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SOME METABOLIC RESPONSES OF AXOTOMIZED NEURONES TO CONTACT BETWEEN THEIR AXONS AND DENERVATED MUSCLE

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SUMMARY

1. The nucleolar and cell body dry mass and nucleic acid content of hypoglossal neurones were measured in adult rats using interference microscopy and ultra-violet absorption microspectrography.

2. The left hypoglossal nerve was transplanted into the ipsilateral sternomastoid. Seventy days later the sternomastoid was denervated by dividing the ipsilateral spinal accessory nerve. This was followed by metabolic changes in hypoglossal nerve cells.

3. The changes induced in hypoglossal neurones by division of the ipsilateral accessory nerve did not occur if botulinum toxin was injected locally at the same time.

4. In other rats the left hypoglossal nerve was anastomosed to the proximal stump of the ipsilateral median nerve simultaneously divided at the level of the wrist. Seventy days later this median nerve was divided in the axilla. This was followed by metabolic changes in hypoglossal nerve cells.

5. These results are discussed in relation to the possible roles of reacting Schwann cells, degenerating axoplasm and denervated muscle in maintaining aspects of the metabolic response of nerve cells to injury.

6. It is suggested that the synthesis of acetylcholine by an axonal ending, or its release, is dependent upon the presence of an adjacent membrane which can respond to it, and that the metabolic changes measured in the nerve cell body are secondary to this response of the axon terminal.

INTRODUCTION

It has been shown previously that the metabolism of motor nerve cells alters after axotomy (Brattgård, Edström & Hydén, 1957; Watson, 1965, 1968, 1969*a*). Some of these metabolic changes can be initiated without loss of axoplasm by botulinum toxin (Watson, 1969*b*). The investigation reported here was undertaken to see whether later axonal events, other

than those attributable to direct injury or to loss of effective contact with muscle, can influence the metabolic activity of the nerve cell body: especially, to find if the presence of denervated muscle in the vicinity of the axonal ending, of Schwann cells reacting to axotomy or of degenerating axoplasm can act in this manner.

METHODS

Animals. White rats were used, aged 3 months at the time of the first procedure. They received standard food pellets and water, supplemented with bread and milk *ad libitum*.

Hypoglossal nerve transplantation. These experiments were undertaken to see whether the presence of denervated muscle in the vicinity of a motor axon previously separated from the muscle fibre it normally innervates could influence the metabolic activity of the motor neurone. Under ether anaesthesia the left hypoglossal nerve was divided in the tongue: the proximal stump was mobilized and drawn gently through the left sternomastoid until it emerged under slight tension. In most rats the nerve was divided as it emerged and the end of the nerve retracted at once to lie within the substance of the muscle. The nerve was not fixed, but was found to lie deep within sternomastoid in 98% of animals when killed 2–3 months later. The remaining 2% of rats, in which the nerve had slipped out of the muscle, are not included in the results reported here. In another group of rats the hypoglossal nerve was drawn through sternomastoid and its end was sutured to the outside of the muscle sheath at least 5 mm from its point of emergence: further investigation of this group was designed to see if the trunk of the nerve itself, or only the axonal endings, would respond to muscle denervation.

In one group of rats the hypoglossal nerve was divided 70 days after transplantation, and the spinal accessory nerve was left intact: this was done to demonstrate that the hypoglossal neurone was still able to react to axonal events under these circumstances. In another group of rats the ipsilateral spinal accessory nerve was divided 70 days after transplanting the hypoglossal nerve, and care was taken not to damage the implanted hypoglossal nerve: the proximal stump of the spinal accessory was mobilized and thrust into precervical muscles to impede regeneration. This group was divided into several subgroups, some of which also received botulinum toxin (see below). In a third group the right spinal accessory was divided to demonstrate that metabolic changes observed in hypoglossal neurones were not simply part of a general response to surgical trauma.

Denervation of sternomastoid is not the only consequence of spinal accessory nerve division: the muscle also contains the distal stump of the degenerating spinal accessory nerve. In order to see if changes induced in hypoglossal neurones were due to products of such degeneration two procedures were undertaken. In the first, both the hypoglossal nerve and the proximal stump of the ipsilateral sectioned vagus were transplanted simultaneously into sternomastoid between 5 and 10 mm apart: 70 days later the transplanted vagus was divided and ensuing changes were sought in hypoglossal nerve cells: under these circumstances the implanted hypoglossal axons are exposed to hypothetical products of Wallerian degeneration in the absence of denervated muscle. In the second procedure botulinum toxin was injected into sternomastoid 70 days after hypoglossal nerve transplantation: under these circumstances the implanted hypoglossal axons are in the vicinity of muscle undergoing changes identical with those of denervation (Watson, 1969*b*) in the absence of Wallerian degeneration.

Four groups of animals in which the hypoglossal nerve had previously been transplanted into sternomastoid received botulinum toxin. In the first, the spinal accessory and hypoglossal nerves were left intact. In the second, the left spinal accessory nerve was divided at the time of injection of botulinum toxin. In the third, the transplanted hypoglossal nerve was divided at the time of injection. In the fourth group botulinum toxin was injected 60 days after dividing the spinal accessory nerve, 130 days after hypoglossal nerve transplantation.

Each rat received 250 pg precipitated unpurified botulinum toxin type A in 100 μl. Ringer–Locke solution. The solution was injected into the inferior part of sternomastoid remote from the implanted hypoglossal nerve. As a control procedure, previously boiled toxin was similarly injected.

Anastomosis of hypoglossal and median nerves. This was done to investigate the capacity of Schwann cells reacting to axotomy, or of degenerating axoplasm to induce metabolic changes in motor neurones. The left hypoglossal nerve was divided in the tongue and mobilized: the ipsilateral median nerve was divided at the wrist and the proximal stump mobilized as far as the brachial plexus. The nerve was drawn up in front of the clavicle to meet the hypoglossal nerve on the anterior aspect of the sheath of sternomastoid. A pedicled sleeve was prepared from the external jugular vein of the same side. The nerve ends were sutured together without tension and the anastomosis was drawn gently into the pedicled sleeve. This method was found to preserve an adequate blood supply to the whole length of both nerves; a good anastomotic circulation was found histologically to arise from vessels of the vascular sleeve, which also prevented significant outgrowth of fibres away from the nerves.

In most rats the median nerve was divided close to its origin from the brachial plexus 70 days after anastomosis. In some rats the hypoglossal nerve was divided 70 days after anastomosis, so that degenerating hypoglossal nerve fibres could be identified within the median nerve.

