J. Physiol. (1969), **202**, *pp.* 611–630
With 1 *plate and* 4 *text-figures*
Printed in Great Britain

THE RESPONSE OF MOTOR NEURONES TO INTRAMUSCULAR INJECTION OF BOTULINUM TOXIN

By W. E. WATSON

From the Department of Physiology, University Medical School, Edinburgh 8

(*Received* 12 *December* 1968)

SUMMARY

1. The dry mass and nucleic acid content of both nerve cell bodies and their nucleoli were measured by interference microscopy and ultra-violet absorption microspectrography respectively: succinoxidase and acetylcholine hydrolase activities were also determined. Autoradiography was used to follow synthesis of deoxyribonucleic acid (DNA) by glial cells, and to follow nucleic acid and protein metabolism in muscle fibres.

2. After injection of botulinum toxin the synthesis of ribosomal RNA by the neurone followed closely the pattern found after axotomy.

3. After injection of toxin neuronal dry mass increased before the rate of ribosomal RNA synthesis was raised. This early increase, which was not due to increased protein synthesis, probably represents a 'damming back' of proteins within the nerve cell body.

4. After injection of toxin no local accumulation of microglial cells synthesizing DNA was found around the affected neurones: it is suggested that this reflects the intact system for intra-axonal transport under these conditions.

5. The affected muscles show increased nucleic acid and protein synthesis.

6. It is suggested that the results obtained indicate that membrane expansion or synthesis which occurs both in muscle and in neurone under these circumstances is the factor responsible for inducing directly or indirectly the changes found in nucleic acid metabolism after injection of botulinum toxin and after axotomy.

INTRODUCTION

After axotomy striking neuronal changes take place. The total dry mass and nucleic acid content both of the nerve cell body and of the nucleolus are markedly altered (Brattgård, Edström & Hydén, 1957; Watson, 1969): the rate of synthesis of neuronal ribosomal RNA is increased (Watson, 1968*a*). Significant changes in neuronal enzyme activities have

been found (Sjöstrand, 1966; Watson, 1966*a*, *b*) and in addition the perineuronal glial cells have been observed to divide (Watson, 1965*a*; Sjöstrand, 1965, 1966).

These investigations have thrown no light upon the mechanisms of induction of the observed changes, and in an attempt to clarify certain aspects of this problem the effects of local intramuscular injection of botulinum toxin upon these same parameters have been observed. Both after axotomy and after injection of toxin, sprouting of the axon occurs (Duchen & Strich, 1968) and neuromuscular transmission ceases (Brooks, 1956): only after axotomy is axoplasm lost. A comparison of these states was therefore undertaken to determine the role of loss of axoplasm in inducing the chromatolytic response.

METHODS

Animals. Observations were made upon male albino rats aged 3 months at the time of injection of toxin, 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*.

Injection of toxin. Under light ether anaesthesia the left half of the tongue was infiltrated with 250 pg of precipitated unpurified type A botulinum toxin in 250 μl. Ringer–Locke solution: the intrinsic muscles were injected perorally, and the extrinsic muscles through the floor of the mouth. Rats were killed between 1 and 100 days after injection by exsanguination under light ether anaesthesia.

Puromycin and actinomycin D injection. Puromycin, a drug inhibiting synthesis of protein by interfering with transcription of RNA at the ribosome (Nathans, 1964), was dissolved to a final concentration of 2 mg/ml. in artificial cerebrospinal fluid, and 250 μl. was injected slowly into a lateral ventricle under light ether anaesthesia. Preliminary experiments showed that this concentration of puromycin reduced the capacity of hypoglossal neurones to incorporate [^{3}H]lysine into protein by more than 90 %. Animals were killed between 5 min and 8 hr after injection and the dry mass of the nerve cell body was determined.

Actinomycin D, a drug inhibiting the synthesis of DNA-primed RNA (Reich, Franklin, Shatkin & Tatum, 1961; Goldberg & Rabinowitz, 1962) was administered as described previously (Watson, 1968*a*). Animals were killed at varying intervals up to 48 hr after injection, and measurements were made of nucleolar nucleic acid.

Preparation of cells. Single hypoglossal neurones free of glia, and the nuclei of other hypoglossal neurones were isolated and randomly selected in the way described before (Watson, 1968*a*). The further preparation of some of these cells and nuclei required for measurement of nucleic acids by ultra-violet absorption microspectrography was the same as before. Other cells and nuclei were prepared for measurement of dry mass by interference microscopy (Davies, 1958): they were fixed in 10 % buffered formalin for between 2 and 6 hr, washed in water, mounted in a sucrose solution of refractive index 1·472, and covered with a coverslip. Some fixed cells were digested with crystalline ribonuclease 100 mg/ml. in McIlvaine's (1921) citrate-phosphate buffer, pH 7·0 (glass electrode), or extracted with chloroform-methanol (Rouser, Bauman & Kritchevsky, 1961), so that extractable RNA and lipids could be measured. Other cells required for Cartesian diver micromanometry were rapidly transferred to a droplet of substrate at 4° C (Watson, 1966*a*, *b*).

