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OBSERVATIONS ON THE NUCLEOLAR AND TOTAL CELL BODY NUCLEIC ACID OF INJURED NERVE CELLS

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SUMMARY

1. The nucleic acid content of neuronal nucleoli and the total cell body nucleic acid content of neurones of the hypoglossal nucleus were measured by ultraviolet absorption microspectrography.

2. After nerve injury both the nucleolar nucleic acid and the total cell body nucleic acid increased: nucleolar changes preceded those of the cell body.

3. The closer to the nerve cell body that the axon was injured the earlier was the onset and the decline of the nucleolar response.

4. Actinomycin D was given to prevent DNA-primed RNA synthesis, and the rate of 'decay' of nucleolar RNA was measured. This rate varied after nerve injury and was closely related to the nucleolar nucleic acid content.

5. The apparent rate of transfer of labelled RNA from the neuronal nucleus into the cytoplasm changed after nerve injury in a manner closely related to the changes in nucleolar nucleic acid content.

6. It was demonstrated by making consecutive nerve injuries or by preventing or delaying nerve regeneration, that the nucleic acid changes were not induced by removal of contact between the neurone and its motor end-plate, and were not repressed by the restoration of such contact.

7. When regeneration was prevented the nucleolar nucleic acid content and the total cell body nucleic acid ultimately decreased to values less than normal: this decrease was greater when more of the axon was initially removed.

8. The results are discussed in relation to the factor responsible for derepression and repression of DNA cistrons for ribosome synthesis in injured nerve cells.

INTRODUCTION

Microspectrographic demonstration that the chromophilic substance of neurones (Nissl, 1892) consists predominantly of nucleoprotein (Caspersson, 1940; Landström, Caspersson & Wohlfahrt, 1941) and the later development of techniques for measuring the ribonucleic acid (RNA) content of single cells or subcellular fractions (Edström, 1964) have permitted quantitative determination of changes in the RNA content of nerve cells during their chromatolytic response to injury (Brattgård, Edström & Hydén, 1957). Most neuronal RNA is ribosomal (Edström, 1957*a*) and there is a considerable body of evidence that ribosomes or their precursors are synthetized or assembled in the nucleolus of all cell types investigated (Perry, 1960; Edström & Beermann, 1962; Birnstiel, Chipchase & Hyde, 1963; Chipchase & Birnstiel, 1963; Edström & Gall, 1963; Brown & Gurdon, 1964; McConkey & Hopkins, 1964). The size of neuronal nucleoli (Edström, 1957*b*) and their nucleic acid content (Edström & Eichner, 1958) increased during prolonged stimulation and a proportional increase was found in total neuronal RNA. Nucleolar enlargement has been observed as part of the response to injury of some motorneurones (Crouch & Barr, 1954), but not of others (Gersch & Bodian, 1943). The investigation reported here was undertaken to study quantitatively the dynamic relationship between the nucleic acid content of the nucleolus, where ribosomes are formed, and the total neuronal nucleic acid, which is predominantly ribosomal, under the rapidly changing conditions of RNA synthesis found in hypoglossal neurones of the rat after nerve injury (Watson, 1965*a*). By comparing the results obtained after injuring the axon at various distances from the nerve cell body, and under conditions which impeded regeneration of the axon—circumstances which were known to alter the severity, timing and duration of the chromatolytic response (Marinesco, 1898; Van Gehuchten, 1903; Geist, 1933; Romanes, 1946; Liu, 1955)—with results obtained from experiments involving repeated injury of the same nerve, an attempt has been made to assess both the role of loss of contact between the motorneurone and its end-plate as a factor responsible for derepression of nucleolar deoxyribonucleic acid (DNA) cistrons for ribosomal RNA, and the role of restoration of such contact as a factor responsible for the repression of these cistrons.

METHODS

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

Nerve injury. Under ether anaesthesia, the left hypoglossal nerve was crushed or divided in some animals as it emerged through the anterior condylar foramen, in others as the nerve

crossed the interval between internal and external carotid arteries, and in other rats the branches of the nerve were injured within the tongue. In other rats the hypoglossal nerve was ligated with silk at the level of the carotid bifurcation either with or without division of the nerve distally and avulsion of the distal portion from the tongue, or else it was simply divided at this level without ligation and the distal part avulsed. In another investigation the branches of the hypoglossal nerve were initially ligated separately within the tongue and divided distal to the ligatures; in order to impede regeneration the nerve was drawn inferiorly from the tongue and sutured to the superficial aspect of the deep cervical fascia anterior to the ipsi-lateral sternomastoid: 100 days later the nerve was crushed at the level of the carotid bifurcation and the response of the nerve cell bodies to this second injury was followed. All operations were performed aseptically.

Preparation of nerve cells. Between 1 and 200 days after operation a rat was lightly anaesthetized with ether and bled to death. The part of each hypoglossal nucleus anterior to the obex was rapidly removed and placed in a sterile solution of 0·25 M sucrose at 2° C. Single nerve cells were rapidly obtained in large numbers free of glia by gentle mechanical disaggregation on an agar coated glass slide (Zeuthen, 1953). The isolated cells (Pl. 1*A*) were drawn into a micropipette and discharged immediately into a droplet of Ringer–Locke solution on an uncoated quartz slide. No deliberate selection of isolated nerve cells was permitted. After about 3 min at 4° C to allow the cells to adhere to the slide they were immersed in Carnoy's fixative for 45 min, and then transferred briefly to absolute alcohol before rapid rehydration. The slide was gently agitated in 1 % perchloric acid at 4° C for 20 min to remove any remaining acid soluble oligonucleotides (Perry, Hell & Errera, 1961), and washed. The cells were mounted in glycerine, refractive index 1·50 (Caspersson, 1950), and covered with a quartz coverslip. Gentle pressure was applied to the coverslip to flatten the neurone to a thickness of about 5 μ (Pl. 1*B*), to reduce errors due to part of the object being outside the plane of focus.

On the 4th, 10th and 21st days after nerve injury the RNA of single neurones that could be extracted by ribonuclease after fixation in Carnoy was measured by the method of Edström (1964).

Preparation of nerve cell nuclei. The part of each hypoglossal nucleus anterior to the obex was removed from other rats, and each was placed in 0·5 μl. of medium containing 0·34 M sucrose, 0·002 M magnesium chloride, 0·001 M potassium chloride and 0·005 M Tris-HCl buffer, pH 6·5 (glass electrode) (Hadjiolov, Tencheva & Bojadjieva-Mikhailova, 1965) without detergent, and homogenized (Potter & Elvehjem, 1936). The homogenate was discharged on to a quartz slide; after about 3 min at 4° C to allow the nuclei to adhere to the slide they were immersed in 10 % buffered formalin for about 1 hr, washed, agitated in 1 % perchloric acid, washed again and mounted in glycerine beneath a quartz cover-slip. Formalin was used instead of Carnoy's fixative as the latter commonly caused gross precipitation of nuclear sap upon the nucleolus, a process clearly seen when fixation was observed continuously by phase contrast microscopy (personal observation). The nucleic acid content of nucleoli prepared in this way was found to remain constant for at least 2 hr, a period considerably longer than the duration of measurements upon nucleoli derived from one rat.

The possible loss of nucleic acid by the nucleolus during isolation of nuclei in the aqueous media was assessed in several normal rats. The animal was perfused with 10 % buffered formalin at the time of death; the fixed hypoglossal nuclei were dissected out and fixed for a further hour. Nuclei were then isolated by homogenization. This method was an attempted approximation to that of McLeish (1963). This procedure was not used routinely as the yield of undamaged nuclei free of cytoplasmic tags was very low.

