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SOME QUANTITATIVE OBSERVATIONS UPON THE RESPONSES OF NEUROGLIAL CELLS WHICH FOLLOW AXOTOMY OF ADJACENT NEURONES

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SUMMARY

1. The dry mass and nucleic acid content of freshly isolated neuroglial cells and of their nucleoli were measured by interference microscopy and ultraviolet absorption microspectrography. The incorporation of tritiated nucleosides and of an amino acid was followed autoradiographically.

2. After hypoglossal axotomy in the adult rat hypertrophy of astrocytes of the hypoglossal nucleus occurred in a biphasic manner. The first phase lasted from days 1–10 and was accompanied by a small degree of astrocytic hyperplasia, and the second from days 20–80. Hypertrophy of oligodendrocytes accompanied the second phase of the astrocytic response.

3. When severed axons failed to reinnervate denervated muscle, the second phase of the astrocytic response was markedly reduced and the hypertrophy of oligodendrocytes did not occur.

4. If the severed axons re-innervated denervated muscle after a controlled delay, the second phase of the astrocytic response and the oligodendroglial hypertrophy was also delayed.

5. Injection of botulinum toxin into the tongue caused changes in astrocytes and oligondendrocytes closely resembling those found after axotomy.

6. Transient astrocytic hypertrophy occurred in the uninjured right hypoglossal nucleus, and had a different time course to the changes occurring on the injured side.

7. The results are discussed in relation to changes in the metabolism and to alterations in the dendritic fields of injured neurones, previously measured in these circumstances.

INTRODUCTION

Division of a peripheral nerve is followed by metabolic changes in the nerve cell bodies (Bråttgard, Edström & Hydén, 1957), by retraction (Cerf & Chacko, 1958) and re-expansion (Sumner & Watson, 1971) of their dendritic tree, and by an associated initial decrease and a later restoration in the number of presynaptic terminals (F. Sutherland & B. E. H. Sumner, unpublished observations). Changes in neuronal metabolism have been measured under these circumstances using quantitative techniques of cell biology (Watson, 1965, 1966*a*, 1968, 1969). Concurrent changes in perineuronal glial cells have been observed histologically (Cammermeyer, 1955; Rapoš & Bakoš, 1959; Watson, 1965; Kreutzberg, 1966, 1967; Sjöstrand, 1966; Friede & Johnstone, 1967; Roessmann & Friede, 1968; Adrian, 1969; Adrian & Smothermon, 1970), but are difficult to interpret because of the limited reproducibility of metal impregnation techniques, and because of the problems of classifying glial cells by nuclear morphology when basophilic stains are used, especially when the nuclei are partially obscured in autoradiographs. Neuroglial cells may be isolated from discrete parts of the brain (Sumner & Watson, 1972): this paper reports the application to such cells of quantitative cytochemical methods, and describes changes which occur in the hypoglossal nuclei of the rat after dividing a hypoglossal nerve, or after procedures designed to clarify some mechanisms of the glial cell response.

METHODS

Animals. Observations were made upon male albino rats aged 3 months at the time of the first procedure, and between 3 months and 10 months at the time of death: they received standard food pellets and water, supplemented with bread and milk *ad libitum*.

Initial procedure. Five experimental groups of rats were studied over a period of 2 years: each group consisted of several series treated similarly at different times: series of rats from each group were designed to overlap and run concurrently with those of other groups to minimize possible variation from seasonal change or from slowly changing observer error. In rats of the first group the left hypoglossal nerve was crushed at one of three sites; centrally at the level of the carotid bifurcation, proximally at its emergence through the anterior condylar foramen, or distally, the branches of the nerve being crushed within the tongue (Watson, 1968). In the second group, the left hypoglossal nerve was implanted into the ipsilateral, normally innervated sternomastoid, to impede reinnervation of denervated muscle. In the third group, the left hypoglossal nerve was implanted into sternomastoid: 70 days later, after the initial response of the hypoglossal neurones to axotomy had subsided (Watson, 1970), the left spinal accessory nerve was divided, bringing the intramuscular axons of the previously divided hypoglossal nerve into contract with denervated muscle. Study of groups two and three allowed the responses of nerve cells and neuroglia to initial nerve injury to be separated temporally from those responses

which followed muscular reinnervation. In the fourth group, 70 days after implanting the divided hypoglossal nerve into sternomastoid, the hypoglossal nerve was crushed. Study of groups two and four allowed the significance of metabolic change in the nerve cell, which occurred under these circumstances (Watson, 1970) to be distinguished from the significance of retraction and re-expansion of the dendritic tree, which was already retracted following the previous axotomy, and underwent no re-expansion until the axon reinnervated denervated muscle (Summer & Watson, 1971). In the fifth group 250 pg botulinum toxin type D was injected into the extrinsic and intrinsic muscles of the left half of the tongue (Watson, 1969) to impede neuromuscular transmission without loss of axoplasm.

Preparation of cells and their measurement. Between 1 and 200 days after the initial procedure, a rat was lightly anaesthetized with ether and bled to death. The part of the hypoglossal nucleus rostral to the obex was removed rapidly and glial cells prepared as described previously (Summer & Watson, 1972). The dry mass of the cell or of its nucleolus was measured by interference microscopy (Watson, 1969): the nucleic acid content was determined by ultraviolet absorption microspectrography (Watson, 1968). In autoradiographic studies tritiated [5-^{3}H]uridine (1·22 Ci/m-mole) was used to follow the incorporation of nucleotide into ribonucleic acid (RNA), tritiated [methyl-^{3}H]thymidine (3·3 Ci/m-mole) was used to indicate synthesis of deoxyribonucleic acid (DNA), and tritiated [4,5-^{3}H]leucine (2·7 Ci/m-mole) was used to follow amino acid incorporation into protein. Isotopes were diluted so that the composition of the final solution resembled that of cerebrospinal fluid (Mitchell, Loeschke, Massion & Severinghaus, 1963): the final activity of the amino acid or uridine solution was 0·8 mCi/ml. and of the thymidine solution 0·2 Ci/ml. 50 μl. was injected into a lateral cerebral ventricle under light ether anaesthesia. Rats were killed 90 min after injecting uridine and 60 min after leucine: previous unpublished studies had indicated that these intervals were optimum for detecting the altered uptake and incorporation which accompanied altered nucleolar nucleic acid content and dry mass. The autoradiographic techniques have been described previously (Watson, 1965; Sumner & Watson, 1972). Suitable controls excluded significant chemical reduction or densitization of the photographic emulsion.

With no technique was selection permitted of the cells measured. Between 20 and 200 cells of each type were analysed in each rat: the first cells seen were measured, and the slides were scanned in a uniform way. In the results presented graphically each point represents a mean value obtained from one hypoglossal nucleus, and the standard error of the mean is also indicated. Only Fig. 1 shows all the measurements made in an experimental series: the remaining Figures show only the astrocytic and oligodendroglial dry mass: the significance of change in other parameters is described in the text.

Identification of cells. Isolated glial cells of the hypoglossal nucleus could be distinguished without difficulty from neurones, and over 95% of the macroglia could be classified as astrocytes or oligondendrocytes. Astrocytes were recognized initially by their characteristic attachment to blood vessels and by their many long, thin, branching processes (Pl. 1*A*). Such cells could be identified apart from the associated vessel (Pl. 1*B*). Ependymal cells over the rostral part of the hypoglossal nucleus were also attached to blood vessels by a basal process, but could be distinguished from astrocytes by the presence of many cilia and the absence of additional long processes (Pl. 1*C*). Oligodendrocytes had a dense oval nucleus, usually with a prominent nucleolus, scanty perinuclear cytoplasm and few sturdy processes which branched occasionally (Pl. 1*D*). When glial cells are covered with a layer of photographic emulsion, identification remains possible (Pl. 1*E*), but is less easy as the characteristic pattern of the branching processes may be slightly obscured. Pericytes

were identified on the blood vessel wall only when seen tangentially: the nucleus was oval and surrounded by thin slips of cytoplasm often containing a few refractile droplets 1–2 μm in diameter (Pl. 2).