Interference microscopy. The dry mass of nerve cell bodies, nuclei or nucleoli, was measured with a Leitz interference microscope (Fluorite optics, objectives 100/1. 36; condensors N.A. 0·50, oil immersion) by determining the integrated optical path difference of the fixed cell

or organelle of known refractive index contained in, and permeated by, a sucrose solution, the refractive index of which was also known (Davies, 1958). A low pressure mercury lamp provided filtered monochromatic light of 546 pm wave-length; white light from a tungsten source, used alternatively, allowed the interference band of zero order to be found. The cell was first examined in a banded field (Pl. 1, fig. 1) to check that the greatest optical path difference did not exceed 110 pm (0·2 λ). The width between bands was then increased until band separation became infinite: under these circumstances the background appeared uniformly illuminated by part of a zero-order interference band. Background intensity was adjusted by altering the optical path difference between the reference beam and the measuring beam by moving a fine transparent wedge within the reference beam until relative phase retardation was such that regional variation in the optical path difference due to the cell caused proportional variation in the optical density of the corresponding image (Pl. 1, fig. 2). Two photomicrographs were taken: the first was of the cell under these conditions (Pl. 1, fig. 2) and the second of a closely adjacent clear field crossed by four or five interference fringes. Photomicrographs taken on 35 mm Plus X film were processed in Kodak 19b developer. The negatives were evaluated with a Joyce–Loebl integrating microdensitometer ($\times$ 22 objective magnification; 1:10 arm ratio; 0–3·0 optical density wedge). The microdensitometer slit width and height corresponded to a field width and height of 200 and 500 nm respectively, referred to the original object plane. The negatives of the cell were scanned in parallel strips 4 μm apart (Pl. 1, fig. 3), referred to the original object plane: those of nucleoli at intervals of 450 nm. Care was taken that variation of background illumination did not exceed 2 % within the measured area.

Quantitative measurement of dry mass by interference microscopy depends upon a number of conditions. Integration of the optical path difference over the total area of the cell body or organelle is possible only when there is a linear relationship between the regional variations in optical path difference of the cell or organelle, and the optical density of the photomicrograph. As this relationship is basically sinusoidal and distorted by the contrast characteristics (gamma) of the film, it can at best be described as an approximation to linearity over a restricted range. Preliminary experiments made upon nerve cells under conditions identical with those of dry mass determination (Pl. 1, figs. 1, 2, 3) show that error from this source does not exceed 10 % provided that the maximum optical path difference of the object does not exceed 110 pm (0·2 λ) and that the level of background illumination is critically selected. These findings conform with those of Davies & Deeley (1956) and of Mitchison, Passano & Smith (1956). The refractive index of the medium used in this investigation reduced the cell's mean optical path difference to less than 55 pm (0·10 λ), and the maximum optical path difference due to small parts of the cell having considerable dry mass concentration (such as the nucleolus) to less than 110 pm.

Calculation of dry mass from the integrated optical path difference, or cell refractance, requires that the refractive index of the fixed cell, and the specific refraction increment (Reiss, 1903; Adair & Robinson, 1930) of its constituents are known, as well as the refractive index of the surrounding medium.

Previous measurements of the refractive indices of fixed cells indicate values in the range 1·53–1·572 (Crossmon, 1949; Oettlé, 1950; Davies, Deeley & Denby, 1957). Values in the upper part of this range are usually obtained when the cells are immersed in oily media (Swift & Rasch, 1956), possibly because of alteration of the state or quantity of bound water (Davies, 1958). Davies *et al.* (1957) found that the refractive index of bull sperm heads was greater in non-aqueous media. The specific refraction increment, that is, the increase in refractive index of a solution for each 1 % increase in solute concentration, is known for protein, lipoprotein and nucleic acids. The specific refraction increment of nearly all soluble unconjugated proteins lies within the range 0·00181–0·00188 (Perlman & Longsworth, 1948; Barer & Tkaczyk, 1954; Barer, 1956) over a wide range of solute concentrations, and is hardly affected by change of pH or solute concentration. Calculated values for specific

refraction increment of very concentrated protein solutions lie within the wider range 0·00151–0·0021 (Davies, 1958). This increased scatter is in part dependent upon the method of calculation used. The measurements made by Davies (1959) and by Davies & Thornburg (1959) indicate that the specific refraction increments of crystalline proteins lie within the lesser range 0·00181–0·00188. The specific refraction increment for lipoprotein is 0·0017 (Armstrong, Budka, Morrison & Hasson, 1947), for deoxyribonucleic acid 0·00175–0·0020 (Vincent, 1952; Northrop, Nutter & Sinsheimer, 1953; Brown, McEwan & Pratt, 1955) and for ribonucleic acid 0·00168–0·00194 (Northrop & Sinsheimer, 1954; Davies, 1958).

In this investigation the refractive index of neurones measured by immersion refractometry (Barer, 1956) in aqueous media was found to be 1·556 (Text-fig. 2). This value, lying close to the centre of the range reported for various cell types, was used for calculating dry mass. Similarly, as extraction procedures indicated that about 70 % of the dry mass of hypoglossal nerve cell bodies is protein, a finding conforming with analyses of Deiter neurones (Brattgård & Hydén, 1952) and hypoglossal neurones (Brattgård, Edström & Hydén, 1957) of the rabbit by X-ray absorption historadiography (Engström, 1956), a value of 0·0018 was used as the specific refraction increment. The use of these values was justified by comparing the values obtained for ribonuclease extractable RNA of nerve cell bodies and of nucleoli using interference microscopy with values obtained using ultra-violet absorption microspectrography. Neuronal RNA was found to be 169 (± 20 S.E.) pg by interference microscopy, and 182 (± 34 S.E.) pg by ultra-violet absorption: the corresponding values for nucleolar RNA were 1·2 (± 0·2 S.E.) and 1·3 (± 0·2) pg respectively (Watson, 1968*a*). The close agreement between these values suggests that the use of the values of 1·556 and 0·0018 for the refractive index and specific refraction increment is correct.

Ultra-violet microspectrography. A Leitz ultra-violet microspectrograph was used to measure the nucleic acid content of neurones and of neuronal nucleoli in the manner described previously (Watson, 1968*a*).

Cartesian diver micromanometry. Enzyme activities were measured in ampulla Cartesian divers (Zeuthen, 1953); the conditions of measurement have been described before (Watson, 1966*a*, *b*). Succinoxidase activity was measured by the method of Hydén & Pigòn (1960). Acetylcholine hydrolase was measured using the medium of Giacobini & Holmstedt (1958); acylcholine acylhydrolase was inhibited with Mipafox.