Tissue preparation and cytological measurements. Some rats were killed between 5 and 50 days after nerve transplantation or anastomosis to confirm that the response of hypoglossal neurones after these procedures resembled the response to axotomy alone. Most rats were killed between 3 and 50 days after the second procedure. The rat was bled out under ether anaesthesia, and the left hypoglossal nucleus removed. Previous papers describe in detail the further preparation of isolated hypoglossal neurones and of their nucleoli both for measuring nucleic acids using a Leitz ultraviolet microspectrograph (Watson, 1968), and for measuring dry mass with a Leitz interference microscope (Watson, 1969*b*), and also the techniques of estimation employed. Each point shown in Figures represents a mean value obtained from measuring twelve to twenty cells or nucleoli obtained from one rat. The S.E. of the mean is also indicated.

Autoradiography. Tritiated [Me-^{3}H]thymidine (specific activity 19·6 c/m-mole) was obtained from the Radiochemical Centre at Amersham. Rats received 100 μl. artificial cerebrospinal fluid (c.s.f.) (Mitchell, Loeschke, Massion & Severinghaus, 1963) containing 10 μc by injection into a lateral cerebral ventricle, to allow deoxyribonucleic acid (DNA) synthesis by neuroglia to be followed. The technique of autoradiography has been described before (Watson, 1965). Suitable control experiments excluded significant chemosensitivity or emulsion desensitization by tissue. Some sections were digested with ribonuclease or deoxyribonuclease before preparing autoradiographs.

Histology. Motor end plates and nerve fibres within sternomastoid were demonstrated using methods described by Duchen & Strich (1968). Nerves after collision anastomosis were stained by Holme's silver method, or for myelin by the method of Aitken, Sharman & Young (1947).

Nerve stimulation. The transplanted hypoglossal nerve was stimulated electrically in some rats between 4 and 20 days after dividing the spinal accessory nerve, using a pulse repetition rate of 12/sec and a pulse width of 1 msec: the intensity of the stimulus was adjusted until it was just adequate to cause the normal sternomastoid to twitch when the electrodes were laid directly upon the intact superficial sheath of the muscle. The hypoglossal nerve was ligated and divided proximally at the base of the skull immediately before stimulation.

RESULTS

Hypoglossal nerve transplantation. Following hypoglossal nerve division and transplantation or anastomosis, significant increases were found in hypoglossal nucleolar dry mass ($P < 0{\cdot}001$), nucleolar nucleic acid

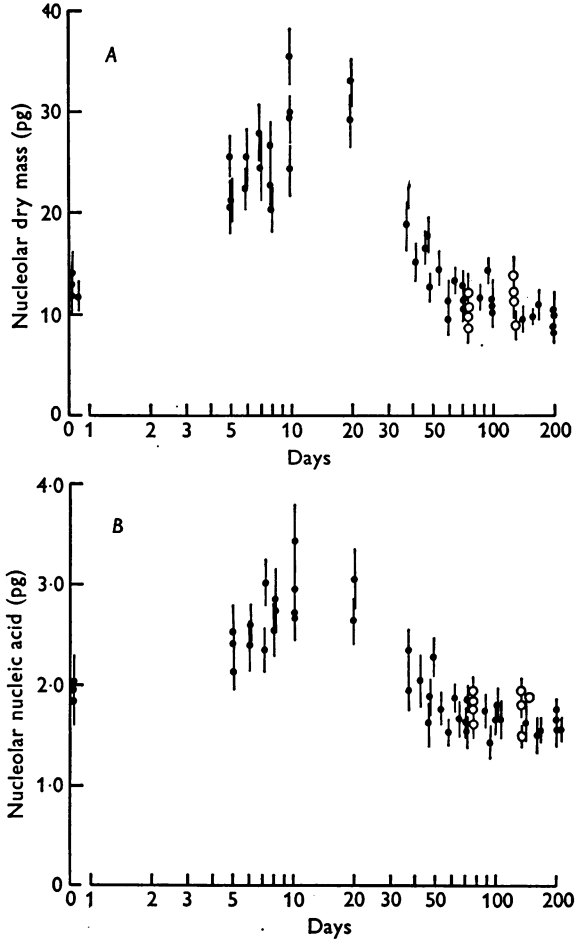

Fig. 1. For legend see opposite page.

($P < 0{\cdot}01$; $> 0{\cdot}001$), cell body dry mass ($P < 0{\cdot}05$; $> 0{\cdot}01$) and cell body nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$) (Fig. 1*A–D*). Seventy days after nerve transplantation all parameters were significantly less than normal ($P < 0{\cdot}05$; $> 0{\cdot}01$). No significant change occurred between the 70th day and the 110th day, the period which for other rats was the first 40 days after the second procedure. In some rats the hypoglossal nerve was divided a second time 70 days after transplantation into sternomastoid. Significant increases were found in nucleolar dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$).

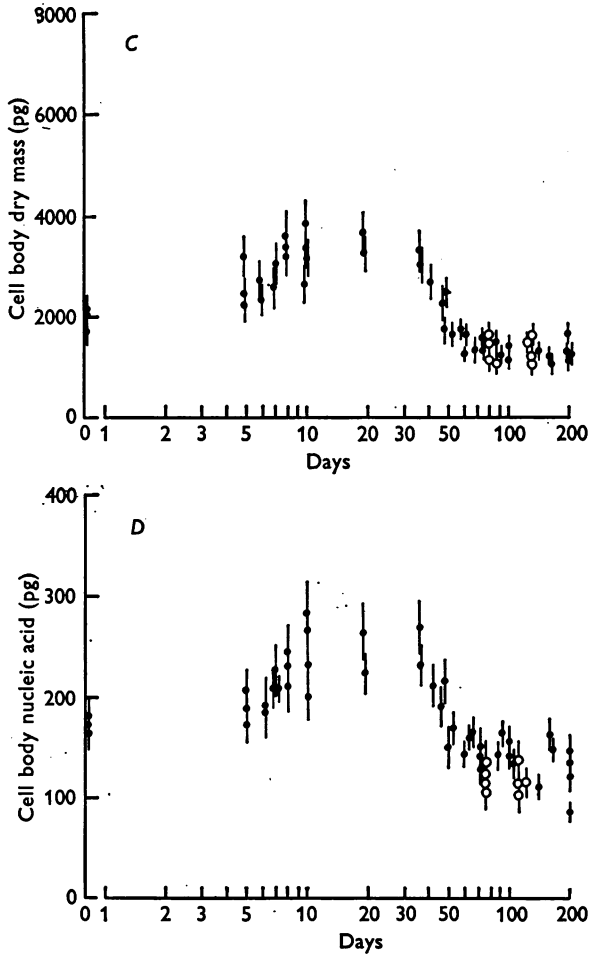

Fig. 1. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since ●, transplantation into sternomastoid; ○, hypoglossal-median anastomosis. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body nucleic acid measured under similar circumstances.

nucleolar nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$), cell body dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$) and cell body nucleic acid ($P < 0{\cdot}05$; $> 0{\cdot}01$) (Fig. 2*A–D*). This response was not influenced by injection of botulinum toxin into sternomastoid at the time of second nerve injury.

