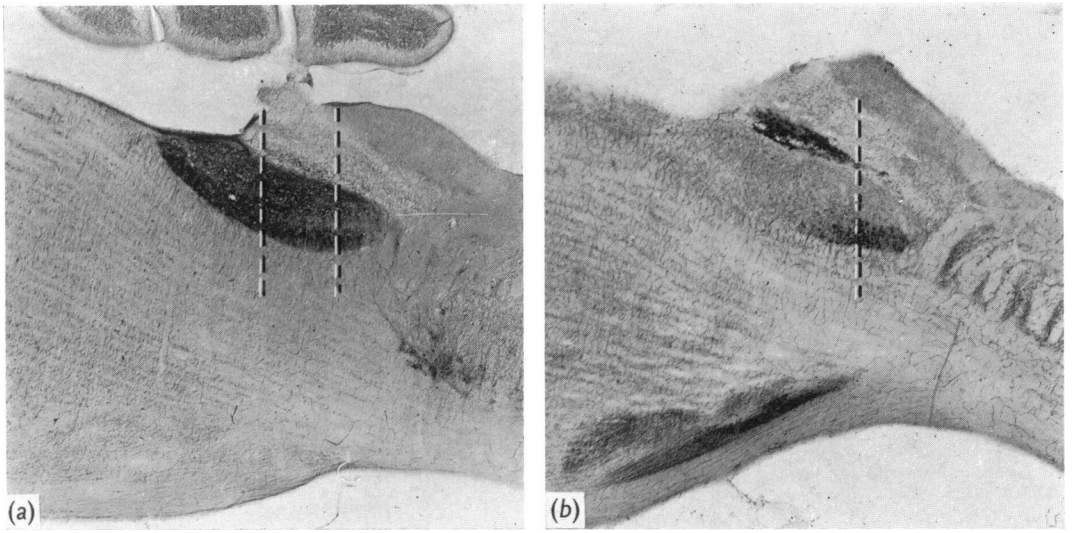

Fig. 1 (*a*). A parasagittal section through the medulla oblongata showing intense AChE activity throughout the entire hypoglossal nucleus which extends caudally from the floor of the fourth ventricle. The vertical lines indicate the levels which are shown in the transverse plane in following light photomicrographs. Fig. 1 (*b*). An adjacent section showing intracellular staining for BuChE in the ventro-caudal part of the nucleus. Comparison with (*a*) reveals that these cells form a distinct cluster and possess both AChE and BuChE activity. ×15.

and the other ventral, with a small ventro-lateral sub-group (Lewis *et al.* 1971). More rostrally, the distinction between the two main groups became less obvious and the ventro-lateral subgroup disappeared. The caudal cells in the ventral group all showed staining for BuChE as well as for AChE (Fig. 1*b*).

With the electron microscope, staining for AChE was seen within the cisternae of the granular endoplasmic reticulum (E.R.) and the intermembranous space of the nuclear envelope (Fig. 2). Between the areas of high content in the cisternae were seen shorter zones in which the staining was lighter and discontinuous. In the nuclear envelope, areas occurred which were completely devoid of stain, but all of the E.R. showed at least a small amount of reaction product. The ribosomes and polysomes associated with the bilaminar sheets did not show AChE activity at any time. Numerous short lengths of E.R. throughout the cytoplasm also became evident when the tissue was stained for AChE. When cut transversely they appeared as either short, darkly stained, rod-shaped bodies or as circular profiles. Pores in the nuclear envelope appeared as discrete gaps in staining when viewed in transverse section or as round clear spaces on a dark field in surface view. Within the Golgi complex reaction product was often present in the saccules (Fig. 2). This staining was frequently most abundant within the dilated ends of the saccules, although in some cases reaction product occurred intermittently throughout the entire cistern.

Staining within the dendrites was similar to that in the cell body. In the wide initial portion of dendrites, stained Nissl bodies and short segments of granular and agranular E.R. were common. Reaction product was also frequently present along

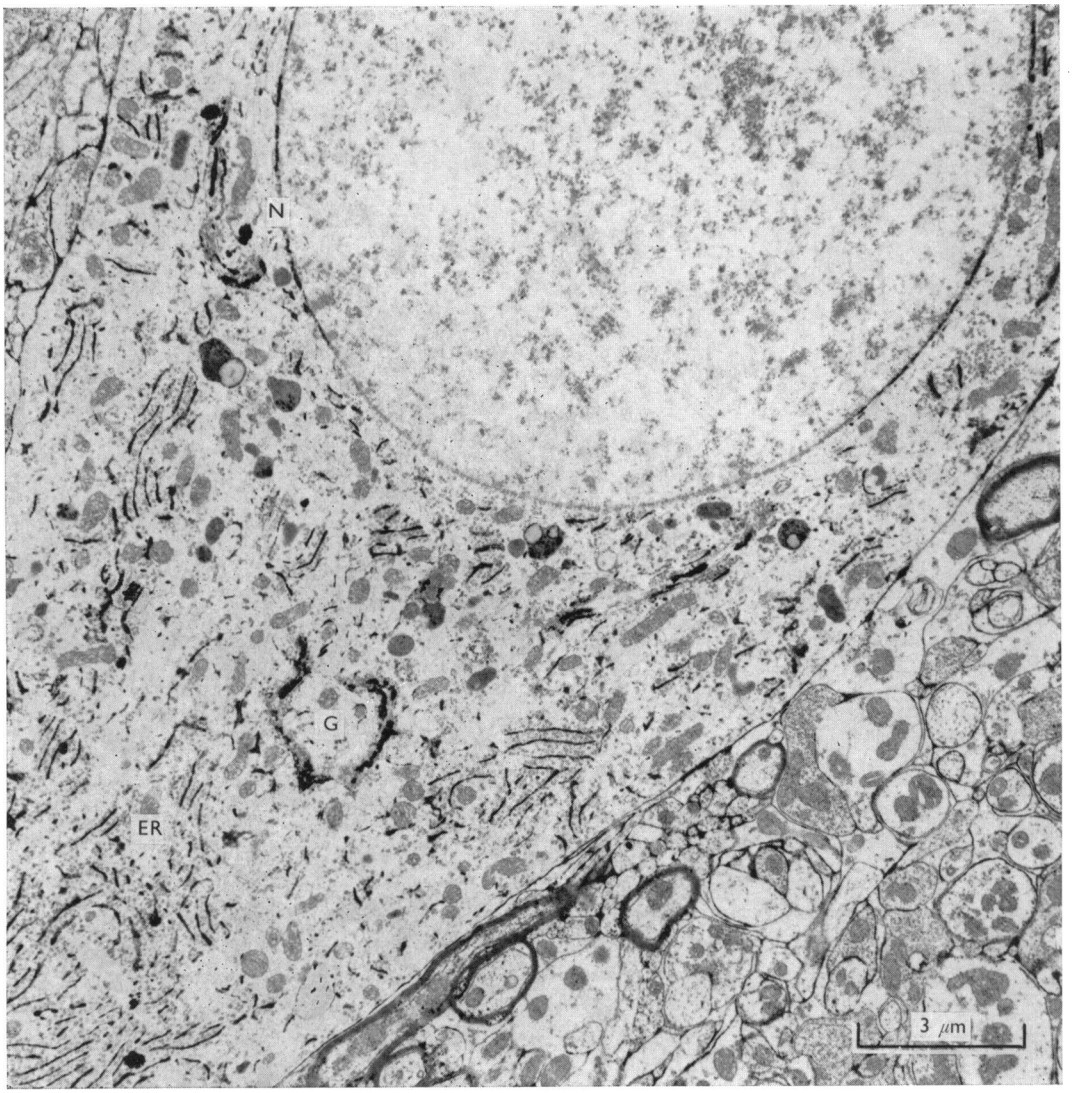

Fig. 2. An electron micrograph of a typical hypoglossal neuron stained for AChE activity. The reaction product occurs primarily within the E.R. cisternae (E.R.), the intermembranous space of the nuclear envelope (N) and the saccules of the Golgi complexes (G). Activity is also present along the membranes of many profiles within the neuropil.

the dendritic surface. In the neuropil, stained clusters of terminal axonal branches were frequently observed, usually consisting of several, small, circular profiles in close apposition, many with the limiting membrane covered with end product. Occasionally, synaptic terminals throughout the neuropil and along the surfaces of the hypoglossal neurons were heavily stained. Reaction product filled the synaptic cleft and extended around the membrane of the terminal bouton. With axo-dendritic synapses staining was frequently present on the dendritic membrane in the general