Autoradiography. Tritiated [^{3}H]compounds were obtained from the Radiochemical Centre at Amersham. The techniques of autoradiography were the same as previously described (Watson, 1965*a*). Some rats were injected intraventricularly with 250 μl. artificial c.s.f. (Mitchell, Loeschke, Massion & Severinghaus, 1963) containing 10 μc [$^3H_{CH_3}$]thymidine (specific activity 19·6 c/m-mole) to allow DNA synthesis to be followed, or 100 μc [$^3H_5$]-uridine (S.A. 27 c/m-mole) to demonstrate neuronal RNA synthesis, or 100 μc of either [$^3H_{4,5}$]lysine (S.A. 6·6 c/m-mole) or [G-^{3}H]methionine (S.A. 95 mc/m-mole), to demonstrate protein synthesis in nerve cells. Other rats received 100 μc [$^3H_{CH_3}$]methionine (S.A. 110 mc/m-mole) after 500 mg puromycin had been injected to inhibit protein synthesis: under these circumstances considerable radioactivity may be demonstrated in nuclear RNA, as a result of methylation of precursors of ribosomal RNA.

Other rats were injected intraperitoneally with 200 μc [$^3H_{CH_3}$]thymidine, 400 μc [$^3H_5$]-uridine or 400 μc [^{3}H]lysine to allow DNA, RNA and protein synthesis to be followed in the paralysed muscles of the tongue. Suitable control experiments were done to exclude significant chemosensitivity or emulsion desensitization by the tissues. Other sections were digested with ribonuclease or deoxyribonuclease before preparing autoradiographs.

Observations made upon hypoglossal neurones when the animal was killed 8 hr after injection of [$^3H_5$]uridine are expressed as the ratio 'grain density in cytoplasm/grain density in nucleus (Cyt./Nuc. ratio)' for the reasons previously discussed (Watson, 1965*a*).

Histology. The diameters of axons of hypoglossal neurones were measured by light microscopy in nerves prepared by the method of Aitken, Sharman & Young (1947). Terminal

nerve fibres within the tongue and their motor end plates were demonstrated by the methods described by Duchen & Strich (1968). Tissue for electron microscopy was prepared by perfusing the rat with Millonig's (1961) fixative, post fixing in 1% osmium, embedding in Araldite (CIBA Ltd.) and staining sections with uranyl acetate. The sections were examined with an A.E.I. EM 6 microscope.

RESULTS

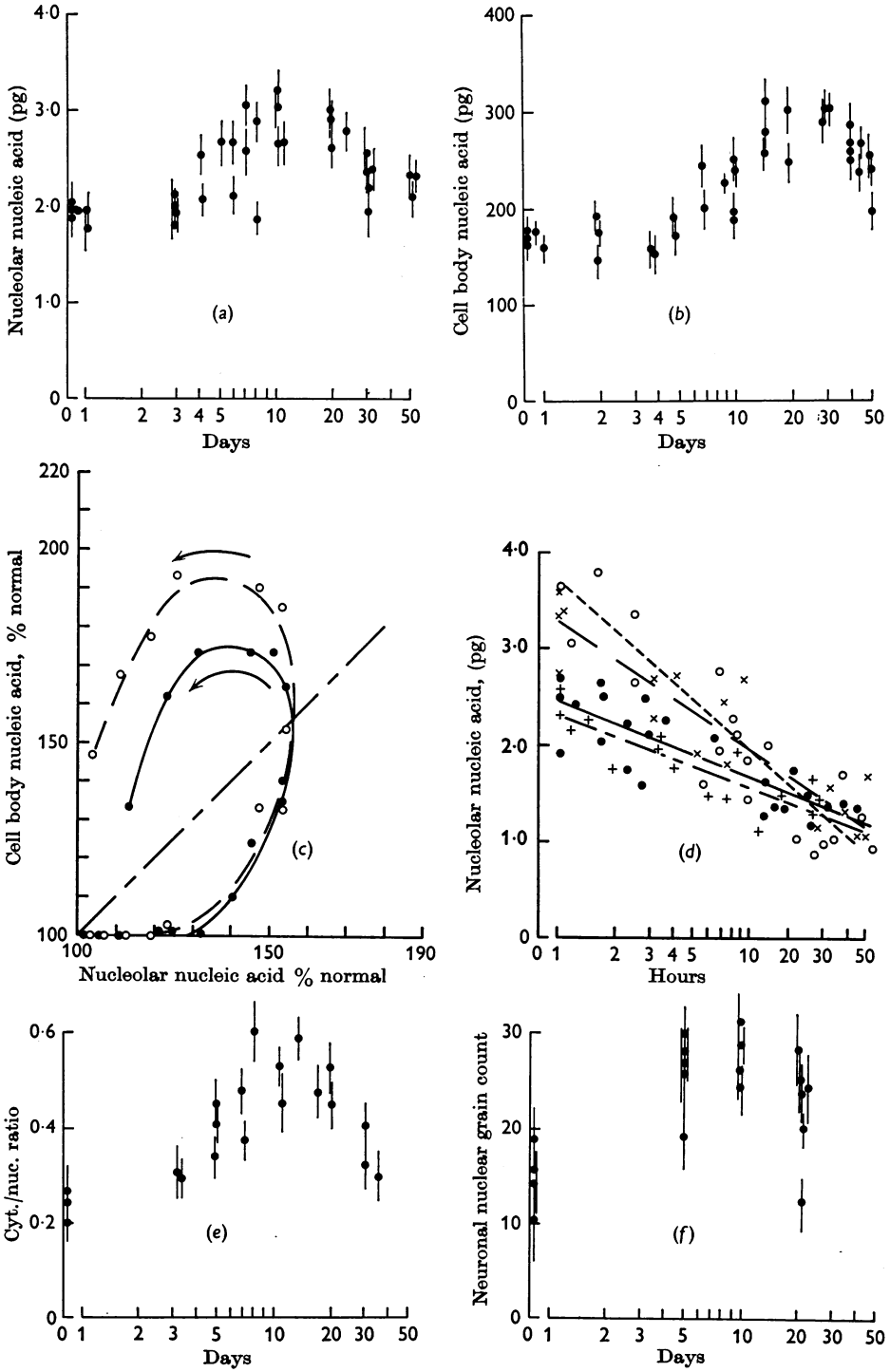
Between 7 and 14 days after injection most rats had a mild transient generalized weakness, appeared poorly groomed and lost weight from 258 ± 5 (S.E.) to 232 ± 7 g (S.E.). Stimulation of the left hypoglossal nerve with square pulses of 5 msec duration caused little or no contraction of the injected half of the tongue between 2 and 25 days after injection. The tongues of some rats contracted weakly on stimulation after the 25th day, but in most the local paralysis lasted 40–50 days. Fasciculation was observed in the intrinsic muscles of the tongue after the third day following injection.