Technical problems associated with measurement

Measurements were made upon the 'total' cell as isolated, and upon its nucleolus: in addition, isotope uptake was studied. Each of these independent methods of indicating glial response had disadvantages: it is for this reason that all three measurements were made, and they were presented as indicating a real change in glia only if the results obtained with all were compatible, and if each independently gave changes of statistical significance.

Measurements upon isolated cells. It is most likely that the glial cells isolated were shorn of a significant proportion of their processes: it is likely that the remaining processes were contaminated by adhering fragments of other cells: unpublished electron micrographs indicate both of these tendencies. It is possible that errors from this source have varied in a way that was not random, as the mutual adherence of glia and nerve cells changed after axotomy (Watson, 1966*b*), a correlate of increased hydrolysis of cell surface substances (unpublished observations). It was for this reason that measurements were not made upon 'total' cell dry mass alone but also upon glial nucleoli, and that autoradiographic studies of [^{3}H]uridine incorporation were made 90 min after isotope injection, a time at which over 90% of the label was still within the nucleus. These measurements were independent of loss of cytoplasm from cell processes, or of contamination of the processes by adhering cytoplasmic fragments of other cells.

Measurements upon nucleodi. Glial cells could be identified confidently only by the quantity and pattern of branching of the cytoplasm (Pl. 1). Identification of isolated glial nuclei as astrocytic, ependymal or oligodendrocytic was found to be impossible. Unlike such measurements in isolated neuronal nuclei (Watson, 1968, 1969, 1970), the nucleolar dry mass and nucleic acid content of neuroglial cells had to be measured within the total isolated cell, including thin covering and underlying layers of perinuclear cytoplasm in addition to nucleoplasm. Initial studies indicated that the values obtained for nucleolar dry mass and nucleic acid content were over-estimated by 18 and by 12% respectively: similar values were obtained in cells reacting after axotomy. No correction has been made for these errors in the results presented.

In 28% of normal astrocytes of the hypoglossal nucleus no nucleolus could be resolved by light microscopy: virtually all astrocytes reacting after axotomy had a prominent nucleolus. If the nucleolar dry mass or nucleic acid content of normal astrocytes having no visible nucleolus were recorded as zero, the mean values for normal astrocytes would probably have been falsely reduced and the statistical significance of the increases following axotomy fallaciously enhanced. The maximum dry mass of a glial nucleolus which would remain below the limits of resolution with the optical system used was calculated to be 20 femtogrammes (fg), and the maximum nucleic acid content 4 fg. It was assumed that all astrocytes in which a nucleolus could not be seen had nucleolar parameters of these values: as this correction raised the value of the nucleolar parameters of the normal astrocyte to a maximum, but had very little influence on the values of the reacting astrocyte, this assumption could reduce the statistical significance of the increases found after axotomy to a degree less than the true significance of the change.

Autoradiography. The incorporation of thymidine by cells to indicate DNA synthesis posed no particular problems, as the cells were heavily labelled and recorded only as labelled or not. Grain counts of cells which had incorporated uridine into nuclear RNA would have been influenced by the absorption of low energy radiation

by overlying cytoplasm, an effect which would have become more marked with an increase in the cell's dry mass. Under these conditions of hypertrophy, however, the nuclear grain count increased, and errors from increased self absorption would only have tended to decrease the statistical significance of any true increase of nucleoside incorporation, and not to have given a falsely positive result. Grain counts over the whole cell following administration of amino acid were open to the double criticism of cell fragmentation and contamination described above and must be accepted cautiously. Problems of interpreting grain counts in relation to routes of isotope administration, pool size, alternative metabolic pathways and macromolecular synthesis have been considered previously (Watson, 1965).

RESULTS

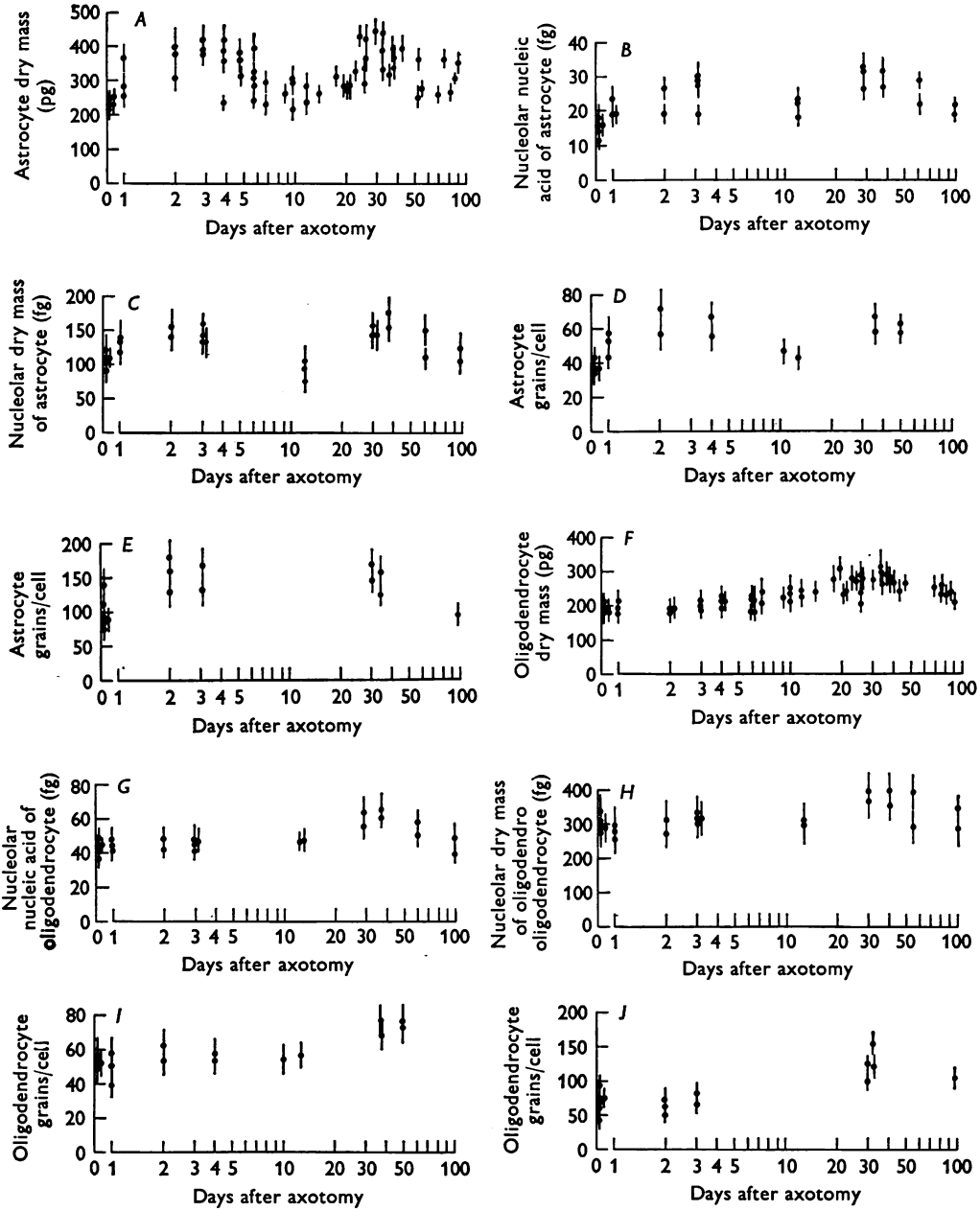
Changes following nerve injury (*rats of the first group*). A biphasic increase in the dry mass of astrocytes followed crushing the nerve at the level of the carotid bifurcation (Text-fig. 1*A*). The first phase lasting from day 1 to about day 10 was significant ($P < 0{\cdot}01$: $> 0{\cdot}001$). The second phase from day 20 to day 80 was also significant ($P < 0{\cdot}01$: $> 0{\cdot}001$). Between days 12 and 16 the dry mass was not significantly raised ($P > 0{\cdot}05$). These increases were accompanied by significant increases in nucleolar dry mass (first phase $P < 0{\cdot}01$: $> 0{\cdot}001$: second phase $P < 0{\cdot}01$: $> 0{\cdot}001$) and nucleic acid content (first phase $P < 0{\cdot}05 > 0{\cdot}01$: second phase $P < 0{\cdot}01$: $> 0{\cdot}001$) (Text-fig. 1*B*, *C*), by a significantly increased rate of incorporation of uridine ($P < 0{\cdot}05$: $> 0{\cdot}01$) into RNA (Text-fig. 1*D*) which could be extracted with ribonuclease and by an increased rate of incorporation of leucine ($P < 0{\cdot}05$: $> 0{\cdot}01$) (Text-fig. 1*E*).