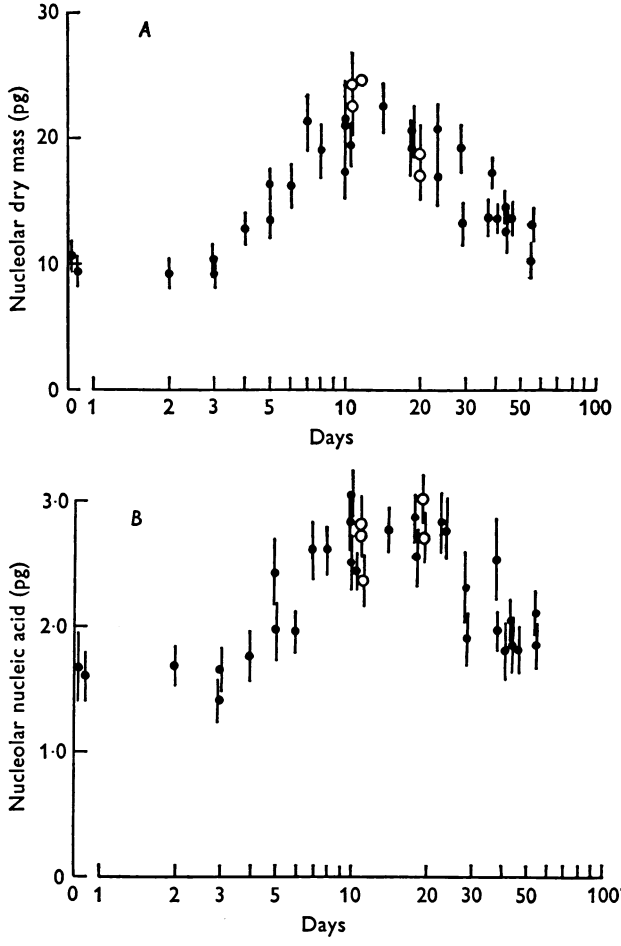

Fig. 2. For legend see opposite page.

Division of spinal accessory nerve. After division of the spinal accessory nerve, 70 days after transplanting the hypoglossal nerve into sternomastoid, significant increases were found in hypoglossal nucleolar dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$), nucleolar nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$), cell body dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$) and cell body nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$) (Fig. 3*A–D*). The latency of these changes resembled that found after dividing the implanted hypoglossal nerve (Fig. 2).

Between 70 and 100 days after dividing the spinal accessory nerves, these parameters were greater than the values shown on day 0, immediately before dividing this nerve ($P < 0{\cdot}05$; $> 0{\cdot}01$).

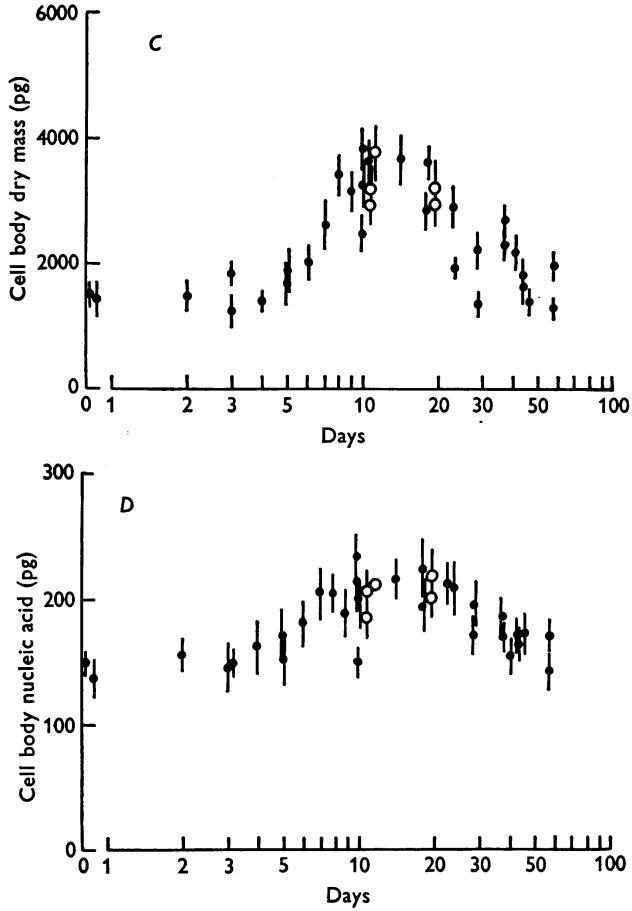

Fig. 2. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since dividing the hypoglossal nerve a second time, 70 days after transplanting it into sternomastoid ●, without botulinum toxin; ○, with botulinum toxin. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body nucleic acid measured under similar circumstances.

As it seemed likely that this metabolic response was induced by changes of denervation in muscle fibres of sternomastoid, rather than by Wallerian degeneration of the distal stump of the spinal accessory nerve, botulinum toxin was injected locally to produce the former without the latter. No

change, however, occurred in any parameter within the first 20 days (Fig. 4*A–D*), the period within which the greatest response was found after dividing the spinal accessory nerve (Fig. 3). After the 30th day significant increases occurred in nucleolar dry mass ($P < 0{\cdot}05$; $> 0{\cdot}01$), nucleolar nucleic acid ($P < 0{\cdot}05$; $> 0{\cdot}01$), cell body dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$) and cell body nucleic acid ($P < 0{\cdot}05$; $> 0{\cdot}01$).

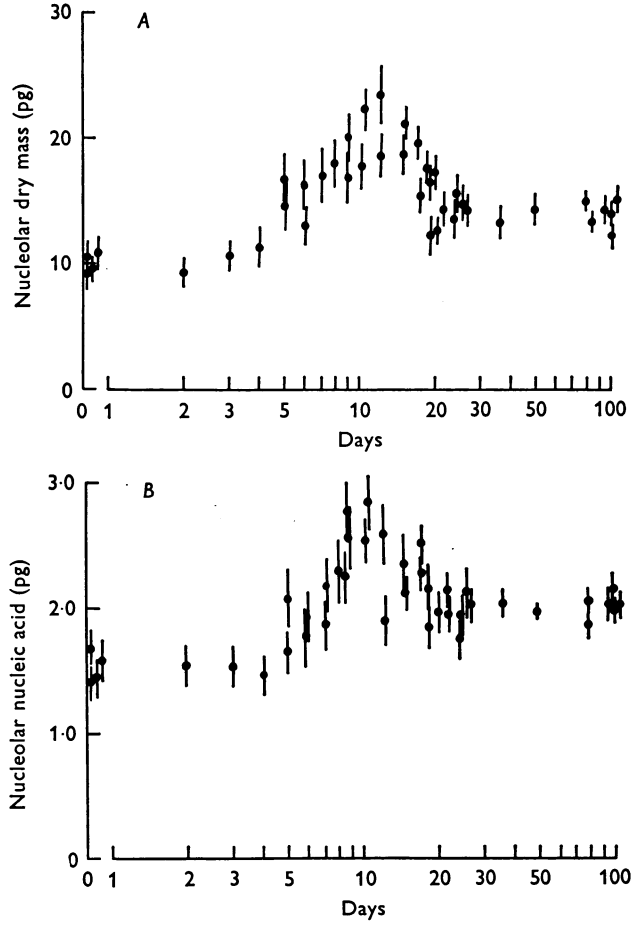

Fig. 3. For legend see opposite page.