Nucleic acid changes. On the 5th day after injection the nucleolar nucleic acid content began to increase, reached a maximum between the 10th and 20th day and then decreased (Text-fig. 1*a*): this increase was highly significant ($P < 0{\cdot}001$). The total nucleic acid content of the nerve cell body began to increase on the 6th day, reached a maximum value about the 20th day and then slowly decreased to reach a normal value about the 50th day (Text-fig. 1*b*): this increase was significant ($P < 0{\cdot}01$, $> 0{\cdot}001$). The nucleolar changes preceded those of the cell body, and the phase relationship between these two parameters is shown in Text-fig. 1*c*, where it is compared with results previously obtained (Watson, 1968*a*) after distal axotomy.

After injection of Actinomycin D the nucleolar nucleic acid decreased (Text-fig. 1*d*). The slope of the calculated regression line representing this rate of decrease was steeper on the 10th and 21st day after injection of botulinum toxin, but was normal on the 4th day. These changes in the slope of Actinomycin D-induced 'decay' in nucleolar nucleic acid were significant ($P < 0{\cdot}01$, $> 0{\cdot}001$).

Eight hours after injection of [$^3H_5$]uridine into a lateral cerebral ventricle, the intraneuronal distribution of labelled nucleic acid was determined autoradiographically, and expressed as the ratio 'grain density of nucleus' (Cyt./Nuc. ratio). Text-fig. 1*e* shows that this ratio varies in a manner closely resembling that of nucleolar nucleic acid content (Text-fig. 1*a*). The increase of this ratio was significant ($P < 0{\cdot}05$, $> 0{\cdot}01$).

Hypoglossal neurones of rats which had received [$^3H_{CH_3}$]methionine by intraventricular injection after protein synthesis had been blocked with puromycin showed heavy labelling of neuronal nuclei: the silver grains lay mainly in the perinucleolar region and a few over clumps of chromatin.



Text-fig. 1. For legend see opposite page.

After injection of botulinum toxin the incorporation of isotope increased significantly (Text-fig. 1*f*) ($P < 0{\cdot}05$, $> 0{\cdot}01$) in a way following closely the nucleic acid content of the nucleolus: over 70 % of this isotope could be removed by initial treatment of the sections with ribonuclease. Injection of labelled methionine of similar specific activity in which the isotope was not confined to the methyl group resulted in a far smaller incorporation into neuronal nuclei under all circumstances.

Changes in dry mass. The refractive index (N_D) of fixed neurones was measured by determining the optical path difference of selected areas of their cytoplasm when they were immersed in sucrose solutions of different refractive index: also, when the range of sucrose concentration was such that the greatest optical path difference did not exceed 110 pm ($0{\cdot}2\,\lambda$) (Plate 1, fig. 1), the integrated optical path difference of the cell body was measured in each solution. The optical path difference, or integrated optical path difference, was expressed graphically against the refractive index of the sucrose solution in which the cell was immersed. The results obtained for each cell, or part of cell, lie on a straight line (Text-fig. 2). This line was extrapolated to find the refractive index at which the optical path difference would become zero: this value, the refractive index of the cell, was found to be 1·556. This preliminary experiment also showed that the fixed cell was rapidly permeated by sucrose solutions: the optical path

---

Legend to Text-fig. 1

Text-fig. 1.(*a*). Nucleolar nucleic acid (pg) is expressed against the time (days) since injection of botulinum toxin. Each point represents a mean value of twenty nucleoli obtained from one rat: the S.E. is also indicated.

(*b*) Cell body nucleic acid (pg) is expressed against the time (days) since injection of botulinum toxin. Each point represents a mean value of twelve–twenty cells obtained from one rat: the S.E. is also indicated.

(*c*) Cell body nucleic acid, expressed as % normal, expressed against nucleolar nucleic acid, similarly shown, to show the similar relationship between these parameters after botulinum toxin (●——●) and after distal axotomy (○——○; Watson, 1968*a*). The arrows indicate temporal direction. The interrupted line is at 45° from the origin.