Ultraviolet microspectrography. A stabilized water-cooled hydrogen lamp was used with a Leitz ultraviolet microspectrograph to obtain absorption spectra within the range 2350–3200 Å from nucleoli and from areas of cytoplasm (achromatic reflecting optics, objective

300/0·85 S, condenser N.A. 0·60; glycerine immersion, refractive index 1·440). The spectrograph slit width corresponded to a field width of 0·7 μ in the object plane; the spectrum was dispersed by a quartz prism and recorded on Ilford N40 photographic plates which were developed in Kodak D 19*b*. A monochromator was used to isolate the 2537 Å line of a low pressure mercury lamp, both to calibrate the absorption spectra and to obtain monochromatic photomicrographs of isolated neurones (Pl. 1*B*) or nuclei (Pl. 2*A*, *B*, *C*): care was taken that unevenness of empty field illumination did not exceed 2 %, and the central part of the field was always used. Both absorption spectra and monochromatic photomicrographs were calibrated by exposing within an empty area of the object field a rotating sector stop having extinction steps each corresponding to an increment of 0·1 optical density units. The conditions of plate exposure and development were standardized so that photographic density increased linearly within the range 0–0·8 optical density units.

Monochromatic negatives of nucleoli, including the reference steps, were scanned with a Joyce–Loebl integrating microdensitometer (× 22 object magnification, 1:50 arm ratio, 0–3·0 optical density wedge): the microdensitometer slit width and height corresponded to a field width and height of 0·1 and 0·2 μ respectively, referred to the original object plane. The area of the nucleolus was scanned in parallel strips 0·2 μ apart. Monochromatic negatives of neurones were scanned in parallel strips 4 μ apart. The integrated absorbance at 2537 Å was corrected in two respects by reference to the absorption spectrum: correction for light loss by scattering was attempted by calculating the light scattered at 2537 Å from the measured apparent absorption at 3120 Å (Walker, 1956), assuming that the light scattered was inversely proportional to the fourth power of the wave-length (Oster, 1948). The integrated absorbance at 2600 Å was calculated from that measured at 2537 Å. An extinction coefficient of 22 was assumed for nucleic acids under the conditions of measurement (Caspersson, 1950). All values presented in this paper are quantitative only within the framework of these corrections and assumptions.

Between twelve and twenty neurones or nucleoli were analysed in this manner in each hypoglossal nucleus examined. In addition, some isolated nuclei were digested with crystalline ribonuclease, 1 mg/ml. in McIlvaine's (1921) citrate-phosphate buffer pH 7·0 (glass electrode) before measuring the integrated absorbance.

Some nucleoli of normal neurones were displaced from their isolated formalin fixed nuclei by gentle oblique pressure on the cover-slip, causing it to slide about 0·5 mm (Pl. 2*B*). The absorbance of these nucleoli was measured to estimate the contribution of nuclear sap lying over or under the nucleus to the apparent nucleolar absorbance when measurements were made within flattened nuclei.

Actinomycin D administration. Actinomycin D, a drug inhibiting the synthesis of DNA-primed RNA (Reich, Franklin, Shatkin & Tatum, 1961; Goldberg & Rabinowitz, 1962), was dissolved to a final concentration of 10 μg/ml. in artificial cerebrospinal fluid (Mitchell, Loeschke, Massion & Severinghaus, 1963), and 0·40 ml. was injected slowly into a lateral cerebral ventricle under light ether anaesthesia every 12 hr: the rat recovered within a very few minutes and behaved normally. Preliminary experiments demonstrated that this concentration of actinomycin D reduced the incorporation by the hypoglossal nuclei of [5-^{3}H]-uridine into nucleic acids by more than 90 %, and autoradiography showed that the capacity of hypoglossal neurones to incorporate the isotope under these circumstances was similarly impaired. Animals were killed at varying intervals up to 48 hr after injection, and measurements were made of nucleolar nucleic acid and of total neuronal nucleic acid.

Autoradiography. Tritiated uridine, [5-^{3}H]uridine (1·22 c/m-mole, Radiochemical Centre, Amersham) was diluted with an electrolyte solution so that the final composition resembled that of artificial cerebrospinal fluid. The final activity of the solution was 0·8 mc/ml. and 0·25 ml. was injected into a lateral cerebral ventricle under light ether anaesthesia. Rats were killed 8 hr after isotope administration: the autoradiographic techniques used were the same as described before (Watson, 1965*a*). The distribution of silver grains over sectioned

neurones was calculated and expressed as the ratio of cytoplasm grain density to nuclear grain density (Cyt/Nuc ratio) for reasons previously discussed (Watson, 1965*a*).

Histological procedures. Hypoglossal nerves prepared by the method of Aitken, Sharman & Young (1947) were examined so that the process of maturation could be followed in the regenerating nerve. Nerve fibres within geniohyoid were examined after supravital staining with methylene blue (Coërs & Woolf, 1959).

RESULTS

Absorption spectra. The absorption spectrum was measured within the range 2350–3200 Å both to estimate the contribution of light scattering to the apparent absorbance at 2600 Å and to see whether any change occurred in the relative absorptions at 2600 Å and at 2800 Å after nerve injury. The absorption spectrum of a typical normal nucleolus is shown in Text-fig. 1. Apparent absorption at 3120 Å was 12% of the peak absorption at 2600 Å. A correction factor was employed which assumed that light scattering was inversely proportional to the fourth power of the wave-length (dotted line, Text-fig. 1), and the estimated contribution of light scattering to the apparent absorbance at 2600 Å was in this case 25% of the measured total absorbance: this was a maximum correction factor for light loss. The minimum value was determined by assuming that loss was independent of wave-length, and hence was the same at 2600 Å as at 3120 Å. The difference between these two factors was about 13% of the measured absorbance at 2600 Å, and this difference represented the possible range of error due to incorrect calculation of the contribution of light scattering to measured absorption. As all values presented below have been corrected using the greater fourth power factor they may be up to 13% too low. The absorption spectrum also showed high absorption at 2800 Å, such that the ratio absorbance 2600 Å/absorbance 2800 Å was 1·34, in the nucleolus shown in Text-fig. 1. This ratio did not change significantly in the nucleolus either after nerve injury or after actinomycin D.

Formalin fixation of the brain by perfusing the rat at the time of death with subsequent isolation of the fixed nuclei gave nucleolar absorption spectra and mean values for integrated nucleolar absorbance at 2600 Å which were not significantly different from those obtained by fixing nuclei previously isolated by homogenization in the aqueous medium. It is therefore believed that no significant loss of nucleolar nucleic acid occurred during the usual process of aqueous isolation before fixation.

The absorption spectrum was also measured in some nucleoli after aqueous isolation of nuclei, but before fixation. The apparent absorbance at 3120 Å was usually less than 5% of that at 2600 Å and the ratio absorbance 2600 Å/absorbance 2800 Å lay within the range 1·70–1·93. Formalin fixation caused both an increase in light scattering, and a decrease in this ratio.

The corrected integrated absorbance of nucleoli extracted from fixed isolated nuclei (Pl. 1*B*) was between 6 and 11 % less than that of nucleoli measured within intact nuclei. It is believed that this difference represented the absorption at 2600 Å of the overlying nucleoplasm of the flattened nuclei. The nucleus was less absorbant than the nucleolus (Pl. 1*A*). Precise

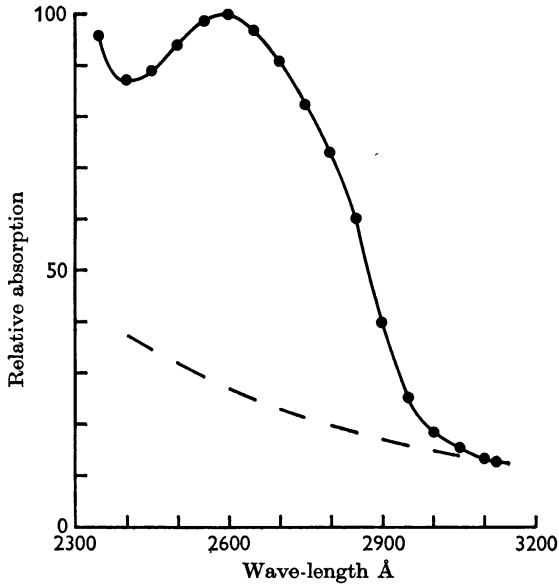

Fig. 1. The relative absorbance of a normal nucleolus is expressed against wave-length. Note the marked peak at 2600 Å. Apparent absorbance at 3120 Å is 12 % of absorbance at 2600 Å. The dashed line indicates the contribution of light scattering to the measured absorbance, assuming that light scattering is inversely proportional to the fourth power of the wave-length.

calculation of this error was difficult, however, because the light path through the object was conical, because the thickness of the nucleus above and below the thickest part of the nucleolus was not known, and because nucleoli were not always circular in the plane viewed microscopically, and were probably seldom spherical. No correction was therefore made for the contribution of the nuclear sap to the apparent nucleolar absorbance, but it is possible that the values obtained are between 6 and 11 % too great.