In order to see if botulinum toxin not only caused changes of denervation in sternomastoid by affecting the terminal axons of the spinal accessory nerve (see Discussion), but also interfered with the capacity of the axon sprouts of the hypoglossal nerve to respond to such denervation, botulinum toxin was injected into sternomastoid when the spinal accessory nerve was divided. Within the first 20 days no significant change was

found in any parameter (Fig. 5*A–D*): a later significant increase occurred ($P < 0{\cdot}05$; $> 0{\cdot}01$). Previously boiled botulinum toxin did not influence the initial response of hypoglossal neurones to division of the spinal accessory nerve, and did not cause a later response (Fig. 5).

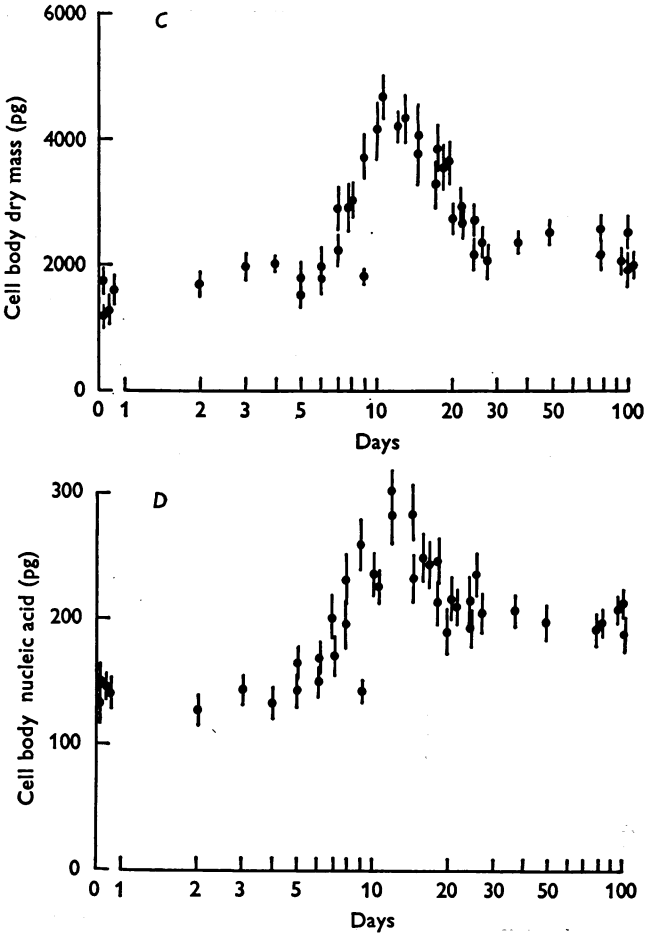

Fig. 3. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since dividing the spinal accessory nerve, 70 days after transplanting the hypoglossal nerve into sternomastoid. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone nucleic acid measured under similar circumstances.

Another group of rats received an injection of botulinum toxin into sternomastoid 60 days after dividing the spinal accessory nerve, 130 days after transplanting the hypoglossal nerve. Nucleolar dry mass ($P < 0{\cdot}05$; $> 0{\cdot}01$), nucleolar nucleic acid ($P < 0{\cdot}05$; $> 0{\cdot}01$), cell body dry mass

($P < 0{\cdot}01$; $> 0{\cdot}001$) and cell body nucleic acid ($P < 0{\cdot}05$; $> 0{\cdot}01$) increased significantly (Fig. 6 *A–D*).

In one group of rats the hypoglossal nerve was drawn through sternomastoid and fixed, so that its axons did not ramify within the muscle. When the spinal accessory nerve was divided 70 days later, the hypoglossal parameters did not change.

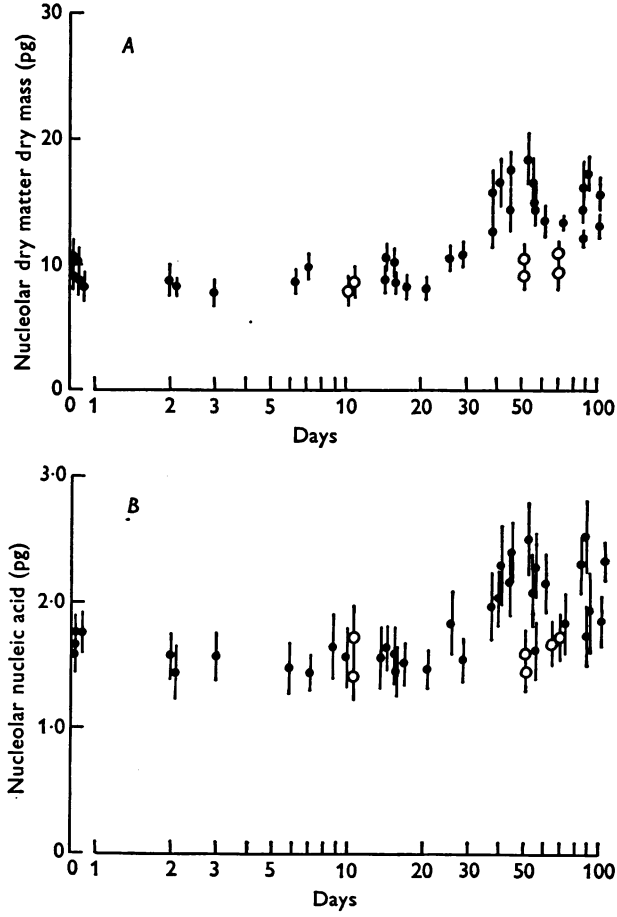

Fig. 4. For legend see opposite page.

Division of implanted vagus. In one group of rats the vagus and hypoglossal nerves had been simultaneously transplanted into sternomastoid. When the vagus was divided 70 days later, the hypoglossal parameters did not change.