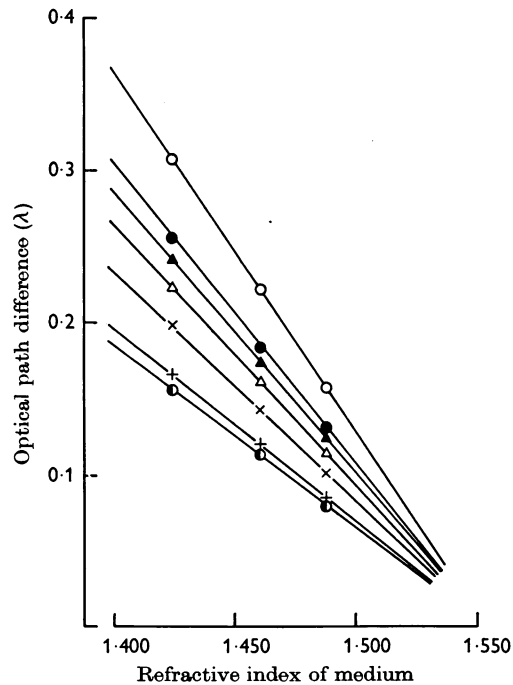
(*d*) Nucleolar nucleic acid (pg) is expressed against the time (hours) since injection of actinomycin D. Each point represents a mean value of twelve–twenty nucleoli obtained from one rat. S.E. omitted for clarity. +— · — · —+ no botulinum toxin, ● —— ● botulinum toxin 4 days previously, ○ - - - - ○ botulinum toxin 10 days previously, and ×— —× botulinum toxin 21 days previously.

(*e*) The ratio 'grain density in cytoplasm/grain density in nucleus' measured autoradiographically in neurones obtained from rats killed 8 hr after injection of [$^3H_5$]uridine is expressed against the time (days) after injection of toxin.

(*f*) The number of silver grains over each neurone nucleus found in rats injected with [$^3H_{CH_3}$]methionine after protein synthesis has been blocked with puromycin, is expressed against the time (days) after injection of botulinum toxin.

difference did not show further detectable change between 5 min and 2 hr after changing the concentration of sucrose.

The nucleolar dry mass increased after injection of toxin (Text-fig. 3*a*), following closely changes in nucleolar nucleic acid (Text-fig. 1*a*): this increase was highly significant ($P < 0{\cdot}001$). The total dry mass of the nerve cell body (Text-fig. 3*b*) followed a pattern different to that of cell body nucleic acid content (Text-fig. 1*b*): it increased to reach a maximum



Text-fig. 2. The optical path difference in wave-lengths (546 pm) measured in selected small cell areas is expressed graphically against the refractive index of the sucrose solution in which measurements were made. Each symbol represents a different part of different cells. Seven representative examples from a total of fifty are shown. Note convergence of the lines to the point of zero optical path difference at a refractive index of 1·556.

value between the 5th and 10th day, remained fairly constant until about the 20th day, and it finally slowly declined in a manner similar to that of cell body nucleic acid content. The relationship between nucleolar nucleic acid content and total cell body dry mass is shown in Text-fig. 3*c*, where it is compared with results previously reported (Watson, 1968*a*, 1969) obtained after distal axotomy. Only after the 10th day is the relationship between these two parameters similar under these two circumstances: before the 10th day, the large increase in dry mass preceding nucleolar

acid change is clearly seen to be different to the results obtained after axotomy.

Extraction of lipid and some lipoprotein with hot methanol-chloroform and of RNA with ribonuclease showed that at least 80 % of the increase in dry mass found on the 5th day after injection was not extractable lipid or RNA.

After injection of puromycin the dry mass of nerve cell bodies decreased: within the first hour the decrease found in normal hypoglossal neurones was 8 % ± 1·5 % (S.E.) (Text-fig. 3*d*). The dry mass did not decrease significantly more rapidly on the 5th day after botulinum toxin ($P > 0{\cdot}05$), but this rate of decrease was greater on the 10th and 21st days after injection (Text-fig. 3*d*) ($P < 0{\cdot}05$, $> 0{\cdot}01$).

Autoradiography of sections of hypoglossal neurones obtained from rats killed 2 hr after intraventricular injections of [^{3}H]lysine showed no significant change in the incorporation of isotope on the 5th day after injection: the grain count was significantly greater on the 10th and 21st days ($P < 0{\cdot}05$, $> 0{\cdot}01$). A similar result was obtained with [^{3}H]methionine in which the isotope was not confined to the methyl group.

Micromanometry. The activity of acetylcholine hydrolase increased after injection of toxin (Text-fig. 4*a*), to reach a maximum value between the 5th and 7th days, and then decreased: this increase was significant ($P < 0{\cdot}01$, $> 0{\cdot}001$). The apparent decrease of this activity to values less than normal between the 15th and 20th days was not significantly different from normal ($P > 0{\cdot}05$).

A significant decrease was found in succinoxidase activity ($P < 0{\cdot}05$, $> 0{\cdot}01$) between the 4th and 20th days after injection (Text-fig. 4*b*).