Nerve injury with permitted regeneration. After a nerve was crushed, changes were found in nucleolar nucleic acid and in total cell nucleic acid which varied according to the site of injury (Text-fig. 2*A*-*F*). Nucleolar nucleic acid increased earlier to reach a peak, remained high for a shorter period and decreased earlier with more severe degrees of injury (Text-fig. 2*A*, *C*, *E*). The difference between the values in Text-fig. 2*A* and in Text-fig. 2*C* is significant ($P < 0{\cdot}01$, $> 0{\cdot}001$), and the difference between

Text-fig. 2*A* and Text-fig. 2*E* is highly significant ($P < 0{\cdot}001$). No significant change was found in the height of the peak. After crushing the nerve proximally (Text-fig. 2*E*) the increase in nucleolar nucleic acid was followed by a significant decrease ($P < 0{\cdot}01$, $> 0{\cdot}001$) below normal values. This decrease was not found after the nerve had been crushed distally (Text-fig. 2*A*). The nucleolar nucleic acid which resisted ribonuclease digestion did not change after axotomy.

Total neuronal nuclei acid, and extractable RNA, increased after the two lesser degrees of nerve injury (Text-fig. 2*B*, *D*), and reached a maximum value rather later after the least degree (Text-fig. 2*B*) than after the intermediate degree of injury (Text-fig. 2*D*): the difference between these results is significant ($P < 0{\cdot}05$, $> 0{\cdot}01$). After the most severe degree of injury (Text-fig. 2*F*) a much smaller increase in total neuronal nucleic acid was observed, a highly significant difference from the results shown in Text-fig. 2*B* and *D* ($P < 0{\cdot}001$). The nucleolar changes shown in Text-fig. 2*A* and *C* preceded at every phase the corresponding changes in total cell nucleic acid (Text-fig. 2*B*, *D*); the maximum nucleolar nucleic acid content was closely related to the greatest rate of increase of total cell nucleic acid, and began to decrease when the total cell nuclei acid had attained a high constant value. As no clear maximum was observed in total neuronal nucleic acid after the most severe degree of injury (Text-fig. 2*F*) it was not possible to relate it to the changes observed in nucleolar nucleic acid (Text-fig. 2*E*).

Histology of the injured nerve showed that myelination of small regenerating nerve fibres was apparent about 8 days after crushing the nerve, and the normal distribution of axon diameters was attained by the 60th day. Examination of the geniohyoid stained supravitally with methylene blue showed ingrowing nerve fibres about the 10th day after injury. At the hyoid end of geniohyoid some sprouting of axons from the terminal parts of the normal hypoglossal nerve could occasionally be seen to innervate some adjacent denervated motor end-plates; this was most usually observed about the 10th day after injury. The ultimate fate of these sprouts was not observed.

Nerve injury with impeded regeneration. In some animals the nerve was ligated centrally in the neck: in a group of these, the nerve was divided distal to the point of ligation and the distal part was avulsed. When the nerve was ligated, but not divided, the total cell nucleic acid response (Text-fig. 3*B*) resembled that found after crushing at this site (Text-fig. 2*D*). If the nerve was also resected the total cell nucleic acid decreased to low values after the 70th day. The nucleolar response to ligation (Text-fig. 3*A*) resembled in timing the response to crushing at this site (Text-fig. 2*C*), but the maximum was significantly greater ($P < 0{\cdot}01$, $> 0{\cdot}001$).

Histological examination of the nerve distal to the point of ligation, and of the geniohyoid showed delayed re-innervation when the nerve was also divided, and virtual absence of re-innervation of muscle after additional division and distal avulsion.

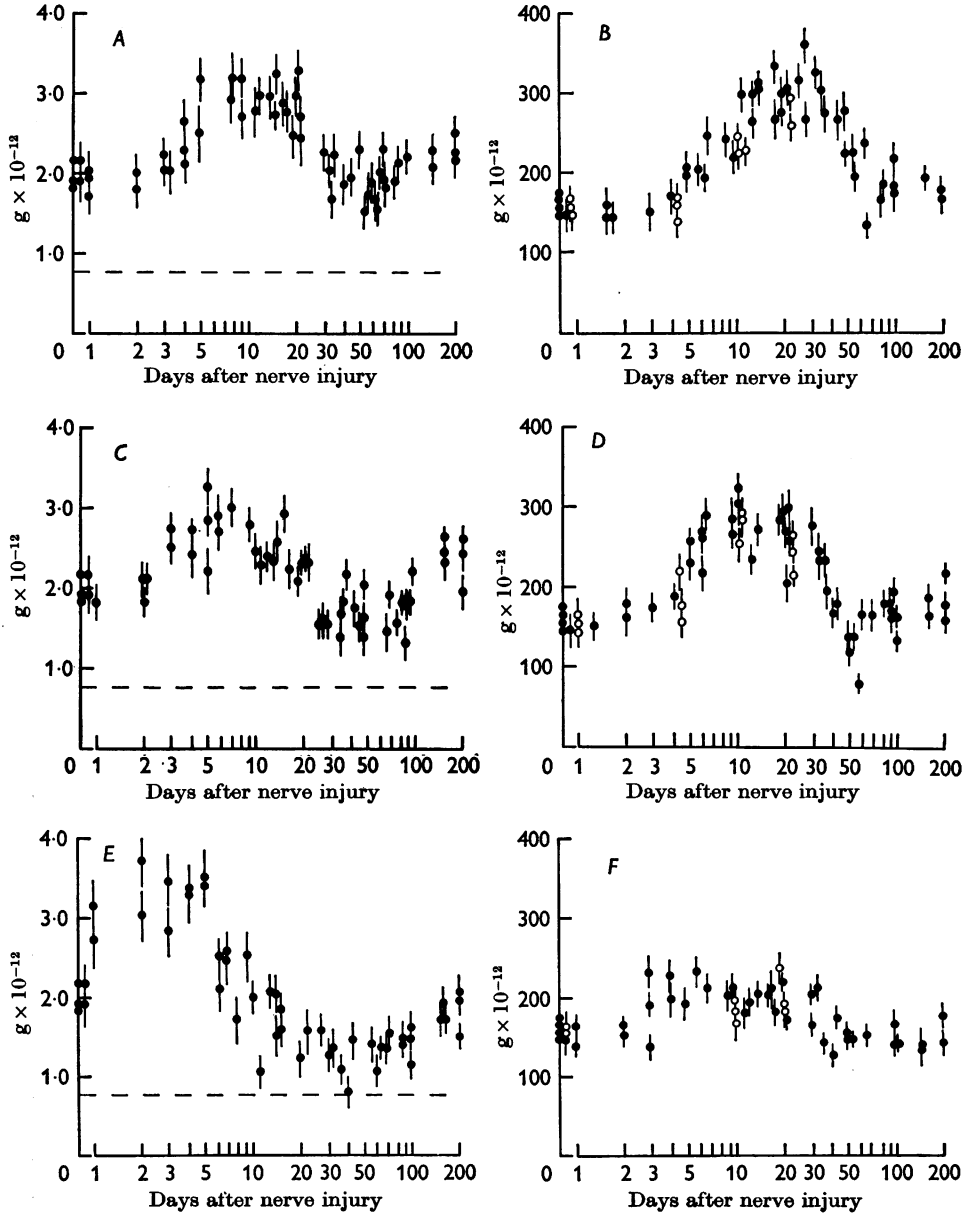

Fig. 2. For legend see opposite page.