The dry mass of oligodendrocytes did not change during the first phase of the astrocytic response, but increased significantly ($P < 0{\cdot}001$) between 20 and 80 days after axotomy (Text-fig. 1*F*). This late increase was accompanied by an increase in nucleolar dry mass and nucleic acid content (Text-fig. 1*G*, *H*) ($P < 0{\cdot}01$: $> 0{\cdot}001$ for each), and by an increased rate of incorporation of uridine ($P < 0{\cdot}05$: $> 0{\cdot}01$) (Text-fig. 1*I*) and leucine ($P < 0{\cdot}05$: $> 0{\cdot}01$) (Text-fig. 1*J*). Ependymal cells remained unchanged.

The latency of the first phase of the astrocytic response was not significantly influenced ($P > 0{\cdot}05$) by the site of nerve injury (Text-fig. 2*A*, *B*).

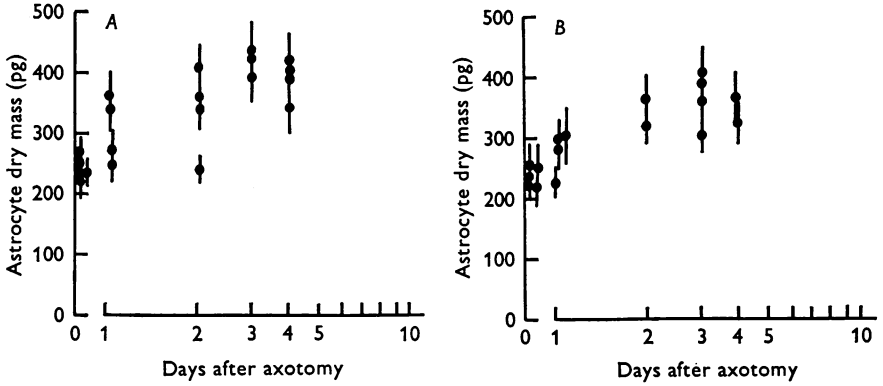
The latency of nucleolar changes and of the increased rate of uridine incorporation was similarly unaffected ($P > 0{\cdot}05$).

Changes following nerve transplantation (*rats of the second group*). The second phase of the astrocytic response was significantly reduced ($P < 0{\cdot}01$: $> 0{\cdot}001$) when re-innervation of denervated muscle was impeded, and the small increase in dry mass found between days 20 and 80 was not statistically significant ($P > 0{\cdot}05$) (Text-fig. 3*A*). No significant change was found during this period in the astrocytes' nucleolar dry mass or nucleic acid content ($P > 0{\cdot}05$ for each), or in the uptake of labelled uridine or



Text-figs. 1*A–J*. For legend see opposite page.

leucine ($P > 0{\cdot}05$ for each). The first phase of the response of the astrocyte between days 1 and 10 resembled that found after axotomy (compare Text-figs. 1 and 3). No significant increase was found in the dry mass of oligodendrocytes ($P > 0{\cdot}05$) (Text-figs. 3*B*), in the dry mass or nucleic acid content of their nucleoli ($P > 0{\cdot}05$ for each) between 20 and 80 days



Text-fig. 2. Changes occurring in the dry mass of astrocytes (pg) of the left hypoglossal nucleus following axotomy of the left hypoglossal nerve at different sites are expressed against the time in days since nerve injury. *A*, nerve injured proximally at anterior condylar foramen. *B*, branches of nerve injured distally within tongue.

after nerve transplantation, or in the incorporation of uridine ($P > 0{\cdot}05$) or leucine ($P > 0{\cdot}05$). Ependymal dry mass did not change.

Changes following nerve transplantation, followed by division of spinal accessory nerve (*rats of third group*). A significant slow increase in the dry mass of astrocytes ($P < 0{\cdot}01$: $> 0{\cdot}001$) (Text-fig. 4*A*) and of oligodendroglia ($P < 0{\cdot}01$: $> 0{\cdot}001$) (Text-fig. 4*B*) followed division of the spinal accessory nerve, and was accompanied by an increase in the nucleolar dry

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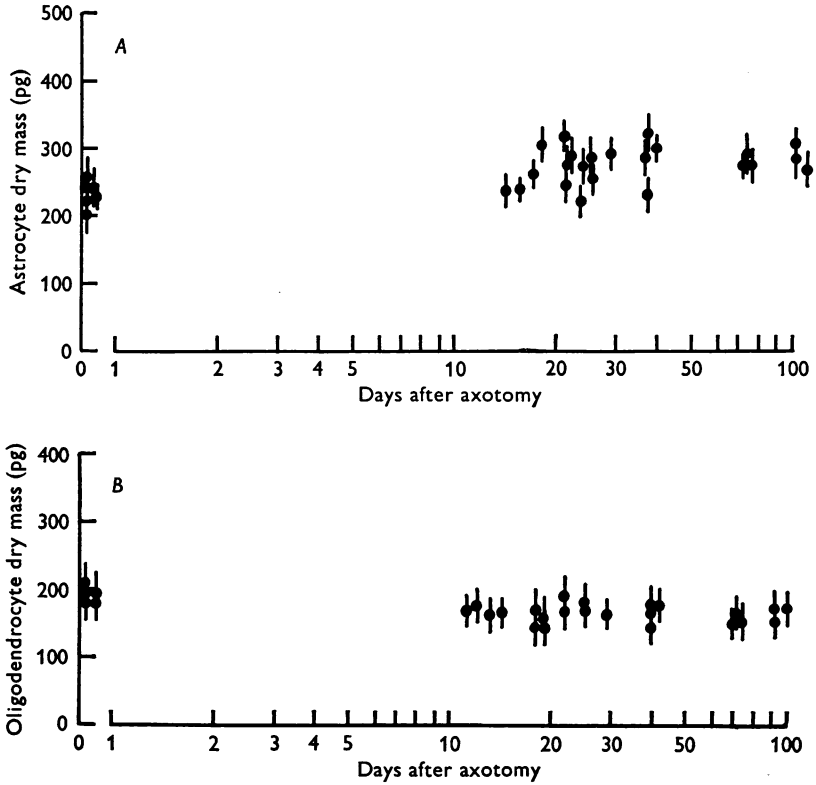
Legend to Figs. 1*A–J*.