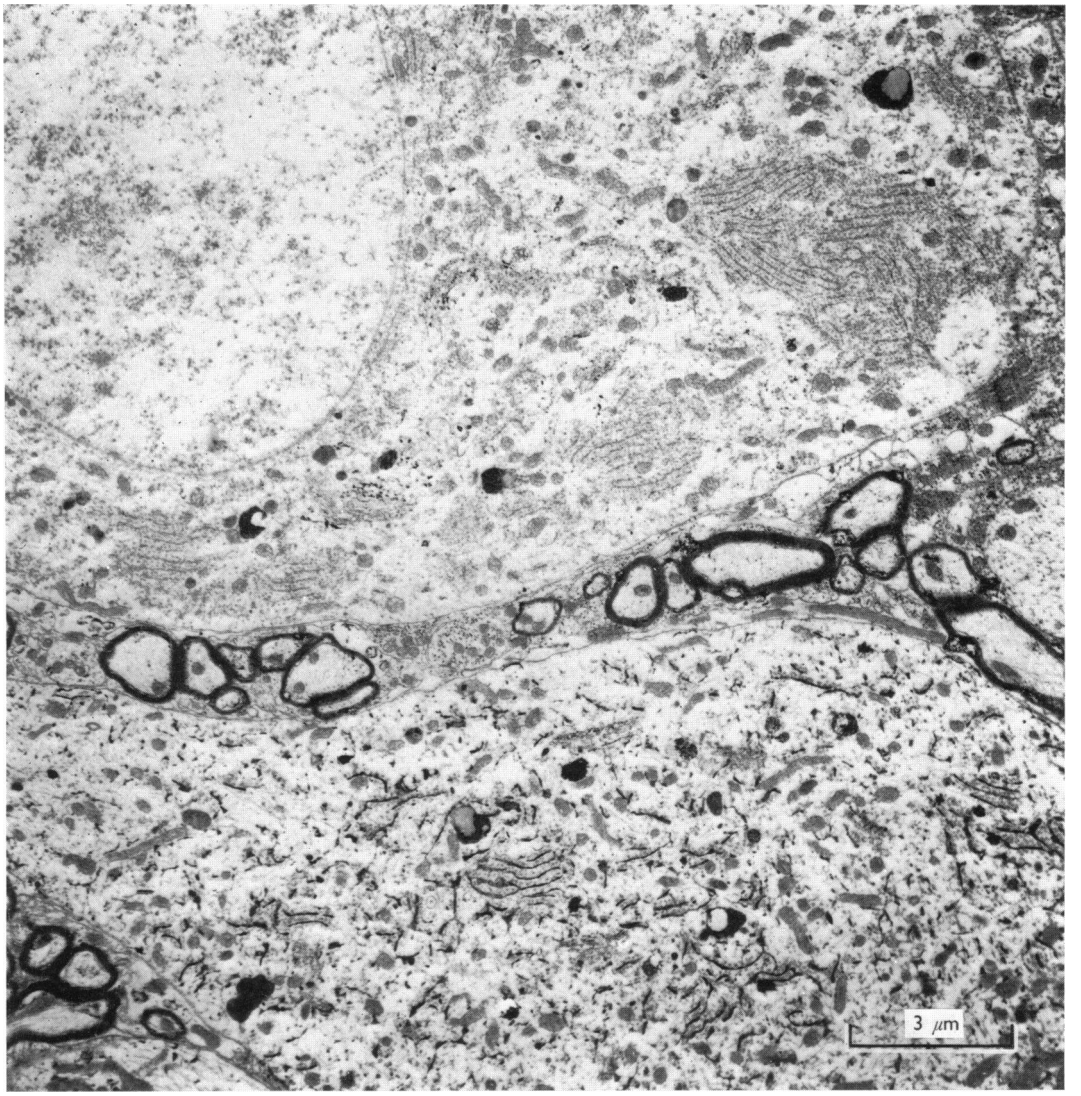

Fig. 3. An electron micrograph showing two cells at the periphery of the ventro-caudal cluster stained for BuChE activity. The neuron at the bottom of the field lies within the cluster and contains abundant reaction product for BuChE throughout its E.R. cisternae. The neuron at the top of the field is typical of the cells outside of the cluster and is devoid of BuChE activity.

area of the synapse. No reaction product was associated with the synaptic vesicles, mitochondria, or other elements within the terminal. Synaptic terminals with no reaction product were also present and frequently occurred in the same general area as well-stained terminals. Moreover, both stained and unstained synapses were often present along the surface of a single neuron.

When the caudal part of the nucleus was stained for BuChE, however, striking differences between the dorsal and ventral cells became apparent. Fig. 3 is an electron

micrograph taken at the periphery of the ventro-caudal cluster, showing both cell types incubated for BuChE within one field. The neurons in the ventro-caudal cluster contained large amounts of reaction product which were distributed in a manner similar to that described for AChE. There were, in fact, no apparent differences between the distribution of the two enzymes within these neurons (compare Figs. 2 and 3). The neurons from outside the cluster, on the other hand, were completely devoid of reaction product for BuChE. Although the intracellular distribution of the two enzymes within the ventro-caudal cells was identical, closer examination revealed marked differences in their extracellular distribution. Reaction product for BuChE was almost completely absent from the neuropil, i.e. no staining was present in association with axonal membranes or axo-dendritic synapses. Similarly, no BuChE was observed in the presynaptic component of axo-somatic synapses.

Changes during the first month after axotomy

Although the degrees of response to axotomy seen in different cells may vary widely in any one animal, a clearly defined range was observable at each stage. The overall trend was particularly obvious from the material prepared for light microscopy, the hypoglossal nuclei being so close to the midline that control and operated sides could be compared directly. The descriptions of the electron microscopic appearances which follow were representative of the majority of neurons examined at each stage.

No obvious changes in activity of either enzyme were seen with the light microscope one day after axotomy, but slight changes were seen in a few cells with the electron microscope, namely irregularities in the outline of the nuclear envelope (Fig. 4*a*), fewer typical Nissl bodies and more whorls and short lengths of granular E.R. (Figs. 4*b*, 4*c*). These changes were most common in the BuChE-containing cluster of cells. By the third day many cells exhibited a distinct layer of granular E.R. underlying the plasmalemma, with somewhat fragmented and swollen E.R. situated more centrally (Fig. 5*a*). There was some loss of AChE activity and a somewhat greater loss of BuChE activity, even from areas of organized E.R. (Fig. 5*b*). By the fifth day the loss of neuronal AChE had become much greater and the loss of BuChE virtually complete (Fig. 6).

At the end of the first week following axotomy the majority of the neurons exhibited chromatolysis. The Nissl substance that remained in these cells was situated in and around the frequent nuclear invaginations (Fig. 8*a*) and subjacent to the plasmalemma where it formed elongate granular masses (Fig. 8*b*). The staining often occurred in short lengths throughout these areas, outlining the fragmented nature of the E.R. cisternae in which it appeared. Many cisternae were poorly stained or were completely devoid of reaction product. The overall decrease in staining intensity seen with the light microscope was thus mainly due to two concurrent processes: a dissolution and peripheral migration of the E.R. (which is the chief site of cholinesterase in the normal cell) and an accompanying decrease in the cholinesterase content of the E.R. which remained. Staining within the Golgi complex and nuclear envelope was also noticeably reduced by this stage, although the Golgi element appeared to be mildly hypertrophic.

During the second week following axotomy the intracellular level of AChE continued to diminish and was accompanied by a decrease in AChE within the neuropil.

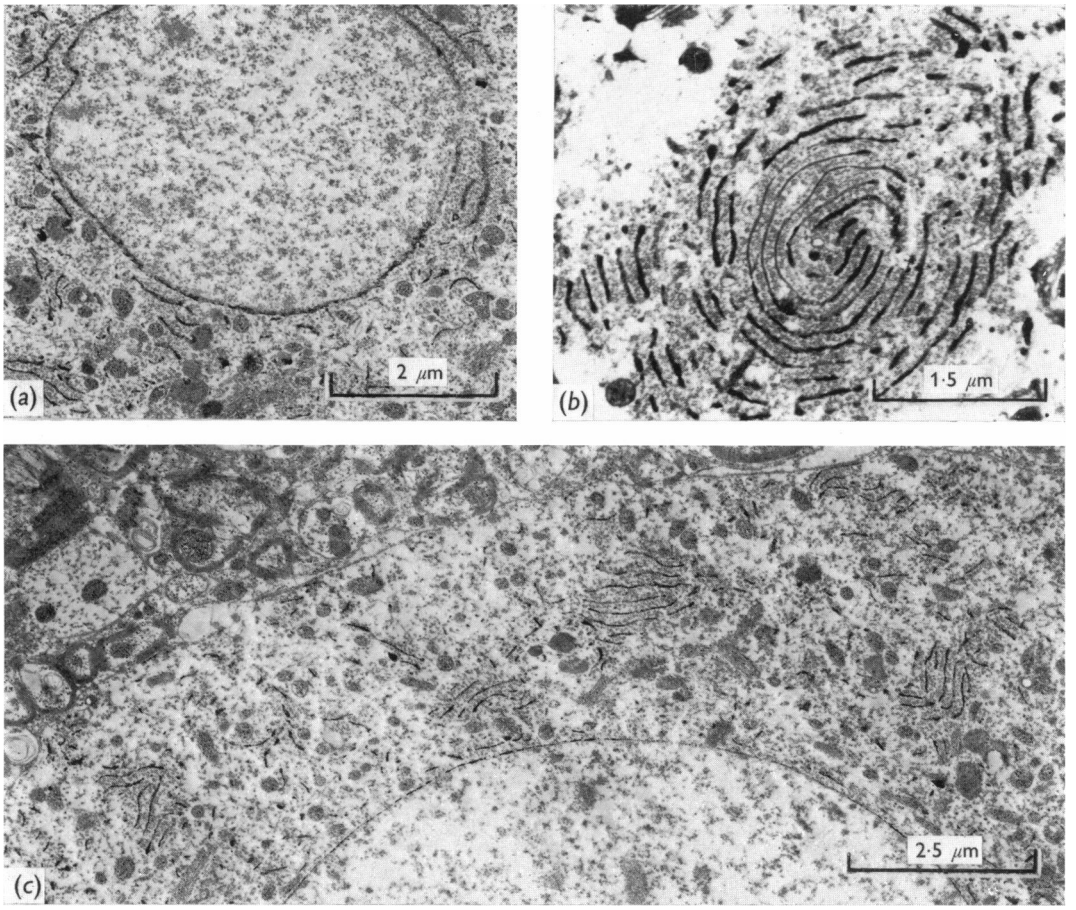

Fig. 4. 1 day. Examples of the earliest signs of reaction to axotomy showing: (*a*) BuChE. An irregular nuclear envelope with a small invagination. (*b*) AChE. A whorl of E.R. which consists of closely packed, concentric cisternae producing a lamellar appearance. (*c*) BuChE. A hypoglossal neuron in which the Nissl bodies are smaller and short lengths of E.R. are more numerous than in normal tissue.