Division of contralateral spinal accessory nerve. The right spinal accessory nerve was divided in one group of rats 70 days after transplanting the left

hypoglossal nerve into the left sternomastoid: the hypoglossal parameters did not change.

Proximal division of median nerve following hypoglossal-median anastomosis. This procedure was followed by significant increases in hypoglossal nucleolar dry mass ($P < 0{\cdot}01$; $> 0{\cdot}001$), nucleolar nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$), cell body dry mass ($P < 0{\cdot}001$) and cell body nucleic acid ($P < 0{\cdot}01$; $> 0{\cdot}001$) (Fig. 7*A–D*).

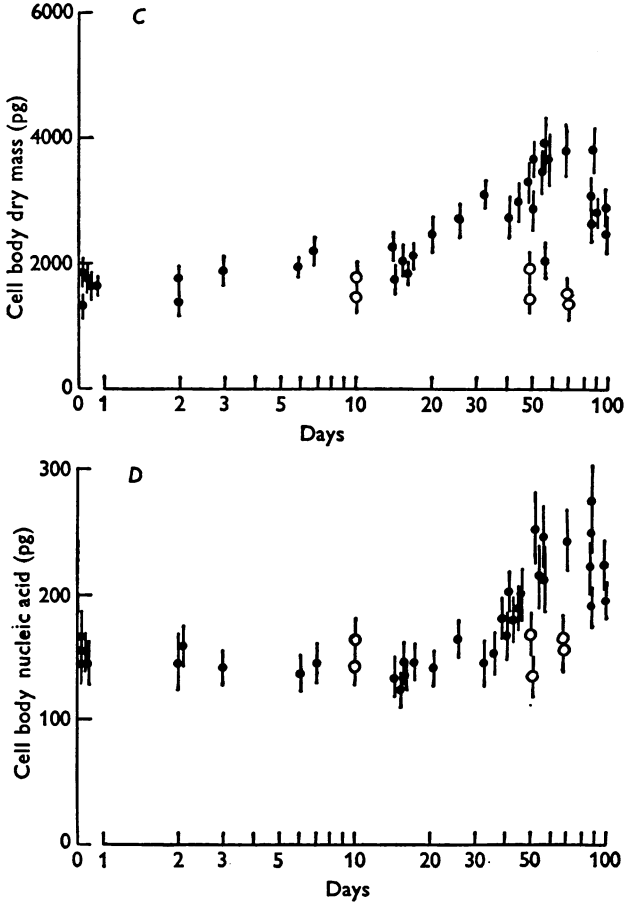

Fig. 4. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since local injection of botulinum toxin, 70 days after transplanting the hypoglossal nerve into sternomastoid. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body dry mass measured under similar circumstances. ●, unboiled botulinum toxin; ○, previously boiled botulinum toxin. Compare with Fig. 3. Note the absence of increase before the 20th day, and its presence after the 30th.

Histology. Seventy days after implanting the hypoglossal nerve into sternomastoid many axons were seen lying between normal muscle fibres. In the absence of further procedure, the appearance of this tangled mass of fibres remained unaltered up to 250 days after transplantation.

After dividing the spinal accessory nerve changes of denervation appeared in sternomastoid, and degenerating spinal accessory nerve fibres

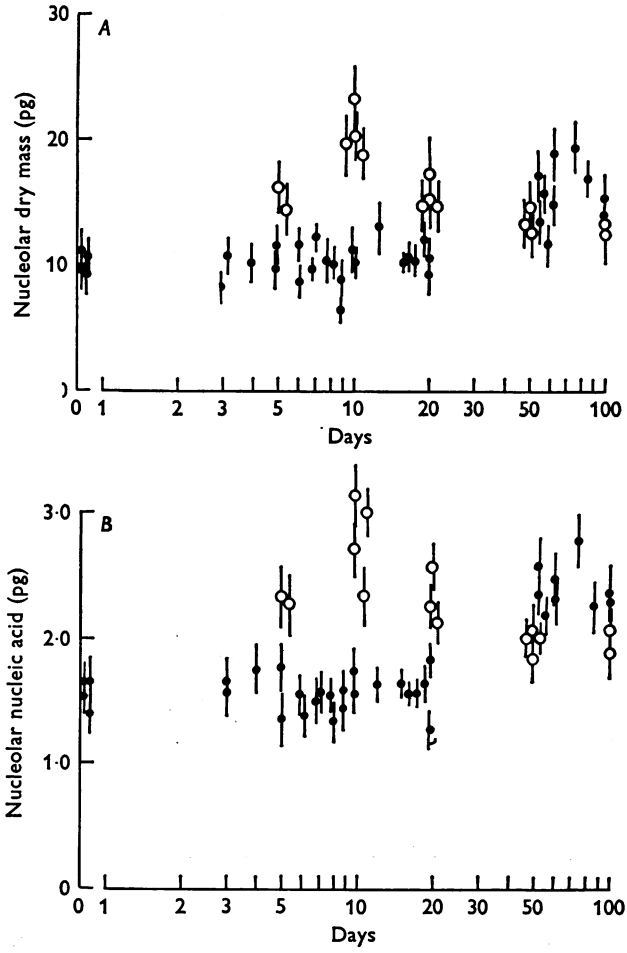

Fig. 5. For legend see opposite page.

were seen. Occasional hypoglossal nerve fibres were first seen passing to motor end plates 8 days after dividing the spinal accessory nerve, and increased steadily in size and frequency up to the 50th day: no other difference could be seen in the gross intramuscular distribution of the tangled mass of hypoglossal nerve fibres over this period. In rats killed

between 100 and 200 days after spinal accessory nerve division hypoglossal nerve fibres were found to pursue a more direct course to the sternomastoid motor end plates and the mass of tangled hypoglossal nerve fibres was greatly reduced.