Text-fig. 1. Changes occurring in the left hypoglossal nucleus following axotomy of the left hypoglossal nerve at the level of the carotid bifurcation are expressed against the time in days since nerve injury. *A*, astrocyte: dry mass, pg. *B*, astrocyte: nucleolar nucleic acid, fg. *C*, astrocyte: nucleolar dry mass, fg. *D*, number of grains/cell in autoradiographs of astrocytes after injecting [^{3}H]uridine. *E*, number of grains/cell in autoradiographs of astrocytes after injecting [^{3}H]leucine. *F*, oligondendrocyte: dry mass, pg. *G*, oligodendrocyte: nucleolar nucleic acid, fg. *H*, oligodendrocyte: nucleolar dry mass, fg. *I*, number of grains/cell in autoradiographs of oligodendrocytes after injecting [^{3}H]uridine. *J*, number of grains/cell in autoradiographs of oligodendrocytes after injecting [^{3}H]leucine.

mass ($P < 0{\cdot}05$: $> 0{\cdot}01$) and nucleic acid content ($P < 0{\cdot}05$: $> 0{\cdot}01$) of astrocytes and of oligondendroglia ($P < 0{\cdot}05$: $> 0{\cdot}01$ and $P < 0{\cdot}01$: $> 0{\cdot}001$ respectively), and by an incorporation of uridine and leucine by both cell types ($P < 0{\cdot}05$: $> 0{\cdot}01$ for each). The ependyma remained unaltered.

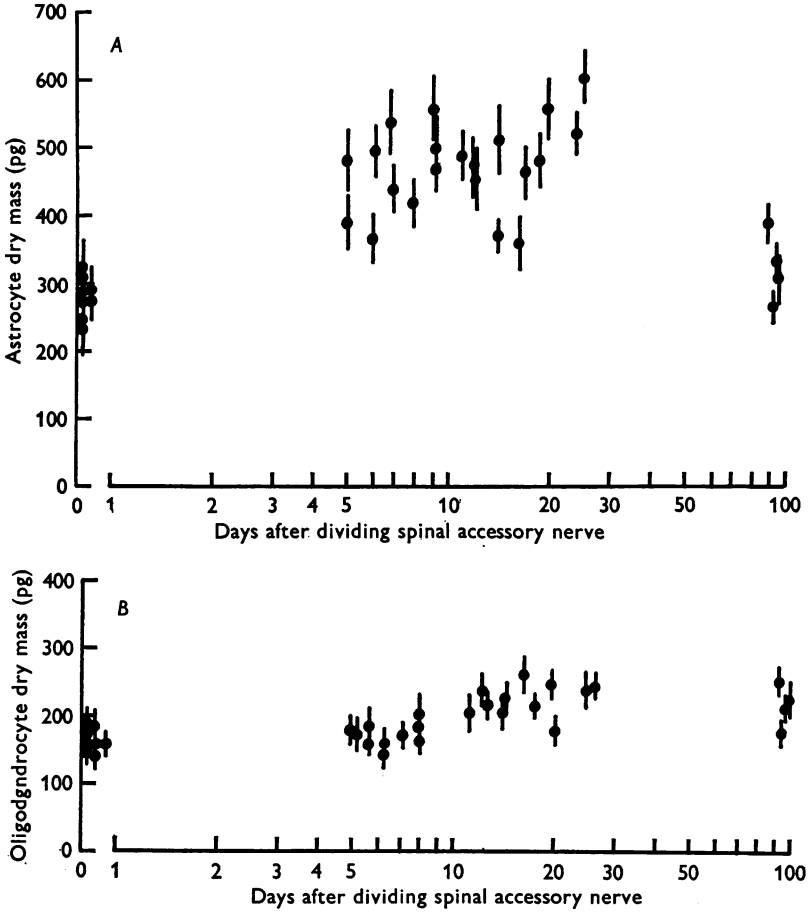


Text-fig. 3. Changes occurring in the left hypoglossal nucleus following implantation of the divided left hypoglossal nerve into the ipsilateral sternomastoid muscle are expressed against the time in days since nerve implantation. *A*, astrocyte: dry mass, pg. *B*, oligodendrocyte: dry mass, pg.

Changes following injury of the hypoglossal nerve 70 days after its transplantation (rats of the fourth group). Neither phase of the response of the astrocyte was statistically significant ($P > 0{\cdot}05$) (Text-fig. 5*A*). No change could be detected in the dry mass of the oligondendrocyte ($P > 0{\cdot}05$) (Text-fig. 5*B*). No significant change was found in the nucleolar parameters of either cell type ($P > 0{\cdot}05$ for each). No isotope studies were made.

Changes following botulinum toxin (rats of the fifth group). Injection of botulinum toxin caused changes in astrocyte dry mass ($P < 0{\cdot}05$: $> 0{\cdot}01$

for the first phase; $P < 0{\cdot}05$: $> 0{\cdot}01$ for the second phase) (Text-fig. 6*A*), nucleolar dry mass ($P < 0{\cdot}05$: $> 0{\cdot}01$ for the first phase; $P < 0{\cdot}01$: $> 0{\cdot}001$ for the second phase) and nucleic acid content ($P < 0{\cdot}05$: $> 0{\cdot}01$ for each phase) and in oligondendrocyte dry mass ($P < 0{\cdot}05$: $> 0{\cdot}01$)

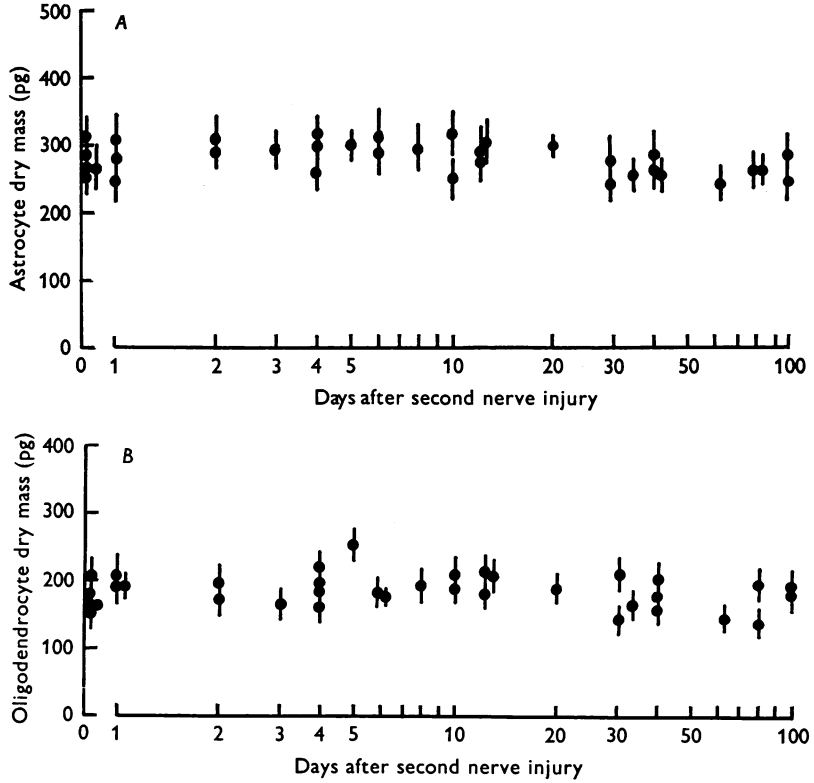


Text-fig. 4. Changes occurring in the left hypoglossal nucleus following division of the left spinal accessory nerve 70 days after implanting the left hypoglossal nerve into the left sternomastoid are expressed graphically against the time in days since spinal accessory nerve division. *A*, astrocyte: dry mass, pg. *B*, oligodendrocyte: dry mass, pg.