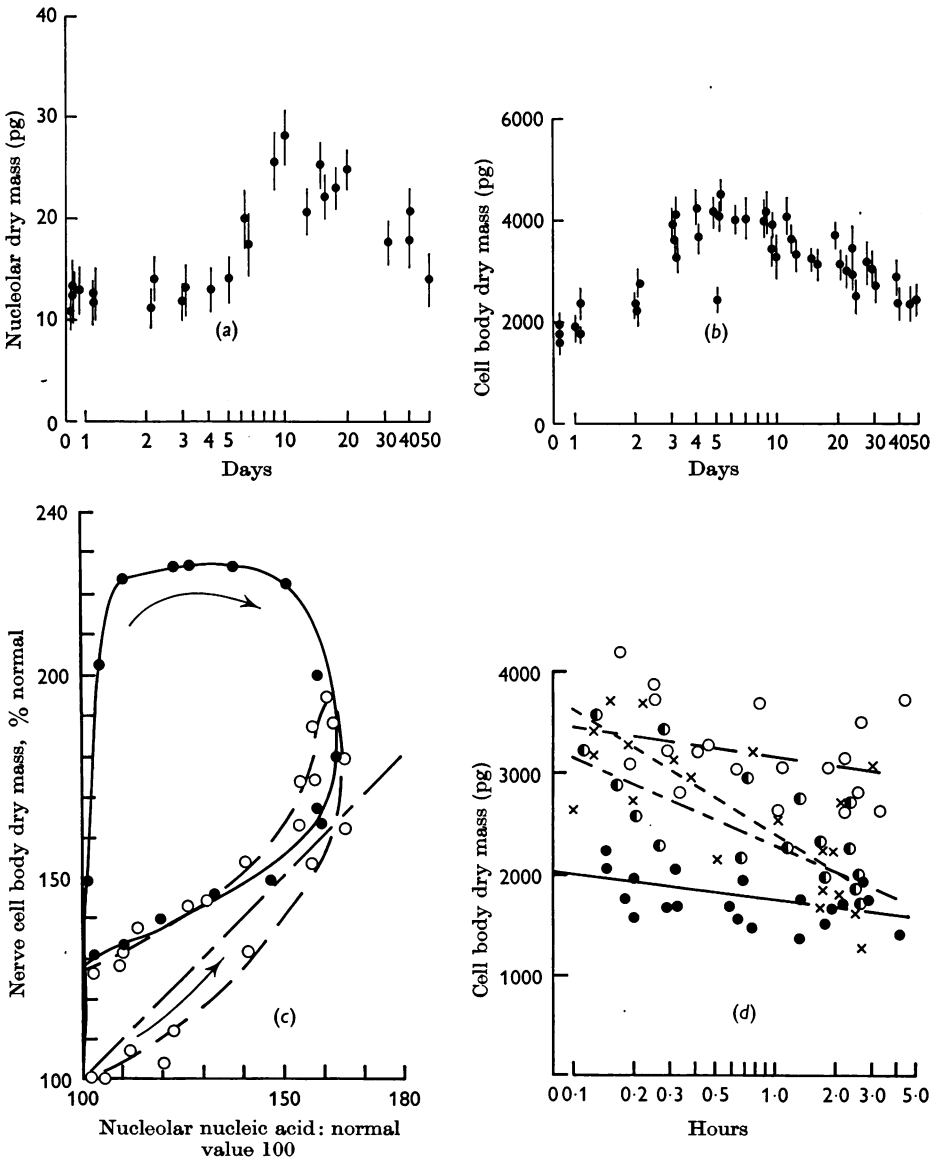
Electron microscopy. No significant changes could be seen in hypoglossal nerve cells before the seventh day after injection: changes were especially sought in mitochondria, endoplasmic reticulum and Golgi apparatus, but none could be convincingly demonstrated. Some fragmentation of the endoplasmic reticulum was first found on the seventh day.

Perineuronal glia. After injection of [^{3}H]thymidine into a lateral cerebral ventricle none was incorporated into the DNA of glial cells of the hypoglossal nuclei at any time after the injection of toxin into the tongue.

Hypoglossal nerve and tongue. No significant change was found in the diameter of axons of hypoglossal nerves within the first 14 days after injection, that is, over the period of the early increase in neuronal dry mass (Text-fig. 3*b*). Sprouting of the terminal part of the motor axon was first seen 6 days after injection of toxin, and became most extensive between 18 and 30 days: within this period many sprouts were more than 500 μm long and commonly passed over adjacent muscle fibres: even after 100 days the normal pattern of muscle innervation had not been completely

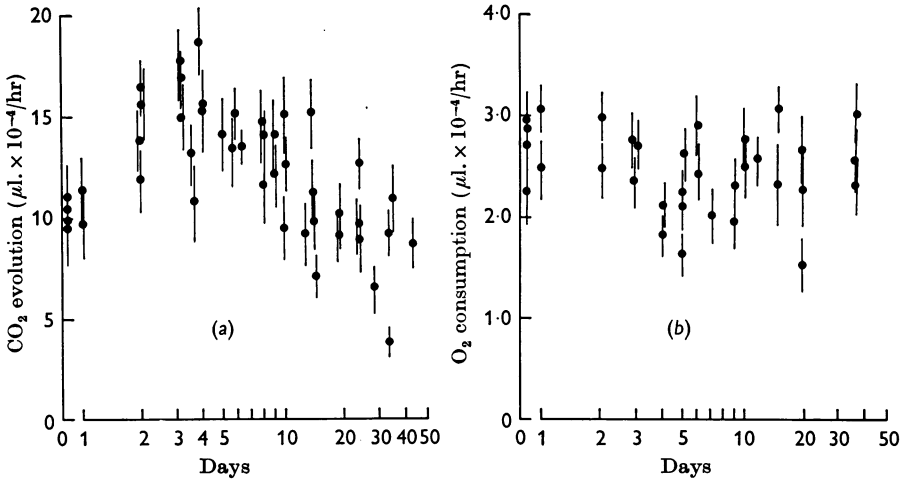
restored. About 5 days after injection the sarcolemmal nuclei of the left half of the tongue became larger and rounder with prominent nucleoli.

Autoradiographs of hypoglossal nerves obtained from rats injected with toxin between 7 and 14 days previously, and with intraventricular [^{3}H]-lysine between 2 and 5 days before death, showed intra-axonal radio-



Text-fig. 3. For legend see opposite page.

activity. Autoradiographs of the tongue after intraperitoneal injection of [$^3H_{CH_3}$]thymidine showed occasional labelling of sarcolemmal nuclei between the 5th and 12th day after botulinum toxin: after [$^3H_5$]uridine had been injected intraperitoneally the incorporation of isotope into RNA was markedly increased within sarcolemmal nuclei between the 5th and



Text-fig. 4 (*a*). Acetylcholine hydrolase activity of single hypoglossal neurones, measured as rate of evolution of CO_2 (μl. $\times 10^{-4}$/hr), is expressed as against the time (days) since the injection of botulinum toxin. Each point represents a mean value of ten–fifteen cells obtained from one rat. The S.E. is also shown.