Thus, by the end of the second week both the intracellular and extracellular AChE were considerably reduced in amount. By the end of the second week the staining within the deep cytoplasm was often restricted to very short lengths of scattered granular E.R. (Fig. 9*a*) and, when intact Nissl substance was present, many of the cisternae were unstained (Fig. 9*b*). Invaginations of the nuclear envelope which contained unstained granular masses were more common at this stage. Dissolution and degranulation of the E.R. lamellae, dispersion of the rosettes to form single granules and an increase in the number of neurofilaments together gave the neuroplasm a mixed fibrous and granular appearance.

During the third week the chromatolytic response of the cells was more marked than at any other stage. Following this peak reaction the staining for AChE began to return and increase in some of the cells. Thus, on the seventeenth day, a distinct

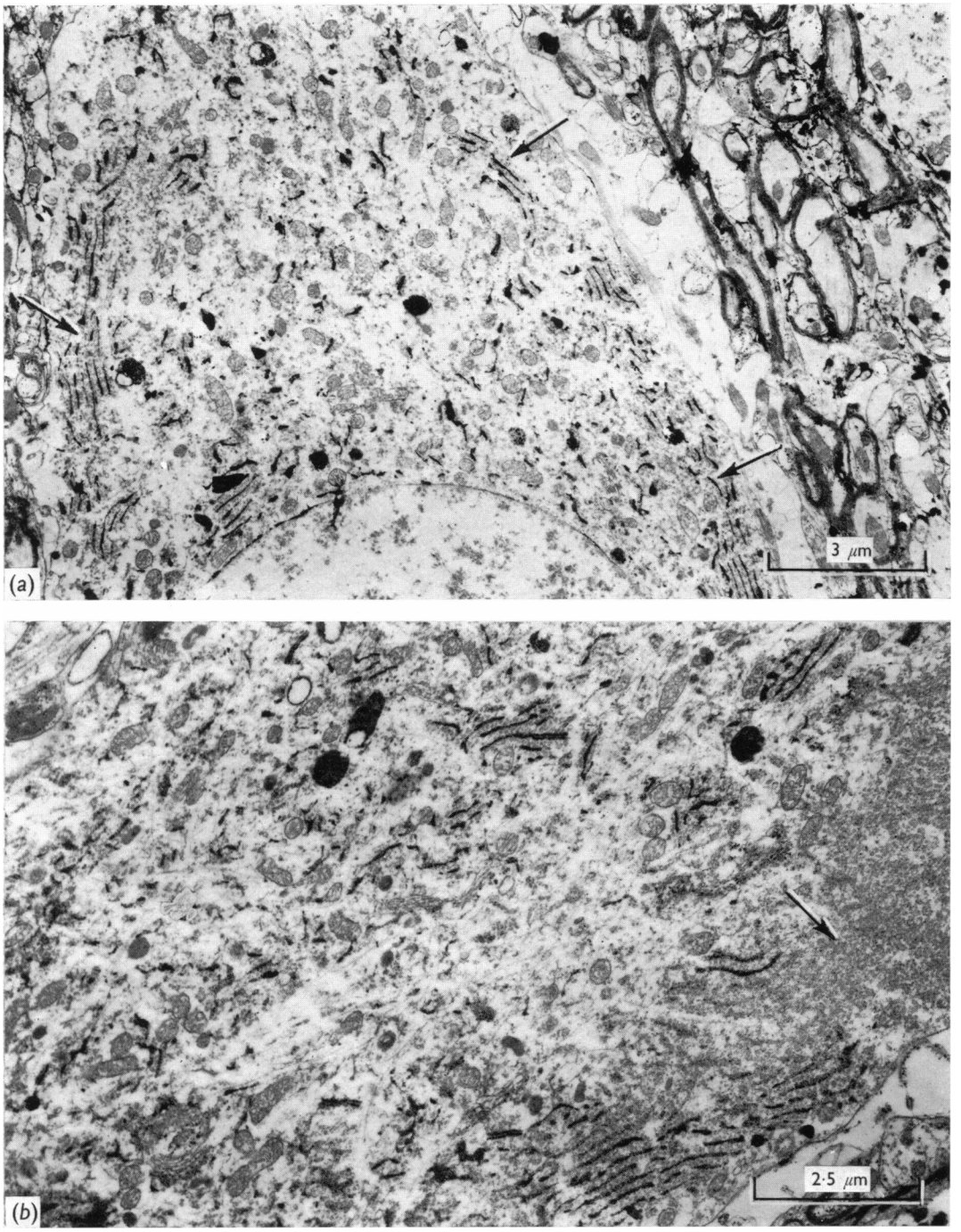

Fig. 5. (*a*) AChE, 3 days. A rostrally situated neuron in which a distinct layer of well stained E.R. underlies the plasmalemma (arrows) whereas the centrally situated E.R. is fragmented and occurs as short, stained lengths. (*b*) BuChE, 3 days. A ventro-caudal neuron containing a peripheral band of E.R. in which the staining for BuChE has been partly lost (arrow).

return of AChE was observed in the cells of the ventro-caudal cluster, whereas at this stage those in the remainder of the nucleus did not show a return of AChE and extracellular reaction product had not increased in any location (Figs. 7*a*, 7*b*). No increase in the BuChE level was observed in the cells which showed a return of AChE (Fig. 7*c*). Staining for AChE within the cells in the rostral part of the nucleus was limited to short scattered segments of E.R. (Fig. 10*a*) and in a few cells the reaction product was absent (Fig. 10*b*). Golgi saccules and nuclear envelopes contained little end product, and unstained fragments of E.R. were common. The membranous component of the E.R. was much reduced in extent, leaving many single ribosomes scattered throughout the cytoplasm. The remaining segments of E.R. were somewhat dilated and a few appeared to have undergone degranulation. By this time the E.R. underlying the plasmalemma had also undergone dissolution and the entire cell presented a homogeneous appearance. Fragments of E.R. in the ventro-caudal cells were well stained when incubated for AChE activity. The return of AChE activity at this stage therefore appeared to be due to a greater utilization of the remaining cisternae of E.R., rather than to the development of new cisternae.

The material prepared for light microscopy was used to assess the progressive changes in enzyme activity within the whole nucleus. The intensity of staining on the experimental and control sides was compared and that on the experimental side assessed in arbitrary units (taking the control side as equal to 20 units). The results are plotted in Fig. 11. The graph emphasizes the slow initial fall in extracellular compared with intracellular AChE during the first week and the return of AChE to the cells of the ventro-caudal cluster during the third week.

During the fourth week there was a marked return of AChE to cells in the rest of the nucleus; however, extracellular AChE and intracellular BuChE remained at a low level (Fig. 12). In spite of the return of intracellular AChE being so obvious with the light microscope (Fig. 12*a*) the cells still looked chromatolytic with the electron microscope. Rough E.R. was more evenly distributed within the cytoplasm than previously, but it consisted of short lengths which were well dispersed and not aggregated into Nissl bodies. The short cisternae stained strongly for AChE, giving an overall fragmented appearance to the cytoplasm (Fig. 14). The ventro-caudal cells were similar in appearance to those elsewhere, but sites of BuChE activity within them remained sparse.

Later changes in the response to axotomy

By the end of the sixth week the level of intracellular staining for AChE had returned almost to normal in most parts of the nucleus, although recovery was less complete in the ventro-caudal area. The intensity of extracellular staining increased rapidly during this period but was still well below that on the control side. In all of the long-term animals differences between the experimental and control sides could be seen, especially in extracellular staining (Fig. 11). Accurate assessment of the degree of recovery was complicated by a decrease in the overall size of the nucleus (Figs. 16, 17). This decrease in size is discussed in more detail below. The return of BuChE staining was very erratic. In most animals the staining returned in only a small proportion of the cells in the ventro-caudal cluster and in these cells enzyme activity remained well below the control level. In one or two animals a rather greater, although still incomplete, return of BuChE was seen (Fig. 13).