(Text-fig. 6*B*), nucleolar dry mass ($P < 0{\cdot}05$: $> 0{\cdot}01$) and nucleic acid content ($P < 0{\cdot}01$: $> 0{\cdot}001$) closely resembling those found after axotomy (compare Text-figs. 1 and 6). These changes were accompanied by significant changes ($P < 0{\cdot}05$: $> 0{\cdot}01$) in the incorporation of uridine and of leucine ($P < 0{\cdot}05$: $> 0{\cdot}01$).

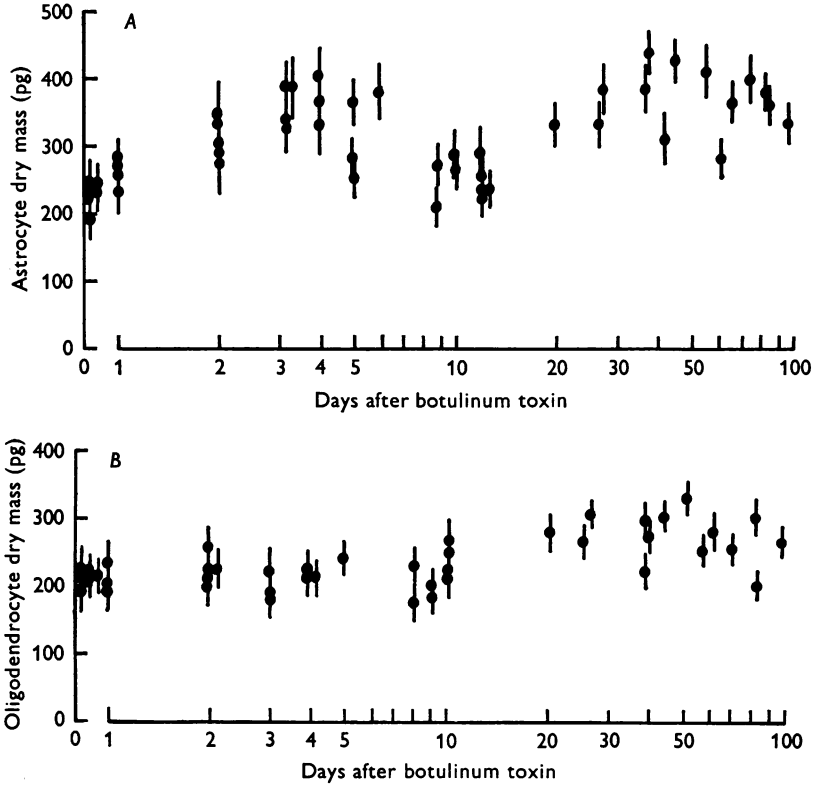
Pericytes and capillaries. A small increase in the dry mass both of pericytes and of capillary endothelial cells was found between days 10 and 80 after axotomy: neither reached statistical significant ($P > 0{\cdot}05$).

Changes in the uninjured right hypoglossal nucleus. Neuroglia of the right hypoglossal nucleus changed after the left nerve had been divided. The timing of these changes did not resemble that of the injured side. The dry

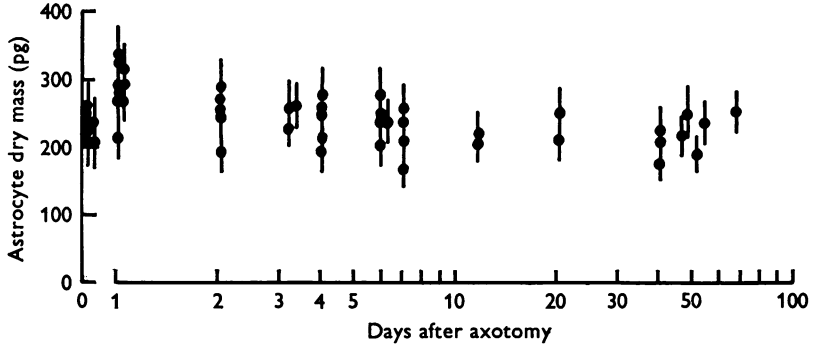


Text-fig. 5. Changes occurring in the left hypoglossal nucleus following second division of the left hypoglossal nerve 70 days after its division and coincident implantation into the left sternomastoid muscle are expressed graphically against the time in days since the second division. *A*, astrocyte dry mass, pg. *B*, oligodendrocyte dry mass, pg.

mass of astrocytes increased to a maximum on the first day after axotomy ($P < 0{\cdot}01$: $> 0{\cdot}001$) and then declined rapidly to reach normal values by the sixth day (Text-fig. 7). This was accompanied by significant increases in nucleolar dry mass ($P < 0{\cdot}05$: $> 0{\cdot}01$) and nucleic acid content ($P < 0{\cdot}001$) and by an increased incorporation of uridine ($P < 0{\cdot}05$: $> 0{\cdot}01$) and of leucine ($P < 0{\cdot}05$: $> 0{\cdot}01$). No later change was found in astrocytes or oligodendrocytes.

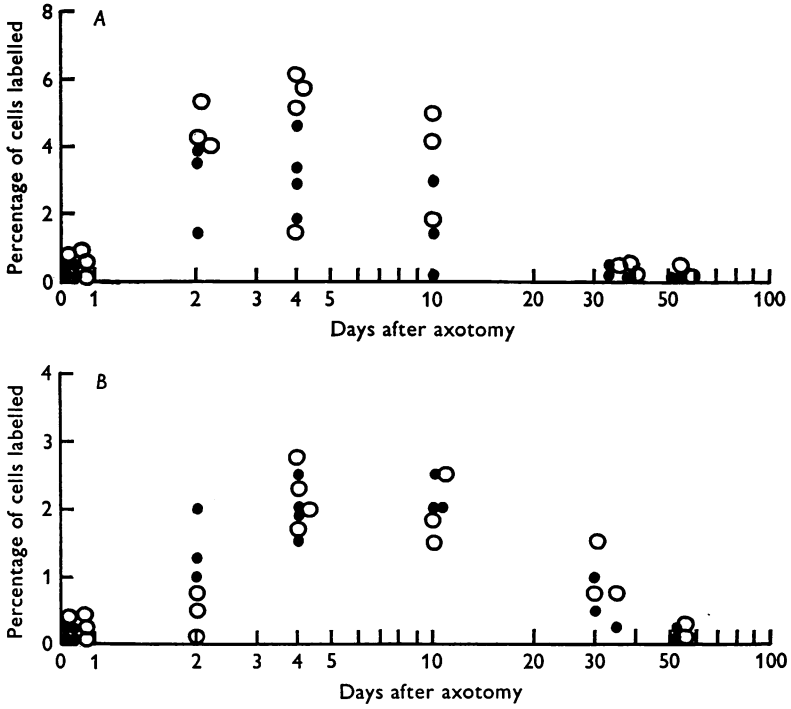


Text-fig. 6. Changes occurring in the left hypoglossal nucleus following injection of botulinum toxin into the left half of the tongue are expressed graphically against the time in days since injection. *A*, astrocyte: dry mass, pg. *B*, oligodendrocyte: dry mass, pg.



Text-fig. 7. Changes occurring in the dry mass of astrocytes (pg) of the uninjured right hypoglossal nucleus are expressed graphically against the time in days since axotomy of the left hypoglossal nerve.

Synthesis of DNA in nucleus of injured nerve. The percentage of astrocytes labelled after injecting [^{3}H]thymidine 4 hr before death increased significantly ($P < 0{\cdot}01$) during the first phase of the astrocytic response (Text-fig. 8*A*): no labelled oligodendrocytes or ependymal cells were seen. The 5 % of macroglial cells that could not be firmly identified as astrocytes or as oligodendrocytes showed a significant increase in DNA synthesis: this



Text-fig. 8*A*. The percentage of astrocytes (●) and of unidentified macroglial cells (○) found labelled in the left hypoglossal nucleus 4 hr after injecting [^{3}H]thymidine is expressed graphically against the time in days since axotomy of the left hypoglossal nerve.