When the nerve was divided centrally without ligation, and with avulsion of the distal part, the response of the total cell nucleic acid (Text-fig. 3*D*) resembled that found after crushing (Text-fig. 2*D*) until about the 70th day: the total cell nucleic acid then decreased to values less than those found after crushing the nerve at this site. The nucleolar response after division (Text-fig. 3*C*) occurred significantly earlier ($P < 0{\cdot}05$, $> 0{\cdot}01$) than when the nerve was crushed or ligated (Text-figs. 2*C*, 3*A*) at this site. The maximum nucleolar response was not significantly different from that found after crushing the nerve, but was smaller than after ligation ($P < 0{\cdot}01$, $> 0{\cdot}001$).

In some animals the nerve was divided as it emerged from the anterior condylar foramen, and was avulsed distally. In these animals too, the nucleolar and total cell nucleic acid declined after the 70th day (Text-fig. 3*D*) and attained values significantly lower than those found when the nerve was divided centrally (Text-fig. 3*D*). In other rats the nerve was ligated in the tongue, divided distal to the point of ligation, and then drawn into the neck: 100 days after injury the total cell nucleic acid was significantly less than those in which the nerve had been crushed (Text-fig. 4, values at zero time) ($P < 0{\cdot}05$, $> 0{\cdot}01$) and less than the normal neuronal content ($P < 0{\cdot}05$, $> 0{\cdot}01$), but greater than in those animals in which the nerve had been ligated centrally with distal division and avulsion $P < 0{\cdot}05$, $> 0{\cdot}01$).

Repeated nerve injury. In some rats the branches of the hypoglossal nerve were ligated in the tongue, divided distal to the point of ligation, drawn down into the neck and sutured without tension to the superficial aspect of the deep cervical fascia anterior to sternomastoid. A hundred days later the nerve was crushed as it crossed the bifurcation of the common carotid artery. Following the second injury the nucleolar nucleic acid content and total cell nucleic acid showed changes resembling those found at the first time of injury (Text-fig. 4, Text-fig. 2*C*, *D*), although the initial 'normal'

Legend for Fig. 2.

Fig. 2. Changes in total cell body nucleic acid and in nucleolar nucleic acid after nerve injury. In *A*, *C* and *E*, the *nucleolar* nucleic acid is expressed against the time in days since nerve injury. In *B*, *D* and *F*, the *total cell body* nucleic acid is similarly expressed. *A* and *B* show the consequences of crushing the nerve distally in the tongue. *C* and *D* show the consequences of crushing the nerve at the level of the carotid bifurcation. *E* and *F* show the consequences of crushing the nerve as it emerged from the base of the skull. The horizontal dashed line in *A*, *C* and *E* shows the nucleic acid content of nucleoli that resisted digestion with ribonuclease: this remained constant after nerve injury ($0{\cdot}78 \pm 0{\cdot}03 \times 10^{-12}$ g). In *B*, *D* and *F*, ● indicates total cell body nucleic acid, and ○ indicates RNA extracted from nerve cell bodies with ribonuclease, and measured by the technique of Edström (1964). The standard error of the mean is also indicated.

values differed. In some of these rats the hypoglossal nerve was stimulated electrically 100 days after the first injury: no muscular response was seen in the tongue or elsewhere. Histologically, no evidence of re-innervation of the muscles of the tongue could be found: in some animals outgrowing fibres could be seen passing along the superficial aspect of the deep cervical fascia from the ligated end of the nerve: their ultimate fate was not determined.

Nuclei with multiple nucleoli. In both normal and injured neurones between 3 and 8% of nuclei possessed multiple nucleoli: this proportion

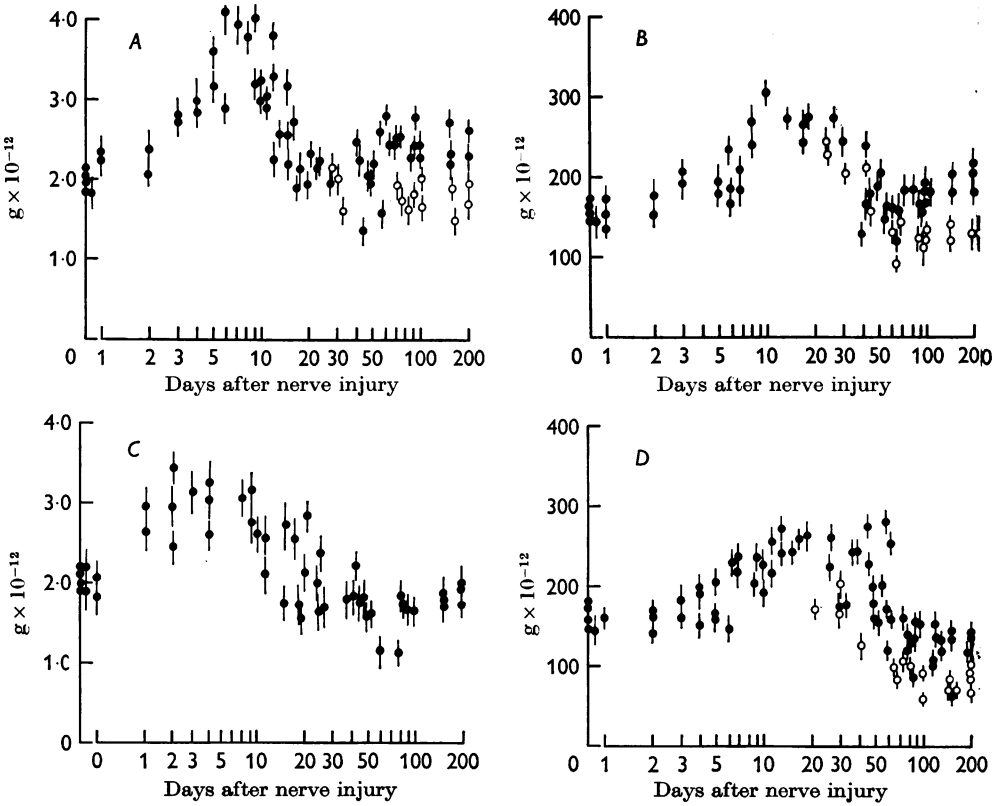

Fig. 3. *A* shows the changes in nucleolar nucleic acid; ● when the nerve was ligated at the level of the carotid bifurcation without distal division or avulsion, ○ when the nerve was ligated at the level of the carotid bifurcation with distal division and avulsion. *B* shows the changes in total cell body nucleic acid corresponding to the nucleolar values shown in *A*. Symbols as in *A*. *C* shows the changes in nucleolar nucleic acid when the nerve was divided at the level of the carotid bifurcation with distal avulsion. *D* shows the changes in total cell body nucleic acid; ● when the nerve was divided at the level of the carotid bifurcation with distal avulsion, corresponding to the nucleolar values shown in *C*, ○ when the nerve was divided as the nerve emerged from the base of the skull with distal avulsion. The standard error of the mean is also indicated.

did not change significantly after injury. Most of these nuclei had two nucleoli, exceptionally three were seen. In nearly all nuclei with multiple nucleoli the nucleic acid content of each of the nucleoli did not differ significantly from that of the other nucleolus within that nucleus. The total nucleolar nucleic acid content of normal neurones having multiple nucleoli