Injection of botulinum toxin into sternomastoid was followed by changes resembling those of denervation in the muscle fibres: sprouting of the terminal fibres of the spinal accessory also occurred. When botulinum toxin

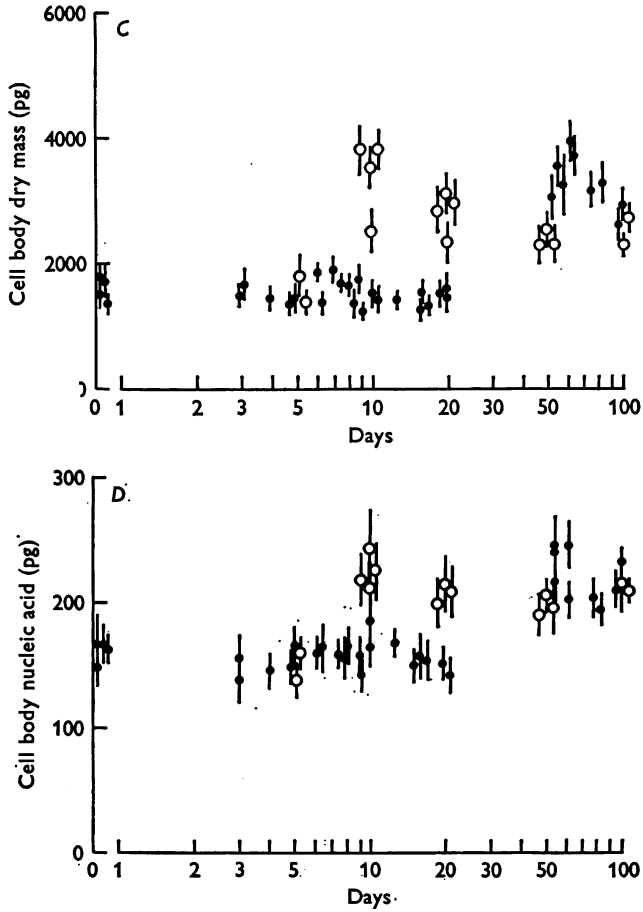

Fig. 5. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since simultaneous injection of botulinum toxin and division of the spinal accessory nerve, 70 days after transplanting the hypoglossal nerve. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body nucleic acid measured under similar circumstances. ●, unboiled botulinum toxin; ○, previously boiled botulinum toxin. Compare with Figs. 3 and 4.

was injected at the same time as division of the spinal accessory nerve similar changes occurred in the muscle. No hypoglossal nerve fibres could be traced to the motor end plates before the 25th day, a time at which this process was regularly well advanced in the absence of botulinum toxin. This delayed pattern of aberrant innervation was associated with the

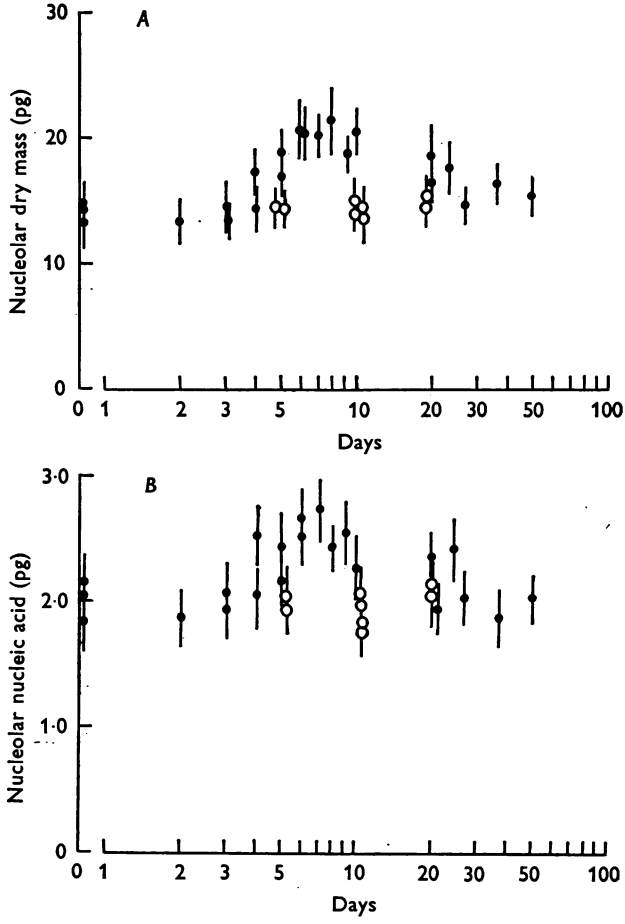

Fig. 6. For legend see opposite page.

delayed metabolic response shown in Fig. 5. Between 100 and 200 days after spinal accessory nerve division and simultaneous injection of botulinum toxin the histological appearance resembled that found after division of the spinal accessory nerve alone.

In 15% of rats occasional regenerating fibres of the spinal accessory nerve could be seen within the serially sectioned sternomastoid after the 18th day following spinal accessory nerve section: no difference was found

in the metabolic behaviour of hypoglossal neurones between these rats and the 85% in which no such regeneration was found.

After hypoglossal-median nerve anastomosis fibres from each nerve penetrated each other for a few millimetres only. When the hypoglossal nerve was divided 70 days after anastomosis, degenerating fibres could not be traced further than 5 mm into the median nerve. When the median

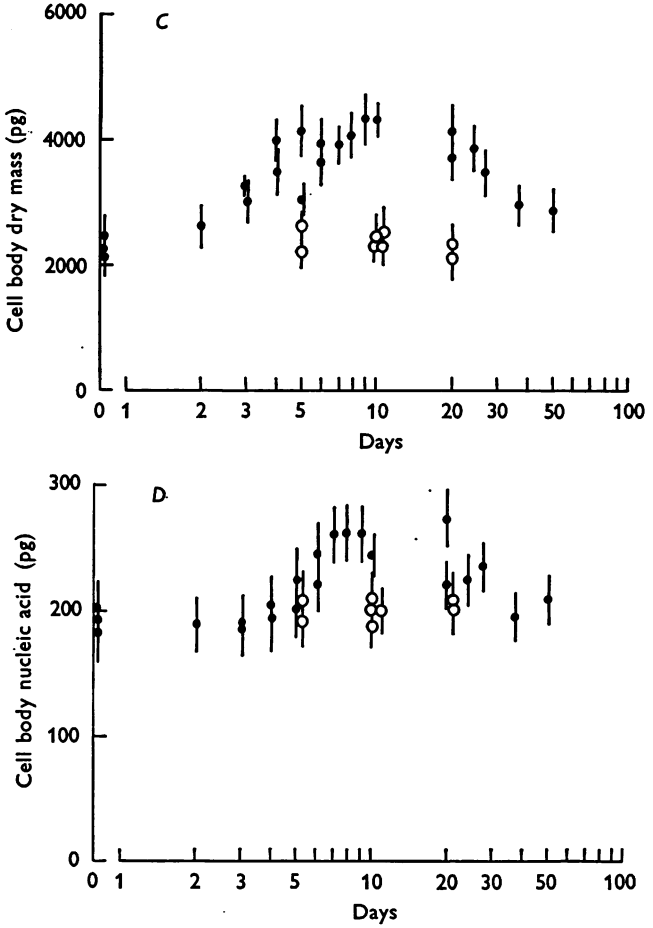

Fig. 6. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since local injection of botulinum toxin. This toxin was injected 60 days after dividing the spinal accessory nerve, 130 days after transplanting the hypoglossal nerve into sternomastoid. *B*. Hypoglossal neurone nucleolar nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body nucleic acid measured under similar circumstances. ●, unboiled botulinum toxin; ○, previously boiled botulinum toxin. Compare with Fig. 4.

nerve was divided proximally 70 days after anastomosis surviving hypoglossal nerve fibres could be seen in the same region of the median nerve. As the period following median nerve division increased hypoglossal fibres could be traced further within the median nerve: an accurate estimate of the apparent rate of hypoglossal axonal growth under these circumstances was impossible to obtain, but was of the order of 1–2 mm/day.