B, the percentage of pericytes (●) and of capillary endothelial cells (○) found labelled in the left hypoglossal nucleus 4 hr after injecting [^{3}H]-thymidine is expressed graphically against the time in days since axotomy of the left hypoglossal nerve.

is indicated separately ($P < 0{\cdot}01$: $> 0{\cdot}001$) (Text-fig. 8*A*). The percentage of labelled capillary endothelial cells and pericytes also increased significantly ($P < 0{\cdot}05$: $> 0{\cdot}01$) (Text-fig. 8*B*). These changes were most marked during the first 14 days: the second phase of the astrocytic response was not accompanied by significant DNA synthesis.

Many labelled macrophages were also seen between 2 days and 14 days after axotomy: some of these were found on the walls of venules, but seldom on capillaries.

DISCUSSION

Methods. The limitations of the methods have been discussed (Watson, 1965, 1968, 1969, 1970): their application to neuroglial cells was not seriously impeded by difficulties of cell identification. In the hypoglossal nucleus the neurones were large and could be distinguished from neuroglia. The 5% of macroglial cells which could not be classified as astrocytes or as oligodendrocytes behaved like astrocytes except for a greater degree of DNA synthesis and a smaller increase of dry mass in the first phase. It is likely that they were a mixed population consisting predominantly of atypical astrocytes but including some oligodendrocytes, an admixture possibly responsible for the smaller increase in dry mass: these cells could include glia of the 'third type' (Vaughn, 1969; Vaughn, Hinds & Skoff, 1970; Skoff & Vaughn, 1971).

Significance of measured change. An increased dry mass may indicate increased synthetic activity or storage, phagocytosis with retained inclusions, or decreased catabolism. The associated increases in nucleolar dry mass and nucleic acid content indicate that the observed increases in dry mass were at least partially a consequence of augmented synthetic activity (Caspersson, 1940; Watson, 1968), an interpretation supported by the raised incorporation of labelled nucleoside and amino acid (Watson, 1965).

Correspondence of neuronal and macroglial changes after axotomy

Metabolic responses of hypoglossal neurones to axotomy have been described previously (Bråttgard, Edström & Hydén, 1957; Watson, 1965, 1966*a*, *b*, *c*, 1968, 1969). A component of the responses due to loss of effective contact of nerve with muscle was separated from a later component due to the re-establishment of contact by implanting the severed hypoglossal nerve into sternomastoid and by dividing later the ipsilateral spinal accessory nerve (Watson, 1970). Retraction of the dendrites followed loss of functional neuromuscular contact (Cerf & Chacko, 1958), and their re-expansion occurred after restoration of such contact with either the same or with different muscle (Sumner & Watson, 1971). The phase of retraction was accompanied by a significant loss of presynaptic terminals, and re-expansion was associated with an increase to their previous density (B. E. H. Sumner & F. Sutherland, unpublished observations). Studies with botulinum toxin (Watson, 1969; Sumner & Watson, 1971) indicated that the metabolic and dendritic responses of the neurone were not due to

cell injury or to loss of axoplasm but to its capacity to establish a functional relationship with muscle.

Astrocytes. The neuroglial changes had many points of correspondence with those of the neurone. The dry mass of astrocytes increased both during the period immediately following loss of neuromuscular contact, whether caused by axotomy (Text-figs. 1*A*, 2*A*, 2*B*) or by botulinum toxin (Text-fig. 6*A*), and also during the re-establishment of contact with the same (Text-fig. 1*A*, 6*A*) or with different (Text-fig. 4*A*) muscle. The correspondence was not precise, however: the first increase in astrocytic dry mass preceded the onset of those neuronal metabolic changes which have so far been measured. The latency of the astrocytic response was not affected by the site of nerve injury (Text-fig. 1*A*, 2*A*, 2*B*), whereas the latency of neuronal change was greater when the axon was injured distally (Watson, 1968). The latency of changes in presynaptic endings of the hypoglossal nucleus following hypoglossal axotomy was not influenced by the site of nerve section and was similar after botulinum toxin (unpublished observations) and corresponded with the primary response of the astrocytes. A similar correspondence was found between an increase in dry mass of astrocytes of the paraventricular nucleus and changes in presynaptic terminals after injecting 6-hydroxydopamine into the cerebral ventricles (Watson, 1972*a*), a drug which was taken up by noradrenergic boutons close to the ventricular surface (Iversen & Uretsky, 1970; Ungerstedt, 1971; Constantinides, Geissbuhler & Tissot, 1971) and destroyed them: under these circumstances a transient decrease in the synthetic activity of the post-synaptic neurone accompanied the increased metabolic activity of the astrocytes. Similarly, following optic nerve section or division of a dorsal root, Wallerian degeneration of the terminal boutons was associated with hypertrophy and hyperplasia of the adjacent astrocytes (unpublished observations). A circumstantial case can therefore be made for the presumptive association of astrocytic reactions with changes in the presynaptic terminals rather than in the post-synaptic neurone. This case is strongly supported by the changes following two consecutive divisions of hypoglossal axons, when re-innervation of muscle was not permitted (rats of group 4) (Text-fig. 5*A*). Under these circumstances metabolic changes occurred in the nerve cells after the second nerve injury (Watson, 1969), but the dendritic field, being already retracted, showed no further retraction (Sumner & Watson, 1971): the absence of astrocytic reaction (Text-fig. 5*A*) strongly suggested correlation of the responses of these cells with the dendritic behaviour of the injured neurones and with alterations in presynaptic endings, but not with metabolic changes as such in the injured nerve cell.

The second phase of the response of astrocytes only followed re-inner-

vation of denervated muscle (Text-figs. 1*A*, 4*A*, 6*A*: compare with 3*A*), and accompanied the restoration of the reduced dendritic field and of the population of presynaptic boutons (Sumner & Watson, 1971), and failed to occur when re-innervation was impeded (rats of group 2) (Text-fig. 3*A*), and dendritic re-expansion did not occur (Sumner & Watson, 1971). This second phase occurred while the metabolic response of the injured cell was decreasing, and after it had attained normal values (Watson, 1965, 1968, 1969).

Oligodendrocytes. The dry mass of oligodendrocytes increased only when contact between nerve and muscle was re-established (Text-figs. 1*F*, 4*F*, 6*F*) and not when such contact was persistently prevented (Text-fig. 3*F*). Their response was dissociated completely from the metabolic response of the reacting post-synaptic cell (Watson, 1968, 1969), but coincided with re-expansion of the dendritic field.

Functional significance of glial changes

Astrocytes. Concurrent change in astrocytes and in presynaptic endings does not necessarily imply direct functional interaction. Responses of astrocytes must be considered in two contexts: first, within the general context of epithelial cells as such, for brain develops as an epithelium and its persisting epithelial attributes can be seen most characteristically in ependymal cells and in astrocytes: each, for example, is related to underlying mesenchyme (Feigin, 1969) (Pl. 1) through the interface of a basal lamina and cells of each type communicate with adjacent cells by gap junctions (Brightman & Reese, 1967, 1969*a*, *b*): secondly, within the context of a highly differentiated epithelial cell possibly having a specialized relationship of functional interaction with adjacent neuronal processes.