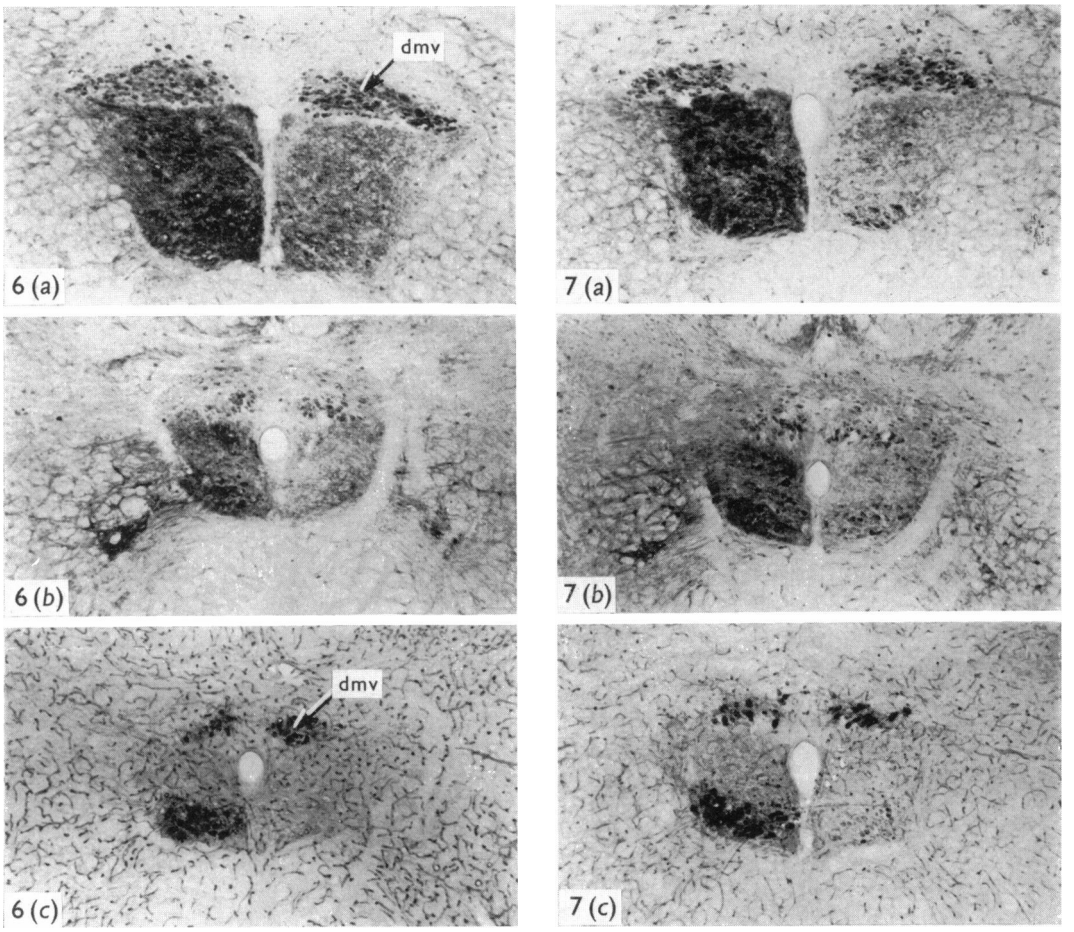

Fig. 6. 5 days. Light microscopic appearance: (*a*) Rostral, AChE. Showing a marked intraneuronal loss of activity and a moderately severe loss in the neuropil. (*b*) Caudal, AChE. Showing a general loss of staining at this level. (*c*) Caudal, BuChE. Showing a dramatic loss of intracellular staining. Note the dorsal motor nucleus of the vagus (dmv) which remains unaffected. × 30.

Fig. 7. 17 days. Light microscopic appearance: (*a*) Rostral, AChE. Showing a level just far enough caudal to include the rostral pole of the ventro-caudal cell group. Note the marked return of AChE activity to the cells within the cluster while those outside it show no return. (*b*) Caudal, AChE. Showing a complete loss of extracellular staining and, as yet, no return of intracellular staining in the dorsal part of the nucleus. (*c*) Caudal, BuChE. Showing a continued absence of intracellular staining. × 30.

In material processed for electron microscopy Nissl bodies were seen within the cytoplasm before the end of the second month, with many cells exhibiting a band of E.R. around the nuclear envelope (Fig. 15*a*). The E.R. in such cells often stained intensely for AChE. Invaginations into the nucleus were still occasionally present, but they were much reduced in extent as compared to the earlier stages. Within the ventro-caudal cells the BuChE level had increased but it was still noticeably less than

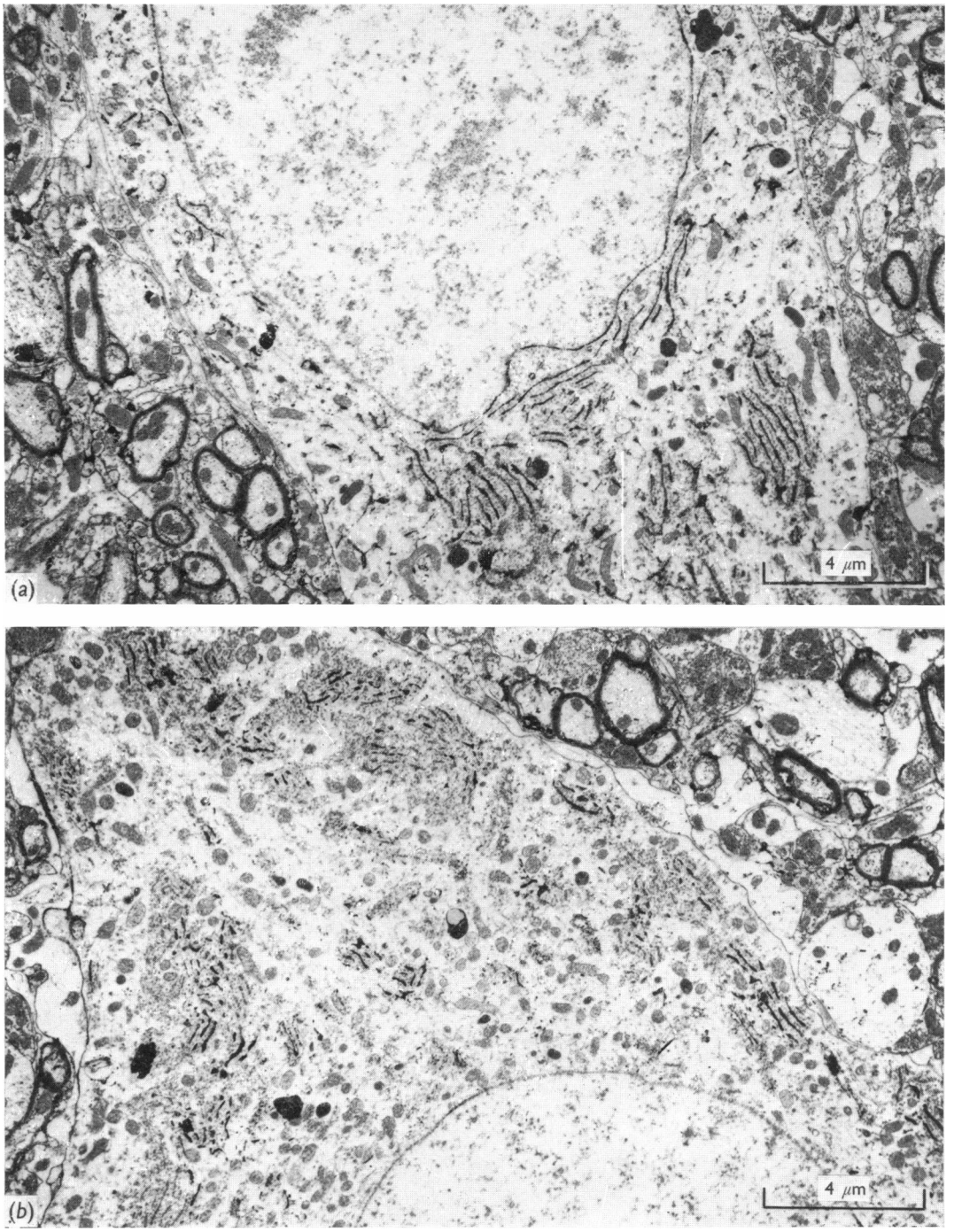

Fig. 8. 7 days, AChE. Two examples of enzyme distribution in chromatolytic neurons: (*a*) showing Nissl substance closely associated with a nuclear invagination, and (*b*) showing a peripheral band of E.R. consisting of elongate granular masses in which the staining is discontinuous.

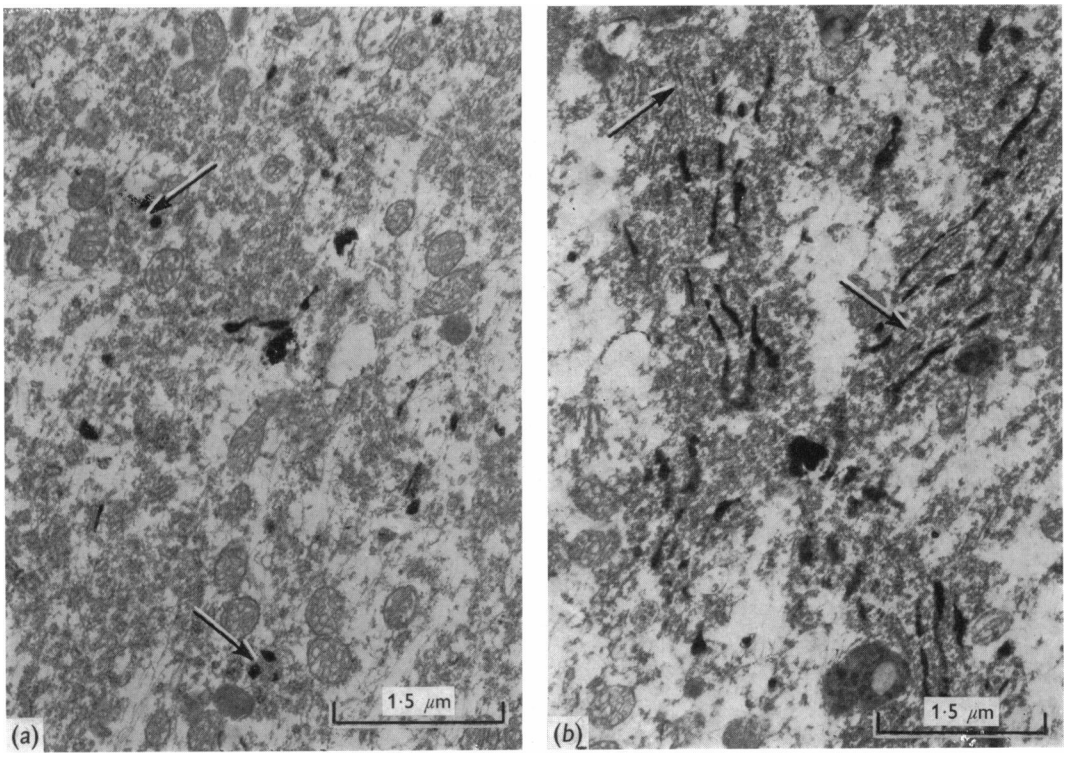

Fig. 9. 13 days, AChE. (*a*) An area from a chromatolytic neuron in which the staining is restricted to very short fragments of scattered E.R. (arrows). (*b*) An aggregate of intact Nissl substance within a chromatolytic neuron. Note the empty appearance of many cisternae (arrows) in which there is no reaction product.