(*b*) Succinoxidase activity of single nerve cells, measured as rate of consumption of O_2 (μl. $\times 10^{-4}$/hr) is expressed against the time (days) since the injection of botulinum toxin. Each point represents a mean value of ten–fifteen cells obtained from one rat. The S.E. is also shown.

Legend to Text-fig. 3

Text-fig. 3 (*a*). Nucleolar dry mass (pg) is expressed against the time (days) since injection of botulinum toxin. Each point represents a mean value of twenty nucleoli, and the S.E. is also shown.

(*b*) Cell body dry mass (pg) is expressed against the time (days) since injection of toxin. Each point represents a mean value of twenty cells obtained from one rat, and the S.E. is also shown.

(*c*) Cell body dry mass, expressed as % normal, expressed against nucleolar nucleic acid, similarly shown, to show the different relationship between these parameters after botulinum toxin (●——●) and after distal axotomy (○— —○; Watson, 1968*a*, 1969). The arrows indicate temporal direction. The interrupted line is at 45° from the origin.

(*d*) Cell body dry mass (pg) is expressed against the time (hours) since injection of puromycin. Each point represents a mean value of twenty cells obtained from one rat. S.E. omitted for clarity. ●——● represents no botulinum toxin, ○ — —○ botulinum toxin 5 days previously, × - - - - × botulinum toxin 10 days previously, and ◐— - — -◐ botulinum toxin 21 days previously.

21st days after injection of toxin, coinciding with the period in which sarcolemmal nucleoli became more prominent: within the same period the incorporation of [^{3}H]lysine and [^{3}H]methionine into protein of the muscle fibres was also markedly increased.

The injection of boiled botulinum toxin caused no significant change in these parameters.

DISCUSSION

Many assumptions are made in attempted quantitative microchemical measurement using the techniques described: the limitations of ultra-violet absorption microspectrography and of autoradiography used in the way described have been previously discussed (Watson, 1965*a*, 1968*a*).

Significance of dry mass determinations. Isolation of nerve cells excludes from measurement nearly all the axon and the peripheral part of the dendritic tree (Plate 1, fig. 2). An attempt has been made to estimate approximately the proportion of the cell's dry mass lying in these excluded areas. The dry mass of axons was measured in transverse sections of known thickness of hypoglossal nerve. The estimated total axonal dry mass was 2200 pg: ultra-violet absorption microspectrography showed an absorption peak at 280 pm, indicating that the axoplasm contains no significant amount of nucleic acid. If it is assumed that the dendritic spread of a hypoglossal neurone resembles that of a spinal motor nerve cell (Aitken & Bridger, 1961; Schadé & van Harreveld, 1961), and that the dry mass concentration of dendrites resembles that of axons, then an estimate of 800 pg may be made for dendritic dry mass. The total dry mass of the neurone may therefore be estimated as 4100 pg, of which only 40 % is measured in the isolated nerve cell body. This measured fraction cannot be considered as a constant proportion of the whole, or as a stable entity which can be measured under varying circumstances, for some fractions of the protein and phospholipid of the cell body exchange rapidly with those of the cell's processes (Droz & Leblond, 1962, 1963, 1964; Miani, 1962, 1963; Watson, 1968*b*). Furthermore, striking changes may occur in the dendrites after axotomy (Cerf & Chacko, 1958). It is very probable, however, that the bulk of neuronal protein is synthesized within the nerve cell body: over 90 % of neuronal nucleic acid is found in the cell body (Hydén, 1960; Utakojii & Hsu, 1965). After injection of tritiated amino acids, no significant activity can be found in the protein of dendrites or of the axons within 1 hr of injection (Droz, 1965). The significance of reported axonal RNA having base ratios (Koenig, 1965*a*) and sedimentation characteristics (Miani, di Girolamo & di Girolamo, 1966) resembling those of isolated ribosomes, and of axonal ribosome-dependent protein synthesis (Koenig, 1965*b*) is uncertain, and the amount of protein possibly synthesized within the axon is very small (Ochs, Johnson & Ng, 1967).

As nearly all neuronal protein synthesis occurs within the nerve cell body, the changes in measured cell body dry mass may be interpreted most readily with reference to the 'decay' of dry mass which follows injection of puromycin. If the neurone is in a reasonably steady state before injecting puromycin, that is, if its dry mass is not changing at a rate comparable with the change that follows puromycin, and if the transport of protein from the cell body into its axon and dendrites and the rate of destruction of protein within the nerve cell body remains unaltered after puromycin (Ochs *et al.* 1967), then the initial rate of 'decay' of dry mass represents the rate of synthesis of protein in the neurone in that steady state. The first condition is satisfied even during the chromatolytic response of the neurone to injury (Watson, 1969): the greatest rate of change of dry mass is then less than 5 % of the initial rate of 'decay' induced by puromycin. After botulinum toxin (Text-fig. 3) the rate of change of dry mass is also less than 5 % of the puromycin-induced rate of 'decay'. The second condition is probably satisfied: there is close correspondence between the altered rate of 'decay' of dry mass (Text-fig. 3) and the uptake of tritiated amino acids by the nerve cell body. A similar relationship was found during the response of the neurone to axotomy (Watson, 1969). Both after axotomy (Watson, 1968*a*, 1969) and after botulinum toxin (Text-figs. 1, 3) the rate of 'decay' of dry mass after puromycin follows closely the rate of 'decay' of nucleolar RNA after actinomycin D, a rate reflecting the rate of synthesis of ribosomal RNA (Watson, 1968*a*).