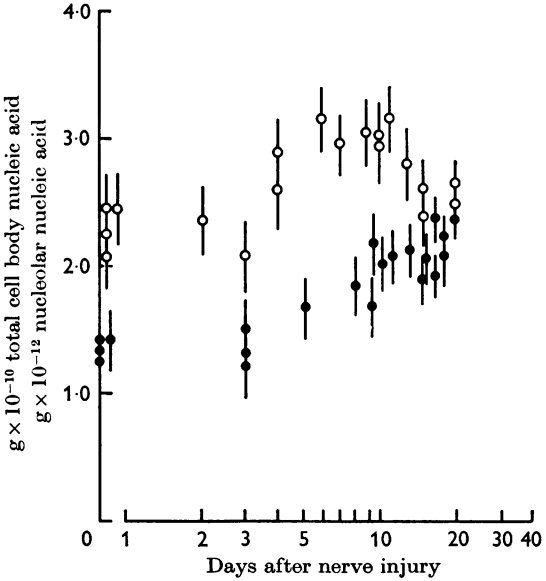

Fig. 4. Nucleolar nucleic acid and total cell body nucleic acid are expressed against the time in days after the second nerve injury. Values at zero time are those found 100 days after the first injury, immediately before the second. ● Total cell body nucleic acid content, $g \times 10^{-10}$. ○ Nucleolar nucleic acid content, $g \times 10^{-12}$. The standard error of the mean is also shown.

was significantly greater ($P < 0{\cdot}05$, $> 0{\cdot}01$) than the total nucleolar nucleic acid of normal neurones with single nucleoli: the total nucleolar nucleic acid extractable by ribonuclease was not significantly different in multinucleolar than in mononucleolar neurones ($P > 0{\cdot}05$). Each nucleolus, whether existing singly within a nucleus, or as one of a pair, had the same amount of nucleic acid resisting ribonuclease digestion ($0{\cdot}78 \pm 0{\cdot}03 \times 10^{-12}$ g, S.E.).

Actinomycin studies. The injection of actinomycin D into a lateral cerebral ventricle caused an initial increase in nucleolar nucleic acid within the first hour, which was followed by a progressive decrease lasting about 48 hr (Pl. 1*C*) expressed in the curves drawn (Text-fig. 5) as a simple logarithmic function. The curves were calculated regression lines: the results are expressed semilogarithmically both because it was expected that the changes would follow some form of logarithmic 'decay', and

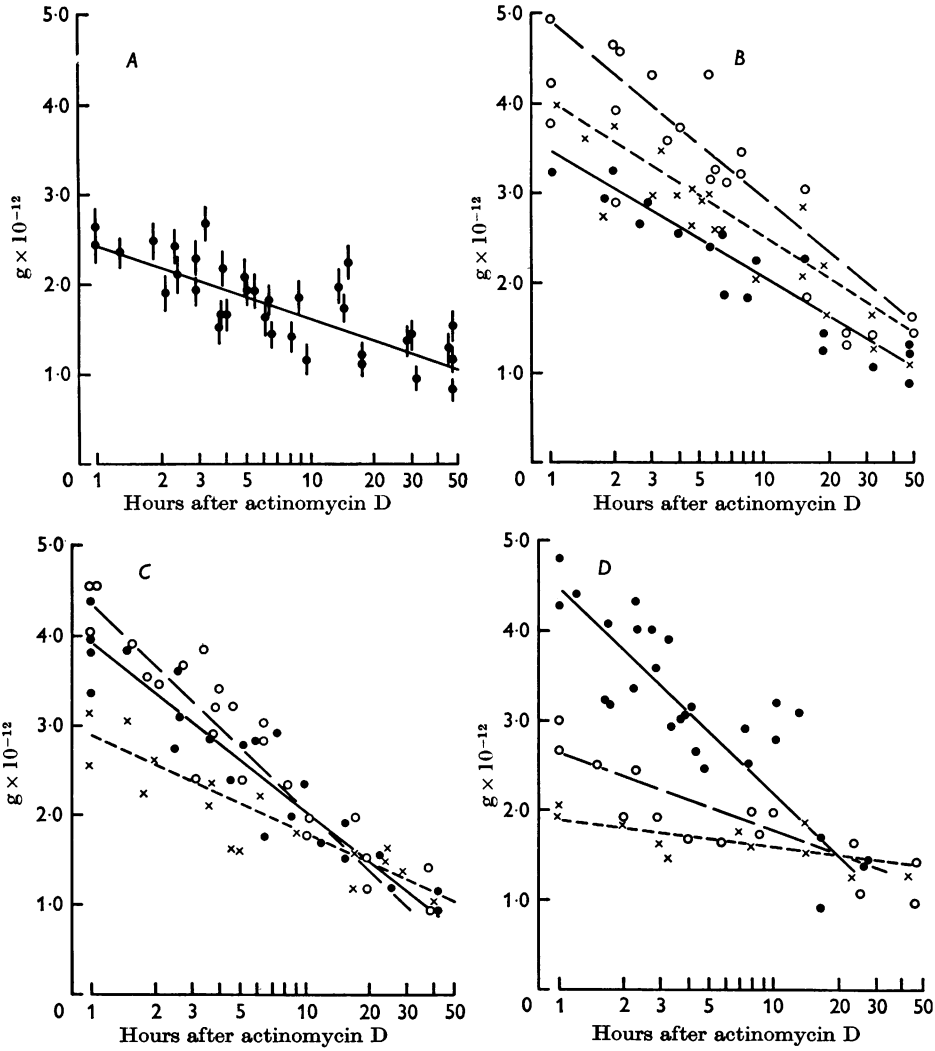

Fig. 5*A*. Nucleolar nucleic acid is expressed against the time in hours since actinomycin D was injected into normal rats. The standard error of the mean is also indicated.

B. Nucleolar nucleic acid is expressed against the time in hours since actinomycin D. The nerve was crushed distally in the tongue. ●——● 4 days earlier, ○— —○ 10 days earlier, ×- - - - - × 21 days earlier.

C. Nucleolar nucleic acid is expressed against the time in hours since actinomycin D. The nerve was crushed at the level of the carotid bifurcation. ●——● 4 days earlier, ○— —○ 10 days earlier, ×- - - - -× 21 days earlier.

D. Nucleolar nucleic acid is expressed against the time in hours since actinomycin D. The nerve was crushed as it emerged from the base of the skull: ●——● 4 days earlier, ○— —○ 10 days earlier, × - - - - -× 21 days earlier.

All lines drawn are calculated regression lines. Standard errors are excluded from *B–D* to maintain clarity.

because the correlation coefficient was smaller with this form of expression. Observations were made on normal nerve cells (Text-fig. 5*A*) and on neurones injured 4 days, 10 days or 21 days earlier by crushing the nerve distally in the tongue (Text-fig. 5*B*), centrally at the carotid bifurcation (Text-fig. 5*C*) or proximally at the anterior condylar foramen (Text-fig. 5*D*).