In the first context the astrocytes may be viewed as forming an epithelium co-extensive with the neuronal elements of brain but not necessarily interacting directly with them. The changes reported here would then have been an indirect consequence of break-down and re-establishment of neuronal connexions and would have played no part in modifying neuronal events. The first phase of the response resembled the reaction of these cells to direct local brain trauma (Osterberg & Wattenberg, 1962; Cavanagh, 1970, unpublished observation), to bouton degeneration associated with Wallerian degeneration in the central nervous system (unpublished observations), and to pharmacological injury (Watson, 1972*a*). Although the nature, latency and duration of the changes resembled closely changes seen in healing wounds of skin and other epithelia (Russell & Billingham, 1962; Ordmann & Gillman, 1966; Ross & Odland, 1968; Croft & Tarin, 1970; Tarin & Croft, 1970), they differ in that the astrocytes were not previously directly electrically coupled to the nerve cells before injury (Orkand,

Nicholls & Kuffler, 1966; Grossman, Whiteside & Hampton, 1969; Kuffler & Potter, 1964; Miller & Dowling, 1970); it is therefore unlikely that the ionic mechanisms of epithelial hyperplasia, hypertrophy and migration (Loewenstein, 1966) may be applied directly in this context. The phase of synaptic dissociation was concurrent with an increased rate of break-down of substances of the cell surface (unpublished observations), and with alteration in the number and distribution of neuronal lysosomes containing N-acetyl-β-glucosaminase (B. E. H. Sumner, personal communication). The social behaviour of cells in culture has been found to be markedly influenced by hydrolysis of components of the cell surface (Burger, 1969, 1970; Burger & Noonan, 1970). Striking hyperplasia and hypertrophy of astrocytes was caused by perfusing the cerebral ventricles with dilute solutions of hydrolytic enzymes, or with products of such hydrolysis, such as amino sugars (unpublished observation), substances known to impair the aggregation of cells *in vitro* (Garber, 1963) or amino acids. It is likely that the dissociation of synapses accompanying the first phase of the astrocytic response was associated with such extracellular hydrolysis both by neuronal lysosomes, and by macrophages which divide (Blinzinger & Kreutzberg, 1968; Adrian & Smothermon, 1970) and are present in increased numbers at this time (Rapoš & Bakoš, 1959; Watson, 1965; Sjöstrand, 1966); a role for calcium is not excluded by this proposed mechanism as this ion is bound extensively to such surface substances (Carvalho, Sanui & Pace, 1963).

Within this context the first phase of the astrocytic response could be an indirect and irrelevant consequence of neuronal changes. It is unlikely that the second phase of the astrocytic response can be interpreted in this way as the considerable macrophage reaction accompanying the first response is far smaller in the second (Watson, 1965); this makes it improbable that the re-expansion of the dendrites accompanied by an increasing number of presynaptic boutons was associated with a greatly increased rate of turnover of boutons with a pattern of extensive selection and removal.

Within the second context, there is no evidence that astrocytes took part in significant phagocytosis of preterminal boutons: it is likely that the intra-astrocytic membrane wrapped boutons (Conradi, 1969) which undergo degradation slowly if at all (Brodal, 1967; Westman, 1969) were not phagolysosomes but endings wrapped in folds of plasma membrane derived originally from the perisynaptic barrier (de Robertis & Gerchenfeld, 1961; Eccles, Eccles & Fatt, 1956; Curtis & Eccles, 1958, 1959; Peters & Palay, 1965, 1966) which had collapsed away from the post-synaptic cell following extracellular hydrolysis. Evidence is lacking that the astrocyte acted as a significant metabolic reservoir for the nerve endings, supported them symbiotically, or transported nutrients to the neuronal elements

from the blood (Kuffler & Nicholls, 1966) and contrary evidence has been provided for invertebrate glia when the neurones were under more normal conditions (Kuffler & Nicholls, 1965). A specific excretory role for the astrocyte is similarly speculative: elevation of the arterial P_{CO_2} to between 60 and 80 mm Hg for up to 7 days caused a smaller degree of astrocytic hyperplasia than was observed here (unpublished observations).

To avoid invoking alternative reasons, it is suggested that the astrocytic changes in the second phase resemble those found early in the right hypoglossal nucleus (Text-fig. 7), and represent increased functional activity (Watson, 1968): the changes in the right nucleus are identical with those found in supraoptic and paraventricular nuclei during dehydration or lactation, or in the anterior horns of the cervical region of the spinal cord following repeated exercise on a treadmill (Watson, 1972*b*). The prolongation of the second phase of the response to axotomy reflects the slow anatomical recruitment of new boutons accompanying dendritic growth (Sumner & Watson, 1971), probably with asynchronous and consecutive alterations of their functional states. The causes of changes associated with enhanced stimulation are not known, although elevation of extracellular potassium (Watson, 1971) or ammonia (unpublished observations) by ventricular perfusion can cause similar changes. The functional consequence of the reaction is also indefinite, although it is probable that new perisynaptic barriers (de Robertis & Gerschenfeld, 1961) were formed in association with the new synapses, and possible that the astrocyte was concerned more directly with stabilizing the potassium concentration (Trachtenberg & Pollen, 1970) within this segregated extracellular microenvironment, or with the detoxication of ammonia (Vrba, Foldbergova & Kanturek, 1958). It is of considerable interest that the dry mass of astrocytes returned to normal values at the end of the second phase despite persisting expansion of the dendritic tree, and a bouton density which remained high. This return was not a consequence of late division of hypertrophic astrocytes, as they did not synthesize DNA at this stage, nor did the number of astrocytes increase. A similar transience of the astrocytic response seen when the activity of a neuronal pool is subjected to a prolonged natural enhancement of its functional state (Text-fig. 7*A*) (Watson, 1972*b*), may have been due either to metabolic change in presynaptic terminals with prolonged use, or to a modification in the pattern of afferent stimulation such that the total intensity of presynaptic activity fell to its previous level but was able to sustain an increased rate of post-synaptic discharge.

Oligodendrocytes. As no function can be ascribed unequivocally to the oligodendrocyte except that of myelination, it is suggested that the increased dry mass observed only under conditions of dendritic expansion

(Text-figs. 1*F*, 4*F*, 6*F*) (Sumner & Watson, 1971) was due to myelination of new presynaptic fibres forming new boutons arising either from local collateral sprouts or from elsewhere, or to increased myelination of fibres already functionally related to the post-synaptic nerve cell in response to a possible increase in activity following re-establishment of functional neuromuscular contact. Such a response would resemble 'maturation' in fibres of a peripheral nerve passing to a functionally overloaded muscle (Aitken, Sharman & Young, 1947).

Ependyma. The absence of change in ependymal cells probably resulted from the persisting stability of its environment due to a sink action of the adjacent cerebrospinal fluid (Davson, 1967): it is not a consequence of an intrinsic lack of reactivity of this cell type (Watson, 1971).