that for AChE (Figs. 15*b*, 16). By the fourth month most of the cells contained well-stained Nissl bodies and Golgi complexes. The lamellae of E.R. were still somewhat fragmented in places, but well developed Nissl substance predominated. When the BuChE-containing cells were stained for AChE the reaction product was distributed in a manner similar to that in the other cells. When they were treated for BuChE, however, the Nissl substance was not well stained. The morphology of the ventro-caudal cells also differed from those more rostrally situated in that many of them possessed extremely irregular nuclear envelopes with numerous infoldings and abundant associated E.R. The intensity of staining for AChE continued to increase until, by six months, it approached normal levels throughout the nucleus. Many of the cells contained well-stained Nissl bodies and the chromatolytic changes seen at earlier stages were no longer evident. Within some of the cells signs of ageing, such as membranous arrays and numerous lipofuscin granules, were evident in the cytoplasm.

In animals kept for longer periods the appearance was either similar to that seen at six months or else showed signs of deterioration. A decrease in extracellular staining was particularly noticeable, but staining generally was somewhat reduced from

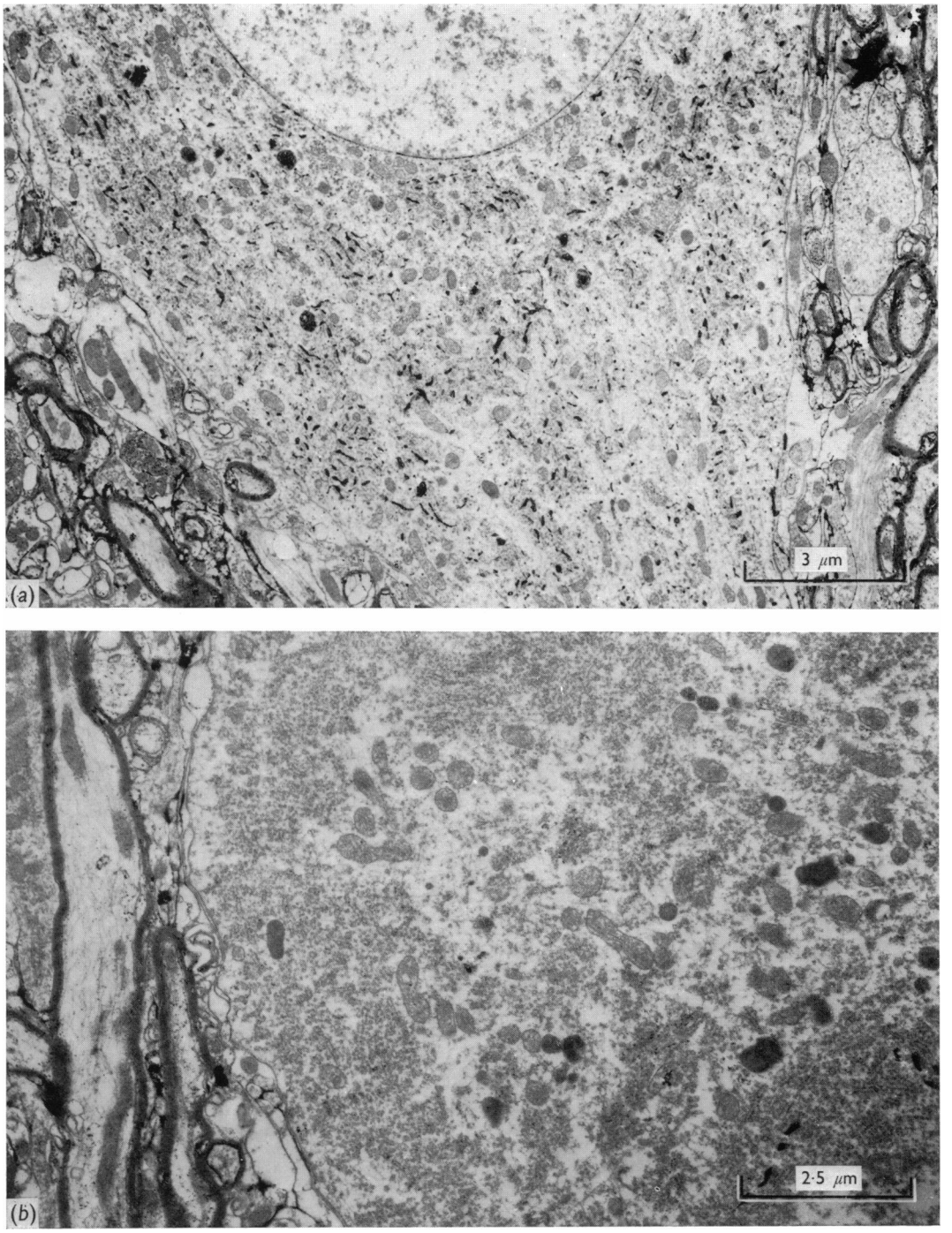

Fig. 10. 19 days, AChE. (*a*) A chromatolytic neuron in which staining is limited to short, scattered segments of E.R. Note the paucity of staining in association with the nuclear envelope and plasmalemma. (*b*) An area from a chromatolytic neuron in which staining is almost totally absent. Note the presence of numerous short lengths of E.R. which are completely devoid of reaction product. Large clusters of granules are abundant throughout the cytoplasm.

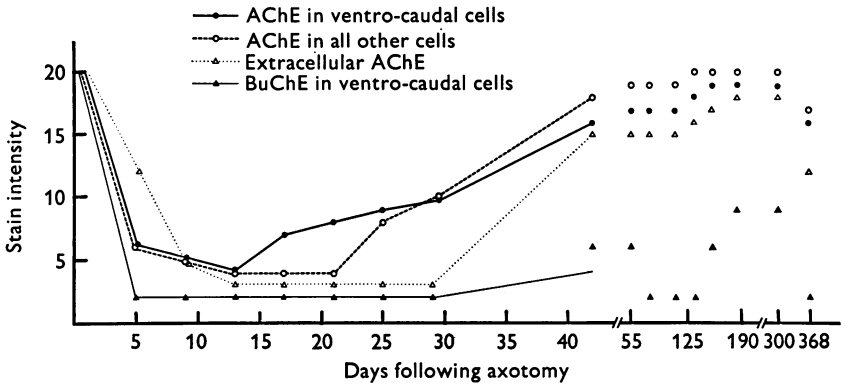

Fig. 11. A graphical representation of the progressive changes in staining intensity for AChE and BuChE at various stages following axotomy. Each point was obtained by comparing control and experimental sides, both under the microscope and on large scale prints.

earlier stages. A consistent feature was the reduced size of the nucleus on the operated side (Fig. 17). With the electron microscope well-stained Nissl bodies were observed within most of the cells in the rostral part of the nucleus (Fig. 18*a*). A few cells still showed subtle chromatolytic changes, and many of the cells were noticeably reduced in size. Although some of the neurons in the ventro-caudal cluster possessed normal Nissl bodies, many of them appeared more chromatolytic than those observed elsewhere in the nucleus (Fig. 18*b*) in that the nuclear envelope was markedly involuted and there were no well-developed Nissl bodies. Within the neuropil, large, dense areas of disorganized myelin were prominent. The profusion of membranes which normally constitutes the neuropil was much less in evidence and the extracellular reaction product was correspondingly reduced in amount.

Cell death was investigated in five animals. In one kept for eleven months a cell count on serial paraffin sections showed a 24 % loss of neurons on the operated side. Serial frozen sections were prepared from the other four animals with survival times between 16 and 22 months. Very obvious cell losses had occurred, as judged from counts made on sections stained for acid phosphatase (in which the neurons stand out very clearly): the total number of neurons on the operated side ranged from 73 % to 48 % of the number on the control side. In the ventro-caudal cluster the neurons with sufficient BuChE activity for them to be countable numbered only between 15 % and 61 % of the neurons that stained for BuChE on the control side.

DISCUSSION

Throughout the chromatolytic cycle in hypoglossal neurons the two most distinctive morphological changes were the dissolution of the granular E.R. and the invagination of the nuclear envelope. Both these chromatolytic changes have been known for many years from light microscopic studies (Lindsay & Barr, 1955; Lieberman, 1971). It was the dissolution of the Nissl substance which led to the term 'chromatolysis' and the nuclear invaginations were termed 'nuclear caps' because of the basophilia of the Nissl substance which frequently lay within them.