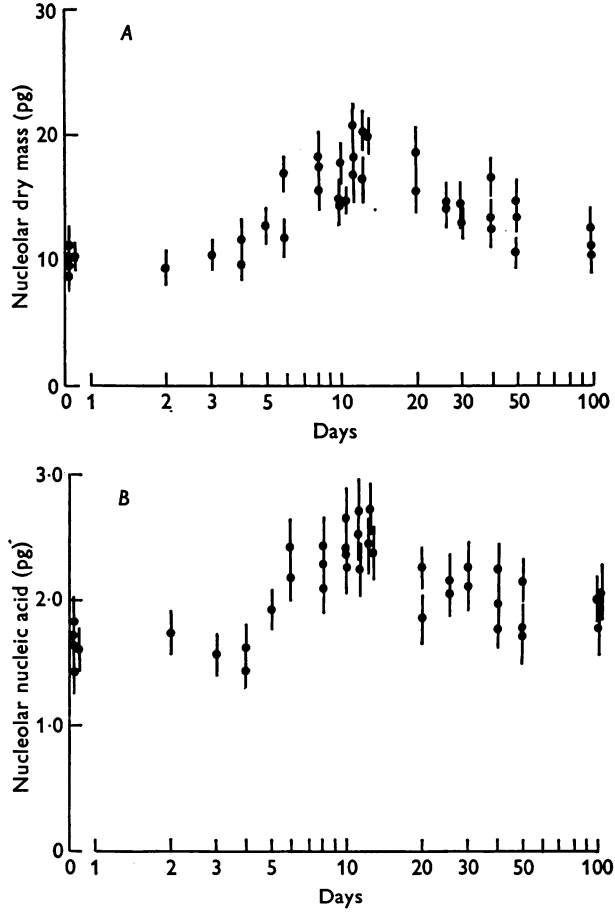

Fig. 7. For legend see opposite page.

Autoradiography. Rats were killed 24 hr after intraventricular injection of [Me-^{3}H]thymidine. Labelled glial cells were found around neurones of the injured hypoglossal nerve between 3 and 13 days after the first injury associated with transplantation or anastomosis, and between 3 and 10 days after second division of the hypoglossal nerve 70 days after transplantation or anastomosis. No labelled glia were found around hypoglossal

neurones after dividing the spinal accessory nerve 70 days after implanting the hypoglossal nerve into sternomastoid, or after proximal division of the median nerve 70 days after hypoglossal-median anastomosis.

Labelled Schwann cells were found throughout the median nerve on the 4th day after proximal division of the median nerve 70 days after anastomosis, 24 hr after intraperitoneal injection of 100 μc labelled thymidine.

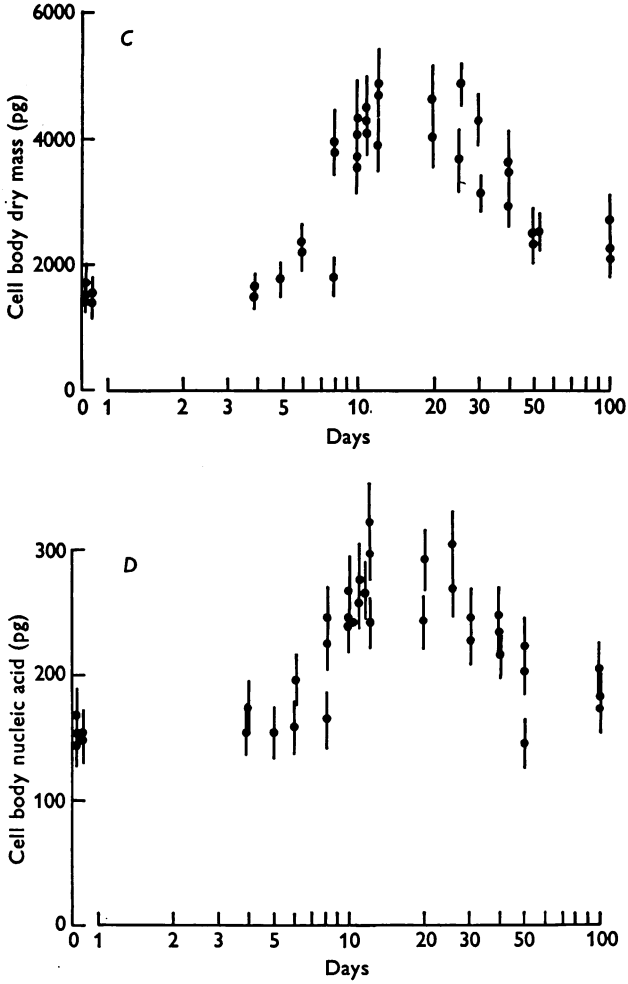

Fig. 7. *A*. Hypoglossal neurone nucleolar dry mass expressed against the time in days since proximal division of the median nerve 70 days after hypoglossal-median anastomosis. *B*. Hypoglossal neurone nucleic acid measured under similar circumstances. *C*. Hypoglossal neurone cell body dry mass measured under similar circumstances. *D*. Hypoglossal neurone cell body nucleic acid measured under similar circumstances.

This indicated that the blood supply to the median nerve was not seriously impaired by proximal division.

Nerve stimulation. Stimulation of the transplanted hypoglossal nerve caused no discernible contraction of sternomastoid before division of the spinal accessory nerve, or before the 6th day following division. Weak contraction was observed in the sternomastoid of one rat out of six on the 6th day and in five out of six rats on the 10th day. By the 18th day, contraction was apparent in all rats investigated, and persisted thereafter. When botulinum toxin was injected at the same time as dividing the spinal accessory nerve no response to stimulation was found in any rat before the 30th day.

DISCUSSION

The scope and limitations of the cytological methods used in this study (autoradiography, ultra-violet absorption microspectrography and interference microscopy), of the methods used for preparing isolated cells and nuclei for measurement and the cytochemical significance of results obtained, have been discussed previously (Watson, 1965, 1968, 1969*b*) and will not be considered further.