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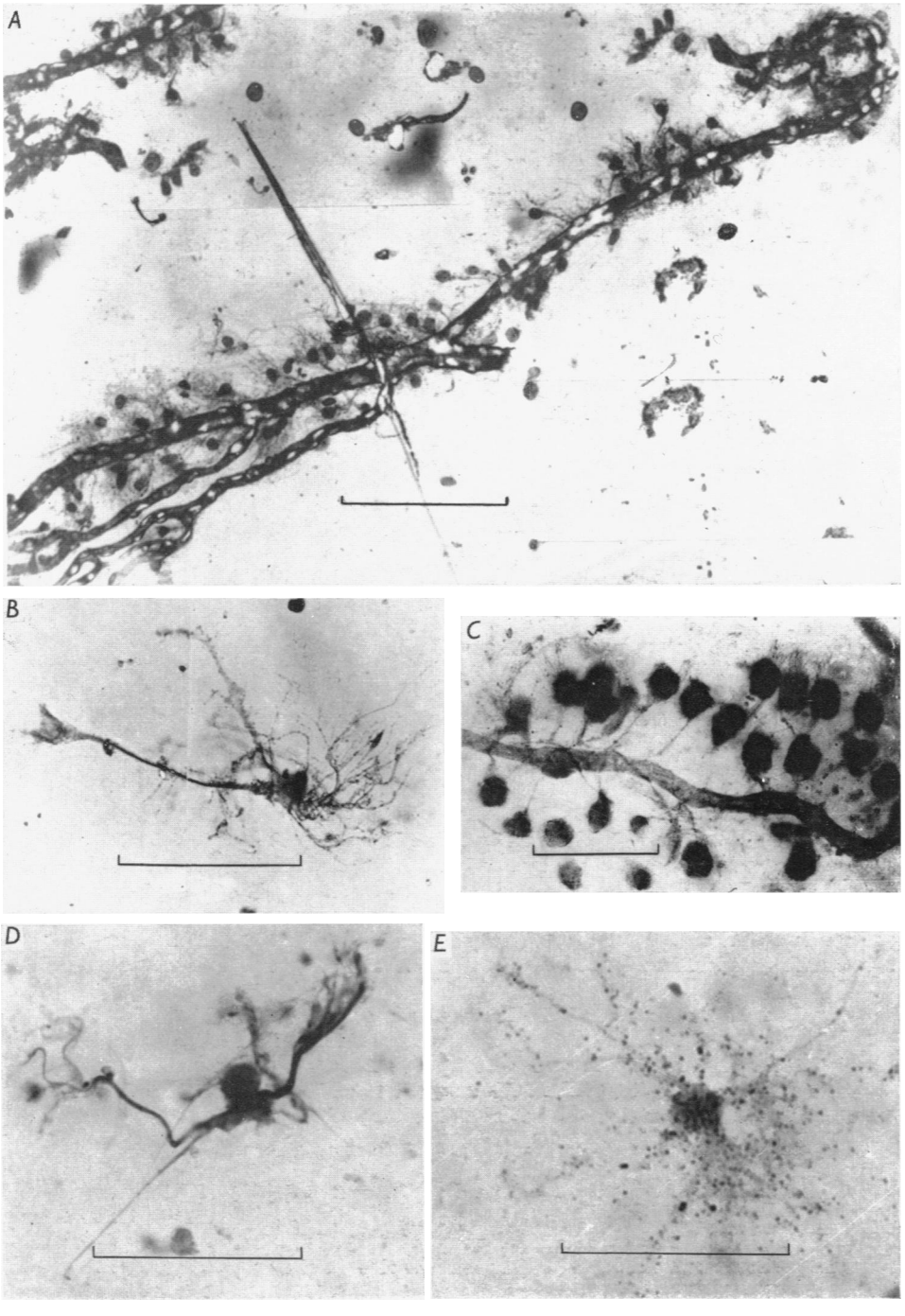
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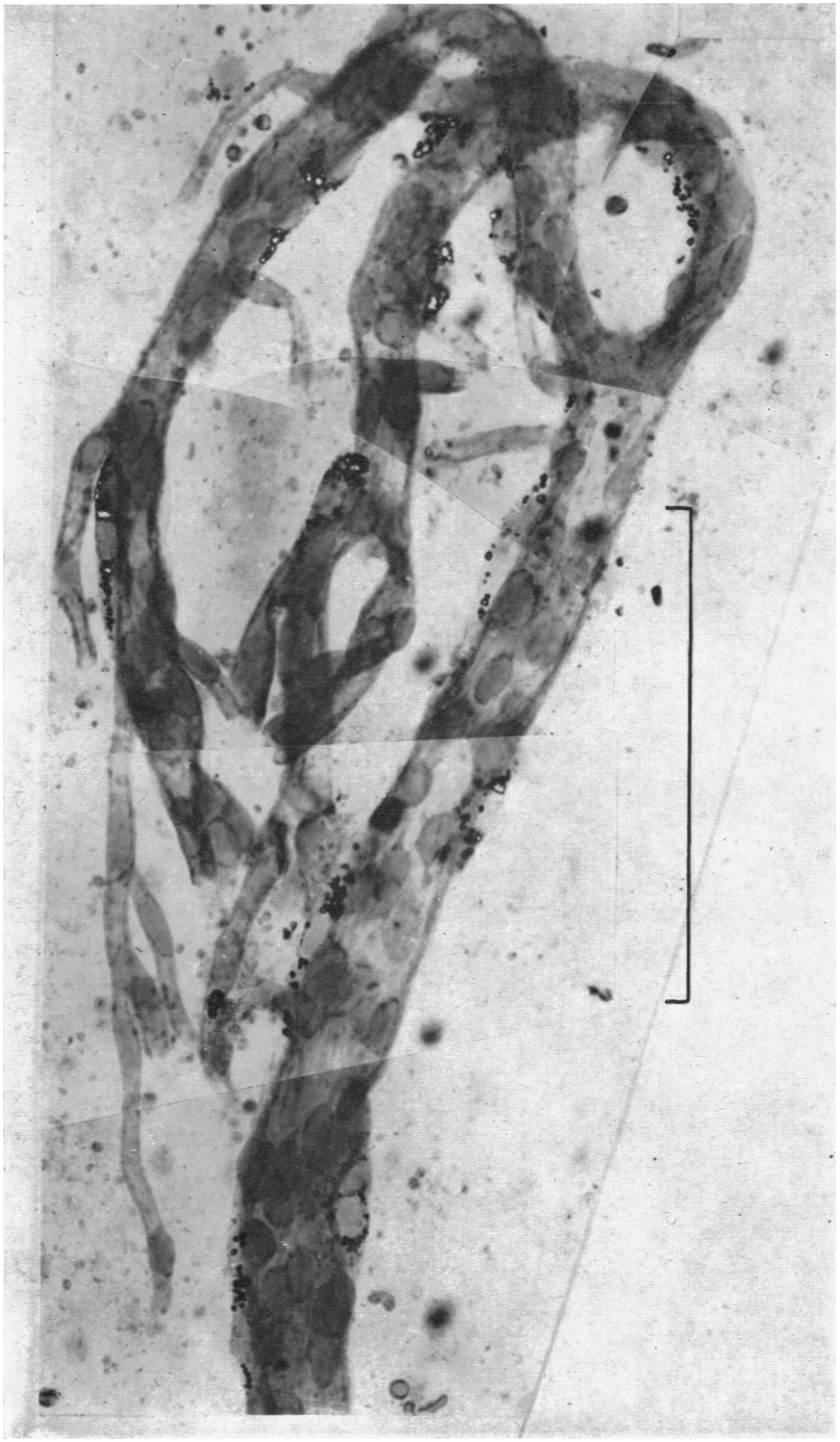

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EXPLANATION OF PLATES

PLATE 1

A, isolated blood vessel surrounded by attached neuroglial cells. These are identified as astrocytes by their thick processes passing to the blood vessel, and by their many branching processes arising from the cell body. Interference microscopy. Scale 200 μm.

B, an isolated astrocyte prepared for measurement of dry mass. Interference microscopy. Scale 100 μm.

C, isolated blood vessel surrounded by attached ependymal cells: these may be identified by their slender vascular processes, by the absence of additional processes arising from the cell body, by the presence of cilia, and by the close apposition of many of the cell bodies. Interference microscopy. Scale 50 μm.

D, an isolated oligodendrocyte prepared for measurement of dry mass. Interference microscopy. Scale 50 μm.

E, autoradiograph of isolated astrocyte obtained after injection of [^{3}H]leucine. Scale 50 μm.

PLATE 2

Blood vessel surrounded by sessile pericytes, having oval nuclei and refractile droplets in their cytoplasm. Interference microscopy. Scale 50 μm.