The negative slope of the regression line calculated for nucleolar nucleic acid was greater in those circumstances in which the nucleolar nucleic acid content was increased, and the differences in timing of the greatest increase of slope reflected closely the differences in timing of elevation of the

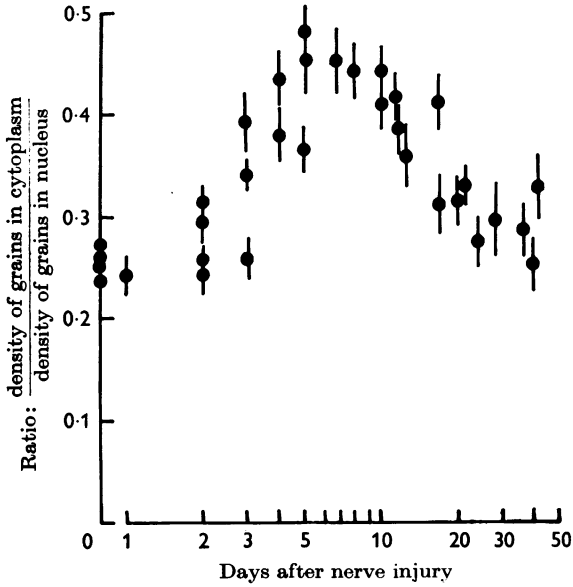

Fig. 6. Ratio: density of grains in cytoplasm/density of grains in nucleus found 8 hr after injecting [5-^{3}H]uridine is expressed against the time in days since the nerve was crushed at the level of the carotid bifurcation. Note that the increase coincides with the increase in nucleolar nucleic acid (Fig. 2*C*). The standard error of the mean is also shown.

nucleolar acid content. For example, after crushing the nerve distally the nucleolar nucleic acid content (Text-fig. 2*A*) was barely raised at 4 days but was high on the 10th and 21st days: the negative slope of the line expressing actinomycin D-induced 'decay' of nucleolar nucleic acid content was similarly within normal limits on the 4th day, but markedly raised on the 10th and 21st days (Text-fig. 5*B*). By contrast, after crushing the nerve at the anterior condylar foramen both nucleolar nucleic acid content (Text-fig. 2*E*) and the rate of actinomycin D-induced 'decay' (Text-fig. 5*D*) were raised at 4 days and low on the 10th and 21st days. No significant

change was observed in the total nucleic acid content of normal or injured neurones after actinomycin D.

Autoradiography. Eight hours after injection of 200 μc [5-^{3}H]uridine into a lateral cerebral ventricle, the intraneuronal distribution of labelled nucleic acid was determined autoradiographically and expressed as the ratio, grain density in cytoplasm/grain density in nucleus (Cyt/Nuc ratio).

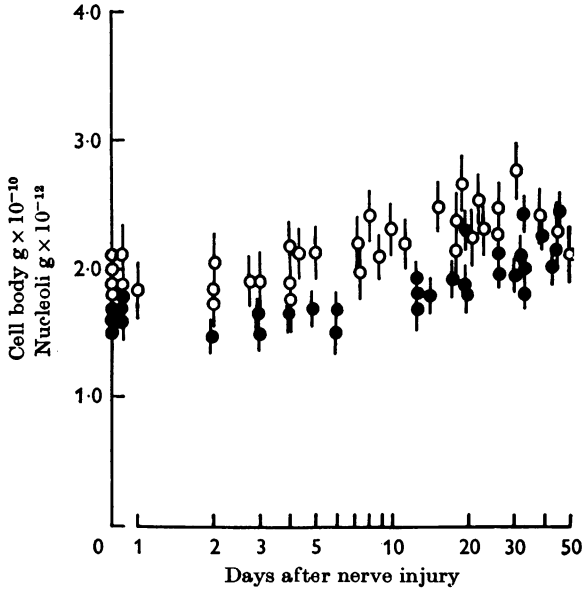

Fig. 7. Nucleolar nucleic acid and total cell body nucleic acid of neurones from the *normal right* hypoglossal nucleus are expressed against the time in days since the *left* hypoglossal nerve was injured. ● Total cell body nucleic acid content, $g \times 10^{-10}$. ○ Nucleolar nucleic acid content, $g \times 10^{-12}$. The standard error of the mean is also shown.

Text-fig. 6 shows how this ratio varied after the nerve had been crushed centrally in the neck. The increase in this ratio coincided both with the increase in nucleolar nucleic acid (Text-fig. 2*C*) and with the increased negative slope of actinomycin D-induced 'decay' of nucleolar nucleic acid (Text-fig. 5*C*). The time at which it attained its maximum value, and at which it declined, similarly correspond with these other parameters.

Right, uninjured hypoglossal nucleus. After injuring the left hypoglossal nerve the nucleolar nucleic acid and total neuronal nucleic acid content increased slightly but significantly ($P < 0{\cdot}05$, $> 0{\cdot}01$) in the right hypoglossal nucleus (Text-fig. 7).

DISCUSSION

Isolation of cells and nuclei. Isolation of nerve cells from brain either by microdissection (Hydén & Pigón, 1960), or by gentle mechanical disaggregation inevitably damages them: the remaining stumps of the dendrites and of the axon are seldom more than 500 μ long, and are commonly shorter: striking changes also occur in the plasma membranes of isolated neurones (Roots & Johnston, 1964). It is probable, however, that these changes do not seriously affect measurement of their RNA content: less than 10% of a neurone's RNA lies within its dendrites (Hydén, 1960), and the concentration of RNA in mammalian axons is very low. Although its base composition (Koenig, 1965*b*) and sedimentation characteristics (Miani, Di Girolamo & Di Girolamo, 1966) resemble those of ribosomal RNA, and in addition, ribosome-dependent protein synthesis has been demonstrated in isolated axoplasm (Koenig, 1965*a*), ribosomes have not been observed.

It is unlikely that the neurone responded actively to the trauma of isolation with significant alteration of RNA metabolism, as isolation was completed within 5 min at 2–4° C. Previous evidence (Brattgård *et al.* 1957; Watson, 1965*a*) suggests that a nerve cell's response to injury occurs over a period of hours or days, and not within minutes. No loss of macromolecular acid-insoluble RNA occurs from nerve cells isolated and fixed in the way described (Edström, 1953; Edström, 1956; Stenram, 1958; Jarlstedt, 1962). Similarly, as there was no significant difference between the nucleic acid content of nucleoli fixed at the time of death before isolation, and of nucleoli fixed after nuclear isolation, it is believed that no loss of nucleolar RNA occurs.

Mechanical disaggregation of hypoglossal nuclei provided a very large number of neurones free from glia. Great care was required to avoid selection from this population of an unrepresentative sample for measurement. Large neurones are especially attractive, and the ability to see smaller cells is critically dependent upon good illumination. The conditions of illumination were kept as constant as possible when disaggregated hypoglossal nuclei of different rats were examined. The first thirty neurones seen of each hypoglossal nucleus were transferred to a quartz slide, and the first 12–20 of these found with the microspectrograph were measured. Similar care was taken to avoid systematic selection of nucleoli.

Microspectrography. The uncertainties concerning quantitative estimation of nucleic acid and protein in cells by ultraviolet absorption techniques (Walker, 1956) arise from the unknown contribution of non-specific light loss to the measured apparent absorption, from the undetermined absorption spectra of proteins and nucleic acids within cells, and

from the doubtful applicability of Beer's Law under these circumstances. Light loss at 2600 Å was derived from the apparent absorption at 3120 Å in the manner described. The precise absorption spectra of nucleic acids and proteins within cells was impossible to determine, because the degree to which absorbing substances react with each other and with other non-absorbing compounds is unknown: the absorption spectra of nucleic acids and proteins in cells may differ from those obtained in solution (Davies, 1954): absorption curves may vary *in vitro* with different solvents (Brode, 1939). Davies (1954) found that fixation of chick fibroblasts caused an increase in their absorbance at 2800 Å. The absorption spectra of neuronal nucleoli described here similarly showed a change in the ratio, absorbance 2600 Å/absorbance 2800 Å from 1·82 (± 0·04) to 1·37 (± 0·05) on fixation. Although precise estimates of the relative concentration of 'standard protein' (Caspersson, 1950) and of nucleic acid, by comparing the absorbance at these two wave-lengths, must therefore be accepted with caution, the absorption spectra indicate that the nucleoli are composed of about 15% nucleic acid and 85% protein, a proportion close to that found biochemically in nucleoli of other cell types (Busch, Desjardins, Grogan, Higashi, Jacob, Maramatsu, Ro & Steele, 1966; Vincent, Baltus & Løvlie, 1966).