Changes in denervated muscle. Mammalian skeletal muscle does not readily accept further innervation (Elsberg, 1917; Steindler, 1916; Aitken, 1950). After denervation aberrant innervation becomes possible, either by re-innervation of previous neuromuscular junctions, or by the formation of new ones (Miledi, 1962; Guth & Zalewski, 1963; Gwyn & Aitken, 1964, 1966). Changes occurring in denervated muscle have been reviewed recently (Gutmann, 1964; Guth, 1968): it remains uncertain which, if any, of the described altered membrane properties of denervated muscle are responsible for this capacity to accept innervation (Nicholls, 1956; Harris & Nicholls, 1956; Drahota, Gutmann & Vrbová, 1957; Desmedt, 1959; Drahota & Hudlicka, 1960; Axelsson & Thesleff, 1959; Miledi, 1960; Birks, Katz & Miledi, 1960).

Changes in neurones on contact with denervated muscle. Between 70 and 150 days after transplanting the hypoglossal nerve into a normally innervated sternomastoid no further gross metabolic change occurs (Fig. 1). This steady state differs from that of normal neurones, for the nucleolar and cell body dry mass and nucleic acid content are less than normal, and the axons are not in functional contact with muscle: the responses of these neurones to a second procedure are therefore not those of normal neurones. Hypoglossal neurones are in some respects especially suitable for investigating the effects of axonal events upon the nerve cell body, for recurrent axon collaterals are absent or sparse (Porter, 1965; Lorente de Nó, 1947).

Hypoglossal neurones having their axons in contact with denervated muscle only show metabolic changes when the axons respond locally by making functional contact with muscle fibres. Under the conditions of this experiment, such contact was made almost exclusively with previously existing motor end plates; this is in agreement with others who have investigated innervation patterns when nerves were implanted soon after muscle denervation (Gutmann & Young, 1944). When botulinum toxin is used to cause changes of denervation in muscle, the axons of the hypoglossal nerve do not respond, and there is no associated metabolic change in the cell body (Fig. 4): after about 30 days, when the toxin begins to be no longer effective (Fex, Sonessin, Thesleff & Zelena, 1966) some hypoglossal axons pass to motor end plates and metabolic changes occur in the cell body. The conditions of this experiment differ from that of Thesleff (1960), who transplanted a nerve after injecting botulinum toxin into a muscle: the implanted nerve was not significantly exposed to toxin, so innervation of the muscle could occur. As botulinum toxin also delays the hypoglossal axonal and cell body response to division of the spinal accessory nerve (Fig. 5), it appears that the toxin is preventing hypoglossal axons from reacting to changes of denervation in adjacent muscle fibres. Botulinum toxin does not prevent the cell body from reacting to axonal events, for metabolic changes occur in hypoglossal neurones when injected at the same time as dividing the transplanted hypoglossal nerve (Fig. 2).

After nerve transplantation a normal response by the hypoglossal nerve cell bodies to local injection of botulinum toxin (Watson, 1969*b*) is obtained only when the hypoglossal axons are already in effective functional contact with muscle (Fig. 6), as a consequence of dividing the spinal accessory nerve 60 days before. The only known action of botulinum toxin is to prevent directly or indirectly the release of acetylcholine from nerve terminals (Ambache, 1948, 1949, 1951; Burgen, Dickens & Zatman, 1949; Brooks, 1954, 1956). As a working hypothesis it is suggested that botulinum toxin has no metabolic effect upon neurones which are not in functional contact with muscle, because they are not releasing acetylcholine, and that the synthesis and release of acetylcholine by the axon is a consequence of the axon's contact with a membrane which can respond to the transmitter substance, in this case the surface of denervated muscle. There is evidence that denervated muscle can cause adjacent cells, other than neuronal processes, to synthesize and release acetylcholine (Birks *et al.* 1960; Miledi & Slater, 1963). The significant properties of the membrane of denervated muscle which can induce such a change, and of the axon which can respond to it, are not known. Botulinum toxin could interfere with this process either by preventing the axon from responding to adjacent denervated muscle and secondarily preventing the synthesis

and release of acetylcholine, or by preventing directly its synthesis or release.

In one group of rats, in which the hypoglossal nerve was passed through sternomastoid so that axons did not ramify within the muscle, denervation of sternomastoid did not cause either histological changes in the nerve or metabolic changes in the nerve cell body. Collateral sprouting can be induced readily within the hypoglossal nerve by partial lesions (unpublished observations). It is possible that collateral sprouting does not occur where the nerve trunk passes through the denervated sternomastoid because its axons are effectively isolated from the muscle by the perineural sheath, a tissue containing cells and junctions which appear histologically to be well suited to such an isolating role (Shanthaveerappa & Bourne, 1962; Gamble, 1964; Sunderland, 1965). Such a boundary would reduce the probability of collateral axonal sprouts being formed along the course of the nerve and passing to any adjacent denervated muscle fibres: as there is evidence of continuous turnover of terminal axons in muscle (Barker & Ip, 1966) it is likely that without such a boundary the patterns of peripheral muscular innervation would become disorderly.

Median nerve division. Metabolic changes occur in hypoglossal neurones when Wallerian degeneration occurs in the median nerve after median nerve division 70 days after hypoglossal-median anastomosis (Fig. 7). It is necessary to exclude direct injury to the transplanted hypoglossal axons before these changes can be considered as a consequence of degenerative or reactive changes in axons, Schwann cells or other constituents of the median nerve. This can be excluded because such direct injury is accompanied by synthesis of DNA by perineuronal glia (Cammermeyer, 1965; Watson, 1965; Sjöstrand, 1965, 1966), both around hypoglossal neurones which were previously normal, and around neurones 70 days after hypoglossal-median anastomosis. No such DNA synthesis was found. As the absence of such synthesis is compatible with the hypothesis concerning glial division put forward previously (Watson, 1969*b*), it will not be discussed further. Histology of the anastomosed nerves also suggests that direct injury of hypoglossal neurones is most unlikely to result from proximal division of the median nerve. Following median nerve division, the length of hypoglossal nerve fibres within the median nerve increases with time. It is probable that this axonal growth of 1–2 mm/day is caused or facilitated by the changes of Wallerian degeneration, and that the associated metabolic changes in the nerve cell body are a consequence of axonal growth (Watson, 1969*b*).

The absence of similar change following vagotomy 70 days after simultaneous transplantation of vagus and hypoglossal nerves into sternomastoid is probably due to the distance between these nerves within

sternomastoid, and strongly suggests that no part of the response of the hypoglossal neurones to division of the spinal accessory nerve is due to degeneration of the distal stump of this nerve.

Relation of these changes to those following axotomy. The results reported, here and previously, indicate that aspects of the metabolic response of the neurone to axotomy can be caused and sustained by several factors, including initial axonal sprouting, axonal growth associated with reacting Schwann cells and degenerating axoplasm, and restoration of effective contact between the axon and denervated muscle. These factors indicate strongly the influential role of the state of the axon terminal in regulating, at least in part, the metabolic activity of the nerve cell body.

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