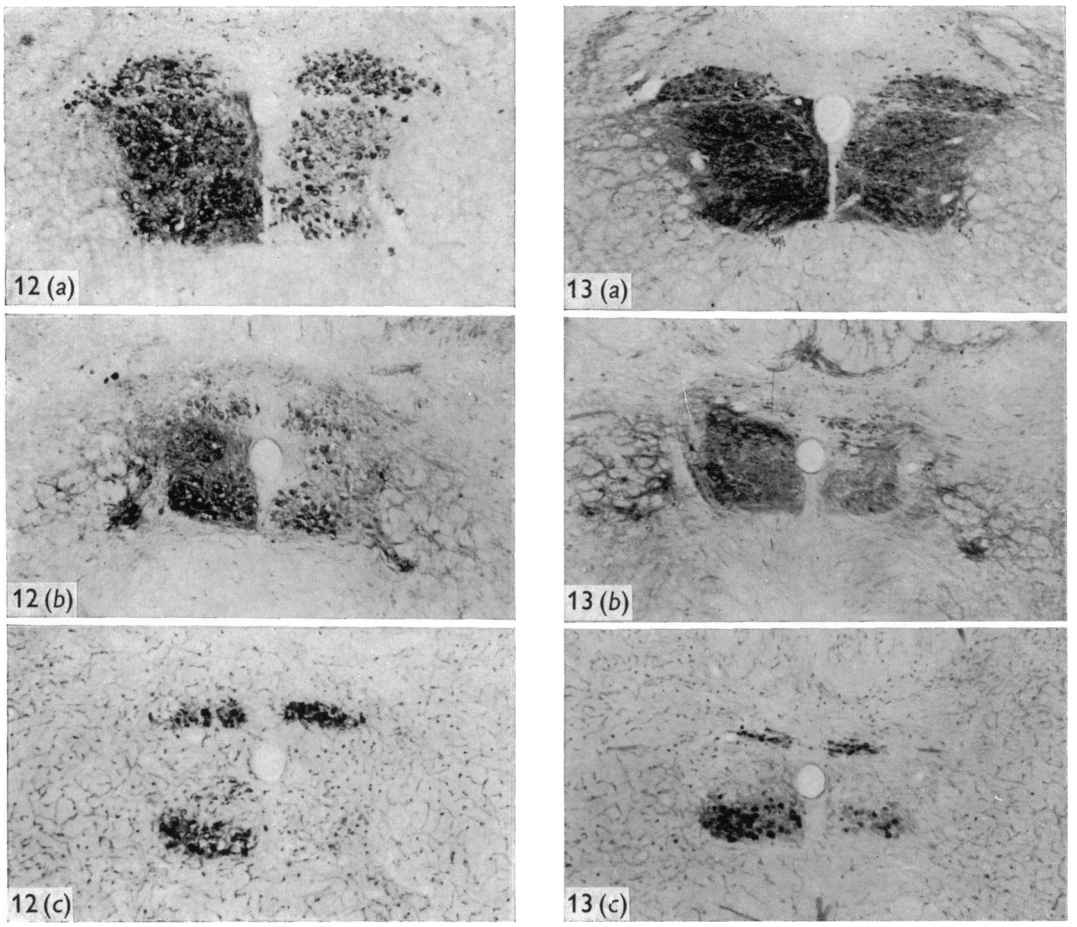

Fig. 12. 28 days. Light microscopic appearance: (*a*) Rostral, AChE. Showing a marked return of AChE activity within the cells, while the extracellular activity remains at a very low level. Note the slight reduction in the size of the nucleus on the operated side. (*b*) Caudal, AChE. Showing a similar pattern for AChE activity in the ventro-caudal cells. (*c*) Caudal, BuChE. Showing an absence of staining for BuChE in the ventro-caudal cells. × 30.

Fig. 13. 42 days. Light microscopic appearance: (*a*) Rostral, AChE. Showing a marked return of both intracellular and extracellular AChE activity. (*b*) Caudal, AChE. Showing some return of AChE activity on the operated side. (*c*) Caudal, BuChE. Showing a small number of ventro-caudal cells in which a moderate amount of BuChE activity has returned. × 30.

In the present study the changes in the granular E.R. were first seen as slight dilatations of the cisternae. This was usually followed by fragmentation and partial degranulation, to give short lengths of granular and agranular E.R. scattered throughout the cytoplasm. Takano (1964) and Kirkpatrick (1968) also observed dilatation the cisternae in chromatolytic neurons and Barron, Doolin & Oldershaw (1967) found both dilatation and degranulation of the E.R. In some cases the Nissl substance formed whorls before undergoing dissolution. The E.R. is evidently a rather sensitive indicator of cell injury, and the changes observed in the E.R. of neurons in this study

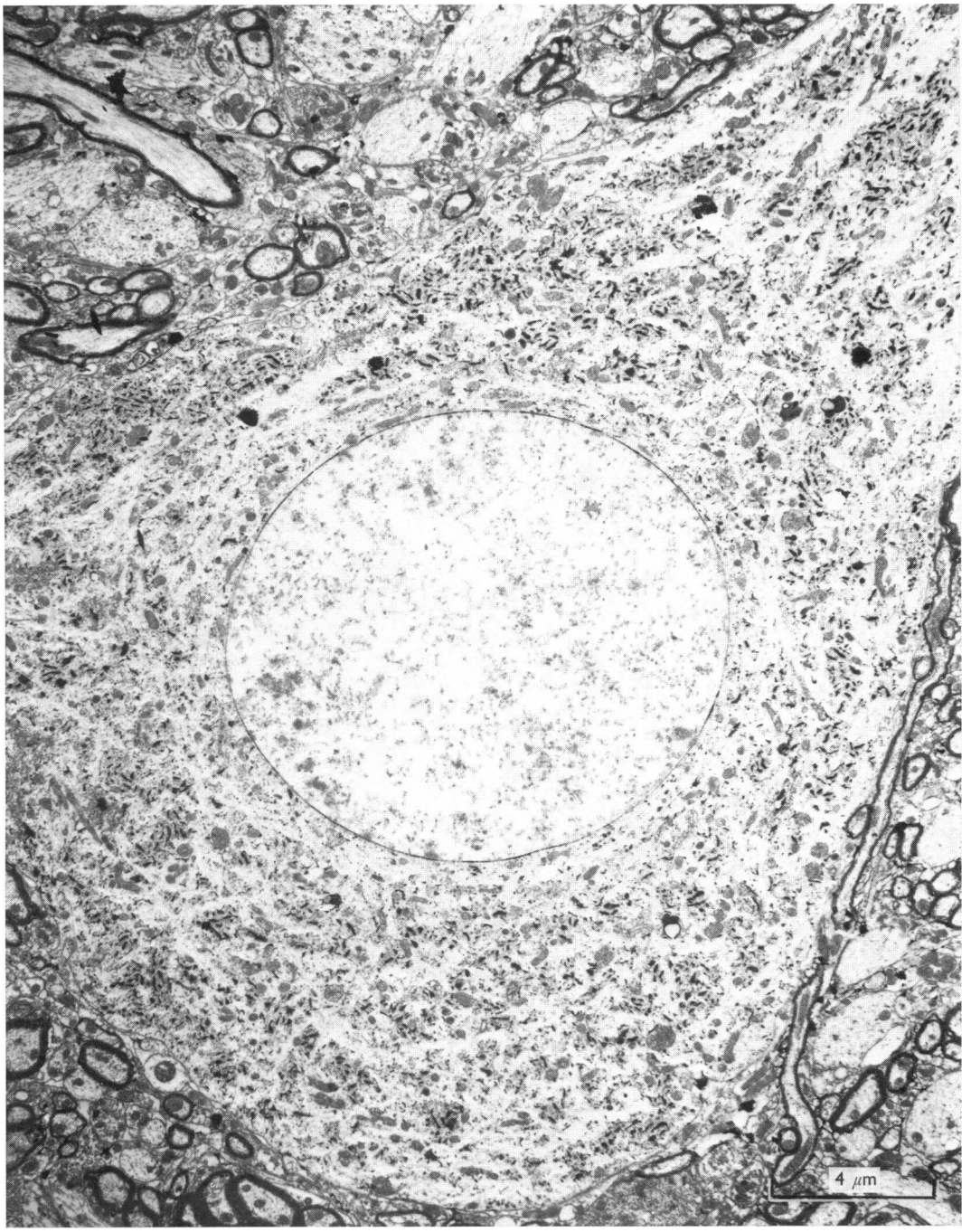

Fig. 14. 28 days, AChE. A rostral neuron in the recovery phase with numerous, short lengths of well stained E.R. dispersed throughout the cytoplasm.