Measurement of integrated absorbance at a wave-length near the nucleic acid absorption peak has provided reproducible measurements of the quantity of nucleic acid in individual cells which were closely correlated with biochemical estimations performed upon large numbers of cells (Leuchtenberger, Leuchtenberger, Vendrely & Vendrely, 1952; Walker & Yates, 1952; Leuchtenberger, Klein & Klein, 1952). Lindström, Zetterberg & Carlson (1966) showed that the absorption spectra of RNA solutions and of concentrated dried microdroplets are practically identical.

Nature of RNA measured. There is good evidence that ribosomes are synthesized and assembled in the nucleolus (Perry, 1966). The nucleolus contains both ribosomal RNA (Pelling, 1965; Edström, Grampp & Schor, 1961) and its subunits (Birnstiel, Chipchase & Hyde, 1963; Vincent, 1964). Destruction of nucleoli prevents formation of cytoplasmic RNA (Perry *et al.* 1961). Synthesis of ribosomes does not occur in *Xenopus* mutants lacking nucleolar organizer DNA and nucleoli (Brown & Gurdon, 1964). DNA-RNA hybridization studies confirm the likely nucleolar origin of ribosomal RNA (Chipchase & Birnstiel, 1963; Birnstiel & Wallace, 1965; McConkey & Hopkins, 1964; Ritossa & Spiegelman, 1965; Ritossa, Atwood, Lindsley & Spiegelman, 1966).

Micro-electrophoretic analysis shows that the base content of RNA (Edström, 1964) extracted from isolated neurones resembles that of brain ribosomes (Jacob, Stevenin, Jund, Judes & Mendel, 1966) and does not

change (Edström, 1957*a*) when the RNA content of nerve cells increases after injury (Brattgård *et al.* 1957), indicating that the additional RNA is predominantly ribosomal. The increased RNA content of nucleoli found after injury coincided with this increasing neuronal content of ribosomal RNA (Text-fig. 2), and the half-life of at least 80% of nucleolar RNA was altered (Text-fig. 5*A–D*). It is therefore believed that the changes observed are changes in the ribosome-synthesizing system of the neurone. Some further support for this belief is supplied by autoradiography (Text-fig. 6). Eight hours after the administration of labelled precursors, the bulk of label found in the cytoplasm has been incorporated into brain ribosomes (Jacob *et al.* 1966). The relative distribution of labelled RNA between the neurone's nucleus and its cytoplasm may therefore reflect the rate at which ribosomes pass into the cytoplasm: the considerable limitations of autoradiography of RNA in the brain, and its application to nerve injury have been discussed previously (Watson, 1965*a*). If variation of the Cyt/Nuc ratio reflects variation in the rate of ribosome transfer into the cytoplasm, and hence the rate of nucleolar ribosomal synthesis, then it should correlate with the rate of ribosome synthesis indicated by actinomycin D-induced 'decay' of nucleolar nucleic acid (Text-fig. 5), which is itself related to the nucleolar content of RNA (Text-fig. 2). Such a correlation was found (Text-fig. 2*C*; Text-fig. 6).

Changes in ribosome synthesis induced by injury. The period of increased ribosomal synthesis coincided with the period of decreased neuronal activity of acetylcholine hydrolase (Watson, 1966) in its time of onset, in the time of maximum effect and in the time of decline. This enzyme is seen histochemically in association with the endoplasmic reticulum (Lewis & Shute, 1966). As the endoplasmic reticulum is undergoing fragmentation at this time (Lewis & Shute, 1965), and as many of the ribosomes of the normal nerve cell are attached to it, it is likely that these observations represent different aspects of the same process, although the precise manner of their interdependence is at present unknown. The shorter period of ribosome synthesis found when the imposed injury was more severe, and the associated smaller increase in the total number of ribosomes possessed by the injured cell is the probable molecular origin of the greater degree of chromatolysis observed with more severe injuries (van Gehuchten, 1903).

The results presented here show the relationship between the nucleolar nucleic acid content and the rate of ribosomal RNA synthesis. The maximum nucleolar RNA, corresponding to the greatest rate of ribosome synthesis, coincided with the greatest rate of increase of total cell RNA, which was predominantly ribosomal: nucleolar synthesis of ribosomal RNA decreased at the time that the total cell RNA stopped increasing, and with

the most severe degree of injury declined to subnormal values. If the rate-limiting step for ribosome synthesis in the normal cell is the degree of repression of the DNA cistrons (Jacob & Monod, 1964) of the nucleolus organizer, then the observed changes represent alteration of this repression. Although the precise mechanisms regulating the repression are not at present known, some conclusions can be drawn from these results: the increased rate of synthesis after injury was not due to loss of contact between the nerve cell body and the motor end-plate, as it occurred when the nerve was injured a second time (Text-fig. 4) after any possible influence of the end-plate had already been removed. The decreased rate of synthesis after a maximum value had been attained was not due to re-established contact between the neurone and the denervated end-plate, as it did not occur any later when such contact was delayed (Text-fig. 3*A*) or prevented (Text-fig. 3*C*) and occurred earlier the further the regenerating process had to travel (Text-fig. 2*A*, *C*, *E*). Neuronal swelling, induced by injury, always preceded the changes in nucleolar nucleic acid and occurred earlier when the nerve lesion was more proximal (unpublished observation): while such swelling could be related to derepression of cistrons (Kroeger, 1963; Kroeger, 1964) and induced swelling of neurones can alter nucleolar morphology (Hild, 1964), it cannot explain the early recurrence of repression with the most severe degrees of injury (Text-fig. 2*E*; Text-fig. 5*D*). Alternatively, there is evidence from experiments involving nuclear transfer, that the formation of nucleoli is controlled in part by the cytoplasm (Gurdon & Brown, 1965). As neuronal injury is primarily cytoplasmic, it is possible that a related mechanism may be concerned.

When axon regeneration was impeded, the reduced rate of ribosome synthesis found more than 70 days after injury could not have been due only to the nerve cell's failure to reach the motor end-plate: if it had been so, the rate of synthesis would have been reduced to an equal degree in all experiments without regard to the initial site of the lesion. It was found, however, that the rate of ribosome synthesis was reduced more when more of the axon was initially removed. It appears that the late low rate of synthesis is related to the final low cell volume: the restoration of normal values more than 70 days after injury with permitted regeneration may be due in part to maturation of the axon (Aitken *et al.* 1947) which increases the total cell volume. Edström & Pigón (1958) have observed a close relationship between cell size and nucleic acid content in uninjured neurones. It is unlikely, however, that the pattern of genetic derepression and repression which occurs in a neurone after axotomy is only a consequence of initial cell swelling and, in some circumstances, of final cell atrophy. The axon is a highly differentiated process, and the cellular mechanism of its replacement is probably related more closely to

changes accompanying regeneration of the cap of *Acetabularia* (Hämmerling, Clauss, Keck, Richter & Werz, 1958), or of the 'head' of *Stentor* (Tartar, 1961) rather than 'simple' replacement of lost homogeneous cytoplasm (Hartmann, 1928).

Changes occurring in the 'normal' hypoglossal nucleus. These changes are not due to the injuring of a few cells which have their axons in the opposite hypoglossal nerve, as the timing of the response differs from that of the injured side. It is unlikely that the changes are due to nerve fibres of the uninjured side sprouting within the tongue to innervate contralateral muscles: this is only occasionally seen and is limited to a few sites, such as the hyoid end of geniohyoid. After injuring the left hypoglossal nerve the whole tongue must be controlled by the right nerve, and it is possible that the changes in the uninjured neurones are those of work hypertrophy. The changes occur at about the same rate as changes in RNA metabolism observed autoradiographically when the functional state of neurones is changed (Watson, 1965*b*).

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EXPLANATION OF PLATES

PLATE 1

A. Freshly isolated neurone from a normal hypoglossal nucleus. Note the adjacent clump of glia. Phase contrast: scale 100 μ.