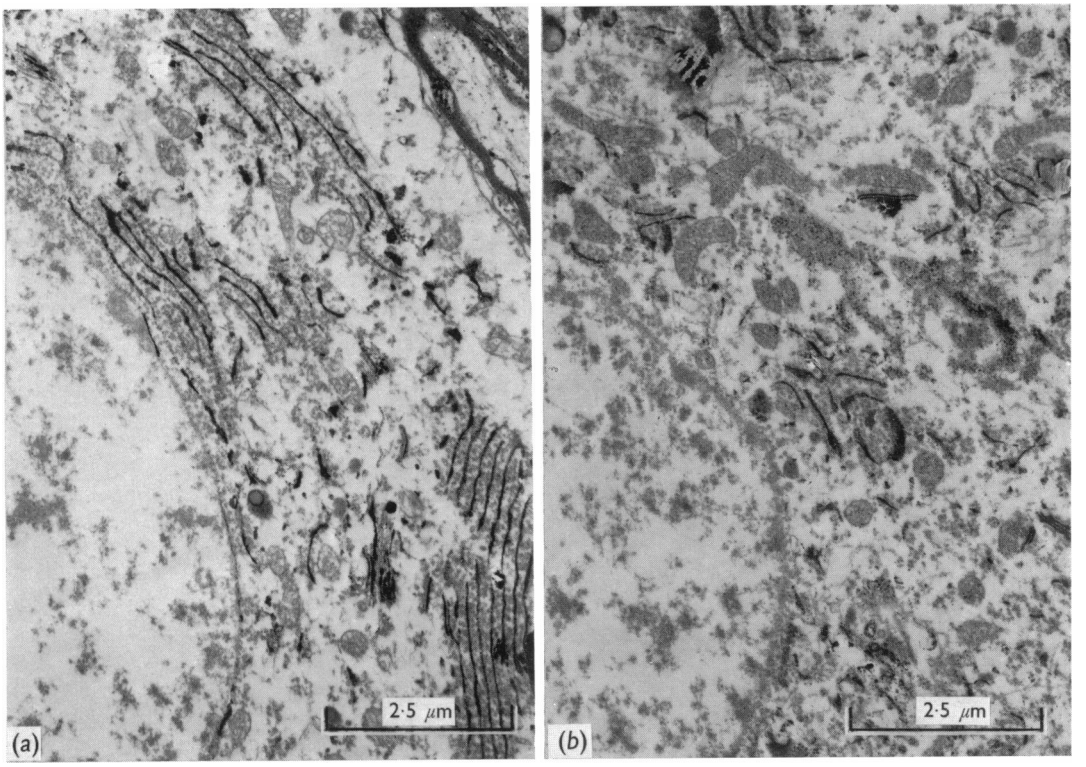

Fig. 15. 56 days. (*a*) AChE in a rostrally situated neuron showing well stained aggregates of E.R. within the cytoplasm. (*b*) BuChE in a ventro-caudal neuron showing that the reaction product is distinctly less abundant than the AChE staining seen in (*a*). The E.R. in the ventro-caudal neuron is also distinctly less well organized.

were similar to those that have been observed in other tissues (Trump & Ericsson, 1965).

Although the size and number of the membranous components of the granular E.R. underwent a decrease during the first two weeks of chromatolysis, the total number of ribosomes in the cell did not appear to change greatly. This observation is in agreement with the electron microscopic findings of Mackey, Spiro & Wiener (1964) and those of Brattgård, Edström & Hydén (1957) who employed quantitative microanalysis to show that the amount of ribonucleic acid per cell remains constant during the reactive phase of chromatolysis. In the present study the polysomes associated with the E.R. were found to undergo dispersal which resulted in a larger number of free ribosomes. Similarly, Price & Porter (1972) reported that ribosomes dissociated from E.R. membranes following axotomy and Barron *et al.* (1967) observed that the rosettes of ribosomes broke up into randomly dispersed single particles. During the third week of chromatolysis we observed that the neuroplasm was exceedingly granular in texture, probably indicating an increase in the total number of ribosomes. This observation agrees with the finding that the total ribonucleic acid content increases after the initial eight to ten days (Brattgard *et al.* 1957). An increased

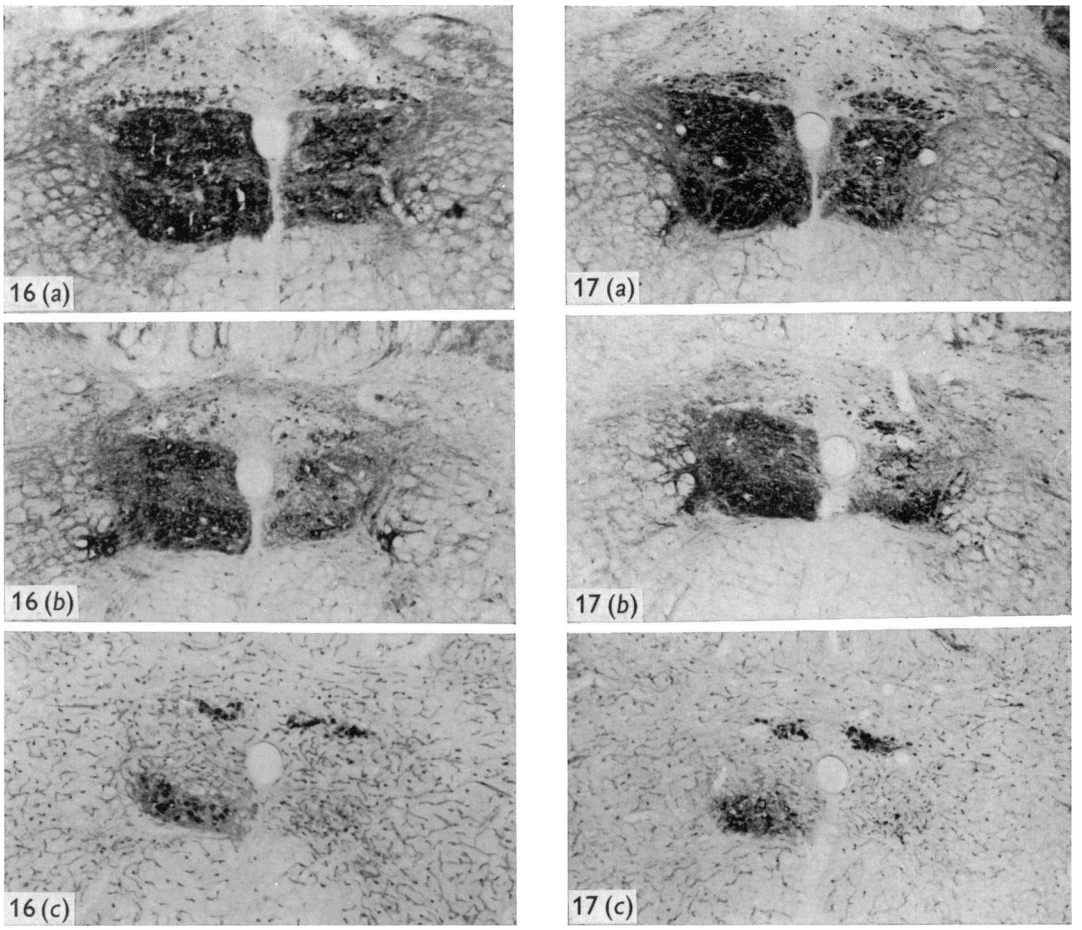

Fig. 16. 80 days. Light microscopic appearance: (*a*) Rostral, AChE. Showing a similar level of intracellular AChE activity on both sides. Note the marked reduction in the size of the nucleus on the operated side. (*b*) Caudal, AChE. Showing some return of AChE activity on the operated side. (*c*) Caudal, BuChE. Showing an absence of staining for BuChE in the ventro-caudal cells. × 30.

Fig. 17. 369 days. Showing three equivalent sections from a long term animal. Note the further decrease in the size of the nucleus on the operated side. × 30.

rate of ribonucleic acid transfer from nucleus to cytoplasm and an increased rate of ribonucleic acid synthesis have also been demonstrated following axotomy (Watson, 1965; Haddad, Iucif & Cruz, 1969). The increase in nuclear activity is probably responsible for the prevalence of nuclear invaginations, with their associated ribosomes and granular E.R., which make up the 'nuclear cap' seen in light microscopic preparations. It is generally agreed that the ribosomes associated with the granular E.R. play a role in the synthesis of protein destined for use *outside* the cell, whereas free ribosomes are involved in producing protein which is employed in maintaining the cell proper. Since chromatolysis is essentially a regenerative process,