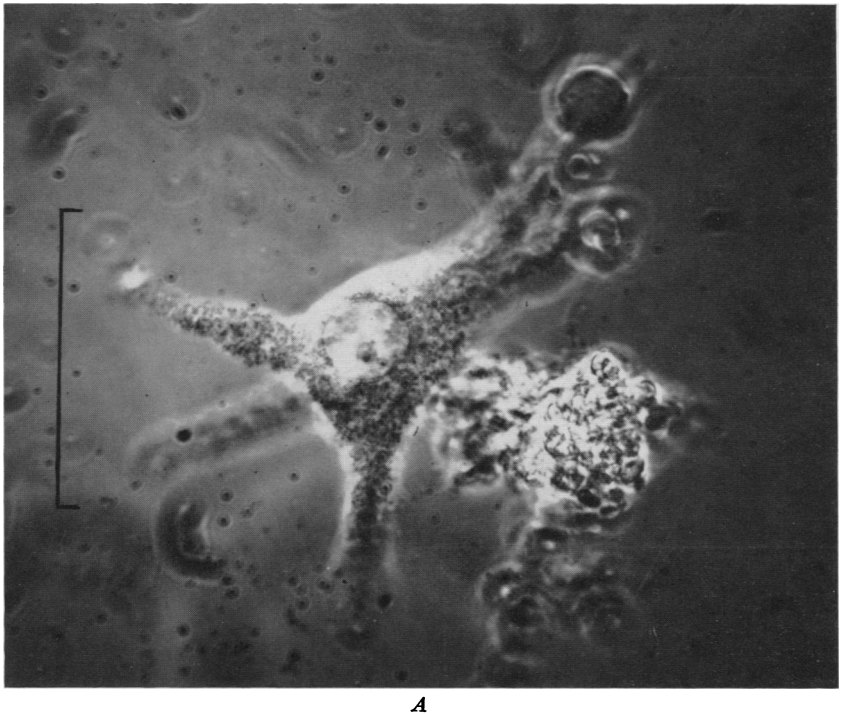
B. Normal hypoglossal neurone which has been fixed in Carnoy. Acid-soluble oligonucleotides have been extracted, and the cell flattened to a thickness of about 5 μ. Monochromatic photomicrograph at 2537 Å. Scale 100 μ.

PLATE 2

A. Nucleus of a normal hypoglossal neurone which has been fixed in formalin after aqueous isolation. Acid-soluble oligonucleotides have been extracted, and the nucleus flattened to a thickness of about 5 μ. Monochromatic photomicrograph at 2537 Å. Scale 10 μ.

B. Nucleus of a normal hypoglossal neurone which has been fixed in formalin after aqueous isolation. Acid-soluble oligonucleotides have been extracted. The nucleolus is seen during extrusion from the nucleus by deliberate oblique pressure upon the coverslip. Thickness about 4 μ. Monochromatic photomicrograph at 2537 Å. Scale 10 μ.

C. Nucleus of an uninjured hypoglossal neurone obtained from a rat injected 48 hr previously with actinomycin D. The nucleus has been fixed in formalin after aqueous isolation and acid-soluble oligonucleotides have been extracted. Note the marked decrease in absorbance of the nucleolus. Thickness about 5 μ. Monochromatic photomicrograph at 2537 Å. Scale 10 μ.



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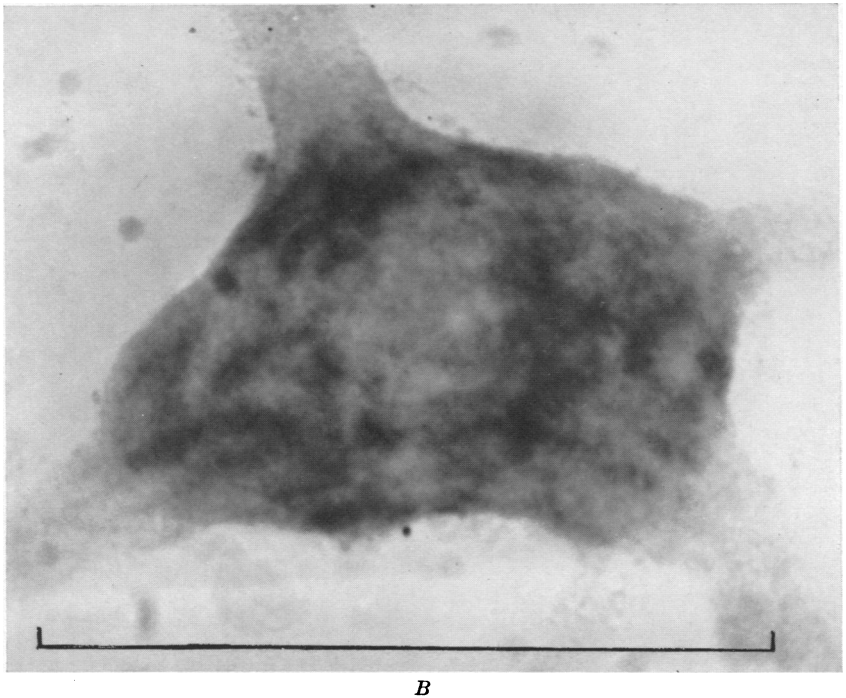

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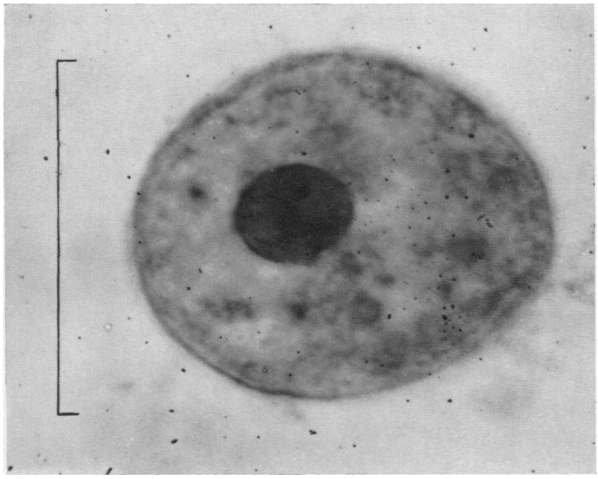
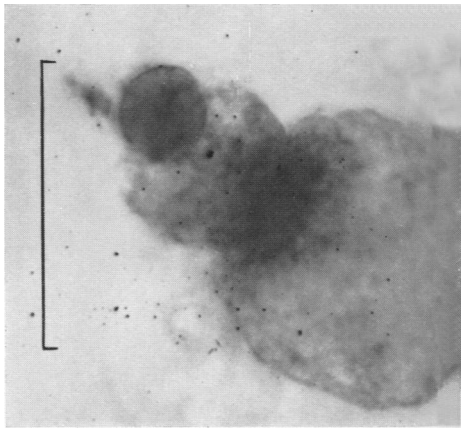
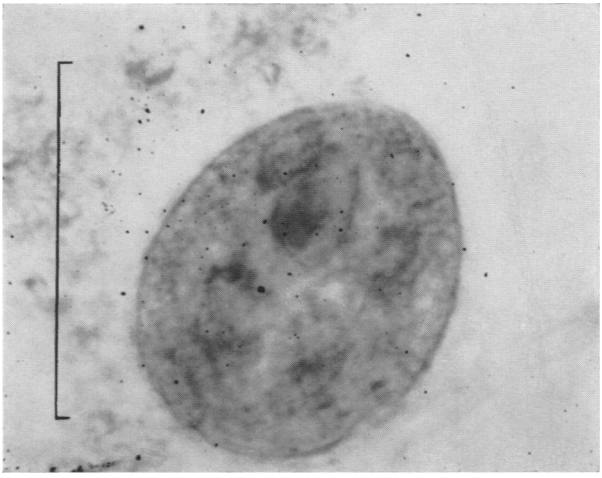


A



B



C