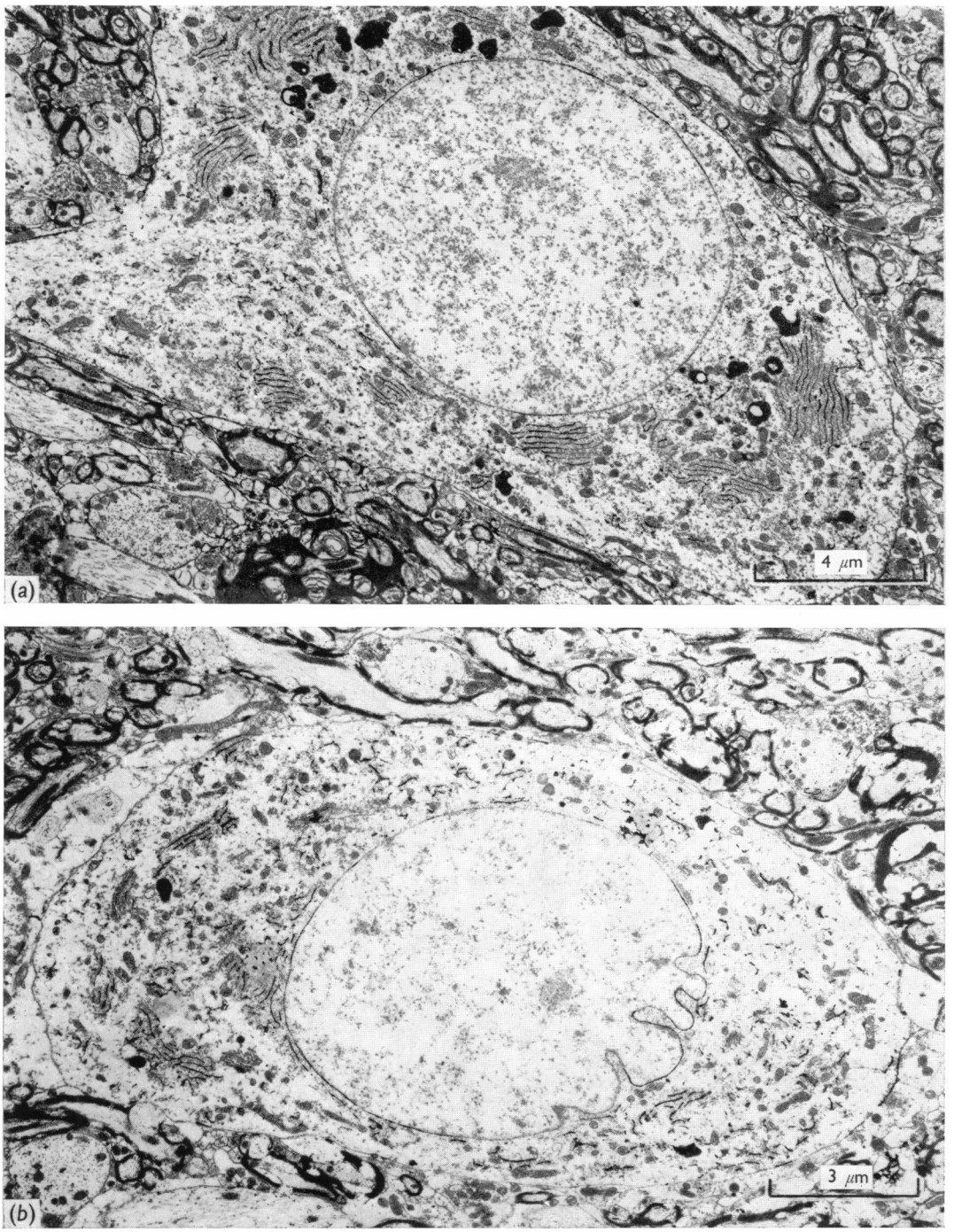

Fig. 18. 374 days. (*a*) AChE in a rostral neuron which contains several well stained Nissl bodies and numerous lipofuscin pigment granules. Note the considerable reduction in the size of this neuron (compare with Figs. 2, 3). (*b*) BuChE in a ventro-caudal neuron showing an absence of well developed Nissl nodies and BuChE activity. The nuclear envelope is markedly involuted and the neuron is considerably reduced in size.

an increase in the production of structural protein would be expected. The reduction in E.R. may thus represent a return to an elementary level of metabolic activity related to the production of new cytoplasm. Undifferentiated, or poorly-differentiated, cells undergoing rapid growth or cell division also show a prevalence of free ribosomes but membranous structures are usually scanty. Since the main function of the neuron is neurotransmission, it is perhaps not surprising that the interruption of this function by axotomy is followed by a complete transformation in the structure of the protein-producing machinery of the cell, placing a greater emphasis on regeneration and reconstitution of the damaged axon.

The distribution of staining for AChE seen in the normal hypoglossal neurons was very similar to that observed in other neurons (Brzin, Tennyson & Duffy, 1966; Lewis & Shute, 1966; Navaratnam & Lewis, 1970), the most distinctive feature being the intense staining within the granular E.R. and the nuclear envelope. Direct evidence that protein synthesis occurs in the Nissl substance of neurons has been provided by using autoradiographic techniques with the electron microscope (Droz, 1965). The demonstration of AChE activity within the cisternae of the E.R. is strong evidence that this enzyme is also synthesized within the Nissl substance.

The results of the present study concerning the changes which occur in AChE activity following axotomy confirm many of the conclusions from light microscopic studies, both in the hypoglossal nucleus (Schwarzacher, 1958) and in other types of neurons (Filogamo & Candiollo, 1962; Navaratnam *et al.* 1968). Schwarzacher (1958) found a more rapid return of AChE activity than we did, but he sutured the cut ends of the hypoglossal nerve together, which would be expected to facilitate reinnervation. The fall in intracellular staining for AChE during the first few days after axotomy is very rapid. This fall is not due entirely to the decrease in the number of E.R. lamellae during the initial period, since intact lamellae without reaction product were a common feature on the third day (Fig. 5*b*). The chromatolytic response thus appears to involve actual loss of AChE from the cell body, over and above that which might result from the dissolution of the E.R. This suggests that the synthetic activity of the E.R. changes at an early stage before there are obvious changes in its morphology. The same order is seen during the recovery phase. During the fourth week the neurons throughout the nucleus showed a marked return of AChE activity when viewed with the light microscope. When viewed with the electron microscope, however, the cells were still distinctly chromatolytic, with reaction product heavily deposited within short lengths of granular E.R. scattered throughout the cytoplasm. It is possible that these E.R. fragments may have persisted from the early stages of chromatolysis, representing residual lamellae of E.R. present before axotomy. In the initial stages of recovery there is a reassociation of ribosomes with the dispersed lengths of unstained E.R., which are not apparently irreversibly inactivated by fragmentation.

Although the Golgi complexes did not undergo drastic changes in number or structure, the reaction product within their saccules decreased. It is generally accepted that the Golgi complex receives protein from the granular E.R.; so the decreased staining within the Golgi saccules may be a direct consequence of the loss of enzyme activity within the E.R. membranes. Reaction product along the plasmalemma was also reduced at peak chromatolysis. In normal neurons the cholinesterase activity

in this location is found at sites of axo-somatic synapses. However, the number of intact boutons over the surface of chromatolytic neurons is distinctly reduced and a greater area becomes occupied by microglial cells (Blinzinger & Kreutzberg, 1968).

During the first few days following axotomy the AChE in the cells was reduced at a somewhat faster rate than that within the neuropil. Similarly, during the recovery phase the intracellular reaction product showed a marked increase during the fourth week, whereas the extracellular staining had not yet begun to return. It is probable that the extracellular AChE which shows a marked reduction is mostly presynaptic, the decrease possibly being a reaction to a loss of function by the target cell.

In the long-term animals a distinct decrease was observed in the size of the hypoglossal nucleus on the operated side. Cell counts from histochemically stained sections revealed a decrease in the total number of hypoglossal neurons to 48–72% of normal. It is noteworthy, also, that the neurons appeared much smaller on the operated side at the one year stage. The overall decrease in size of the nucleus is due therefore, at least in part, to a decrease in the number and size of the neurons. The persistence of the small neurons may reflect the existence of uninjured axon collaterals which serve to maintain the functional integrity of the neuron, but at a reduced level of cell size.

BuChE activity in the long-term animals showed a moderate return at best, and was virtually absent in several cases. Although steps were taken at the time of operation to prevent reinnervation, it cannot be ruled out as a possibility in the long term experiments, and may account for the few cases in which the return of BuChE activity was observed.

In general, the changes observed within the ventro-caudal cells with regard to both morphology and BuChE distribution were similar to those observed with AChE in the remainder of the nucleus. The staining diminished due to a concurrent dissolution of E.R. and loss of intracisternal reaction product. Staining also decreased in amount within the nuclear envelope and Golgi lamellae. All this suggests that BuChE and AChE are similar in nature and possibly have related functions. Despite the basic similarities between the two enzymes, however, some important differences were seen during the chromatolytic cycle. Certain structural changes, such as dissolution of the E.R. and nuclear invagination, appeared earlier in the ventro-caudal cells and persisted for longer. Changes were apparent within these latter cells as early as one day and as late as one year after operation. One of the more striking differences between the two cell types was the early return of AChE to the ventro-caudal cells (on the seventeenth day – none of the cells in the remainder of the nucleus showed a return of AChE until several days later). Moreover, after the return of AChE rostrally, the level of AChE in the ventro-caudal cells was found to be less than in the remainder of the nucleus. Clearly, the pattern of change of AChE in neurons which contain both enzymes is different from that in cells which contain AChE alone. It therefore appears likely that some close functional relationship exists between the two enzymes. Finally, these responses of the cells in the ventro-caudal cluster are similar to those observed in cells of the dorsal motor nucleus of the vagus, which also contain large amounts of BuChE (Fig. 6*c*), viz. the responses to axotomy are very rapid, BuChE returns only very erratically, and cell death is extensive (Lewis

et al. 1972). Possibly, therefore, there is some functional affinity between the two groups of neurons. BuChE, unlike AChE, is not inhibited by excess amounts of acetylcholine (Dirnhuber & Lovatt Evans, 1954) so its presence may be necessary in neurons which undergo high rates of stimulation and are exposed to more than usual amounts of acetylcholine.

SUMMARY

Within the hypoglossal nucleus large amounts of acetylcholinesterase (AChE) activity are present in all the neurons, whereas intracellular butyrylcholinesterase (BuChE) activity occurs only within a ventro-caudally situated cluster of cells. AChE activity within the neurons occurs mainly in the cisternae of the granular endoplasmic reticulum but there is some in the intermembranous space of the nuclear envelope and in the Golgi complexes. In the neuropil, reaction product is seen along some axonal and synaptic membranes. The distribution of BuChE in the ventro-caudal cells is identical with that of AChE except that BuChE activity is absent from the neuropil.

The level of intraneuronal AChE activity falls rapidly during the first few days after axotomy. The fall is due partly to a dissolution and peripheral migration of the E.R. but also to a decrease in AChE content of the E.R. that remains. Return of staining begins in the 4th week and continues as the E.R. reassembles. Staining in the neuropil falls more slowly, but recovers less completely. The ventro-caudal group of cells shows the same kinds of change, but more dramatically. BuChE activity returns only erratically and never completely. The similarity in normal distribution, and in response to axotomy, of the two cholinesterases suggests that their functions are related.

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