Botulinum toxin. Botulinum toxin, type A or D, prevents transmission at the neuromuscular junction (Dickenson & Shevky, 1923) by directly or indirectly preventing the release of acetylcholine from motor nerve terminals (Ambache, 1948, 1949, 1951; Burgen, Dickens & Zatman, 1949; Brooks, 1954) without impairing conduction within the nerve fibre (Brooks, 1956). As this investigation was undertaken as part of a study of the response of the nerve cell to injury, with special reference to the factors responsible for inducing the chromatolytic response to injury, it is necessary to compare the changes found in the parameter measured in this investigation with those found after axotomy: in both circumstances there is loss of functional contact between neurone and muscle, but after injection of toxin there is no associated loss of axoplasm.

Relation of nucleic acid changes to those following axotomy. The nucleic acid content of the nucleolus and cell body increases after axotomy, and this increase occurs later the more distally from the cell body the axon is injured (Watson, 1968*a*): after botulinum toxin the timing of this increase corresponds to that of a distal axonal injury, an observation which correlates well with the anatomical site of the pharmacological lesion. In both circumstances the nucleolar changes precede those of the cell body and are

correlated with an increased rate of synthesis of nucleolar RNA, judged by the associated increased rate of actinomycin D-induced decay of nucleolar RNA, by the associated increase in Cyt./Nuc. ratio after injecting [$^3H_5$]-uridine, and by the increased methylation of precursors of ribosomal RNA (Sirlin, Jacob & Tandler, 1963). These close similarities in the timing of several aspects of the nucleic acid response between the neurone after distal axotomy and the neurone after botulinum toxin, strongly suggest that some similar process of induction of synthesis of ribosomal RNA is occurring in both circumstances, and indicate that the quantity of axoplasm removed is related only to the timing of the response (Watson, 1968*a*), and that loss of axoplasm itself is not a necessary condition for the actual induction of ribosomal RNA synthesis.

Two potential hypotheses concerning the factor common to both states which is responsible for this induction can be dismissed. The first, that depolarization is responsible and occurs after botulinum toxin in the nerve terminals in a manner analogous to an injury potential following axotomy, is not compatible with the observations of Brooks (1956) concerning the pharmacological nature of the lesion. The second, that loss of 'effective contact' between neurone and muscle is the common factor is not compatible with the finding that ribosomal RNA synthesis can be induced by a second axonal injury after 'effective neurone–muscle contact' has already been removed by the first nerve injury (Watson, 1968*a*).

A working hypothesis can be based upon the finding of Duchen & Strich (1968), confirmed in this study, that extensive sprouting occurs of the terminal motor axon after local injection of botulinum toxin. Sprouting is also found after axotomy (Young, 1942). It is suggested that the initial act of sprouting itself contains the factor responsible for inducing these changes and that the induced changes in nucleic acid and protein synthesis are required for sustaining it: some support for this comes from the observation (unpublished personal data) that changes similar in nature and timing occur in neurones when their undamaged axons undergo collateral sprouting. In each of these three circumstances sprouting is associated with increased synthesis of ribosomal RNA. If the act of axonal sprouting is equated with an act of expansion or of synthesis of cell membrane, then this hypothesis has the further advantage that it can be applied to cells other than neurones and, for example, is compatible with the changes found in muscle after denervation (Axelsson & Thesleff, 1959; Miledi, 1960) or after botulinum toxin (Thesleff, 1960; Josefsson & Thesleff, 1961) (see *Changes in muscle* below).

Relation of changes in dry mass following toxin to those of injury. The changes in dry mass are not similar in these two circumstances (Text-fig. 3). Before the tenth day after injection of toxin there is an increase of dry

mass which is not found after axotomy, which precedes the increased synthesis of ribosomal RNA, and which is not associated with an increased rate of synthesis of neuronal protein, estimated from the initial rate of 'decay' of dry mass induced by puromycin (Text-fig. 3). As it is possible that such an increase in neuronal protein represents a 'damming back' of axoplasm (Weiss & Hiscoe, 1948) normally released or destroyed at the nerve ending, the diameters of axons in the hypoglossal nerve were measured: no change was found. The increased cell body dry mass cannot therefore be considered as a passive consequence of outflow obstruction.

Two further possibilities were tested: firstly, that the increased dry mass did in fact represent such a 'damming back', but that the expected axonal swelling was prevented by a co-ordinated decrease in the rate of input of protein into the axon, so that the primary site of protein accumulation was in the nerve cell body; secondly, that the increased dry mass represented such a 'damming back', but that this was associated with a continuing bidirectional intra-axonal transport of migrating organelles and protein (Lubińska, Niemierko, Oderfelt, Szwarc & Zelena, 1963; Kerkut, Shapira & Walker, 1967; Watson, 1968*b*). As [^{3}H]lysine injected intraventricularly continued to migrate down the axons of the hypoglossal nerve even when this early accumulation of neuronal dry mass was greatest, the last hypothesis is preferred. Some further evidence that this early increase of dry mass represents a form of 'damming back' comes from the finding that the activity of acetylcholine hydrolase, an enzyme that almost certainly passes along the axon (Lubińska, 1964), increases during this period (Text-fig. 4*a*). The failure of the mitochondrial succinic oxidase enzyme complex activity to increase as well (Text-fig. 4*b*) is difficult to explain, for there is evidence of bidirectional passage of mitochondria in axons of cultured neurones (Nakai, 1956; Pomerat, 1960). It is possible either that the systems of transport for proteins and for organelles respond differently under these circumstances, or that the partial autonomy of mitochondria in their regulation of protein synthesis (Roodyn, 1965) may be responsible for this difference.

After the tenth day following injection of toxin, the changes in dry mass resemble those found after axotomy (Text-fig. 3); further discussion of these changes is unnecessary, as they are secondary to the changes in nucleic acid metabolism already discussed.

Neuroglia. The failure of labelled glial cells to appear in the hypoglossal nucleus following intraventricular injection of [$^3H_{CH_3}$]thymidine after injection of toxin into the tongue is in striking contrast to their appearance in large numbers after axotomy (Cammermeyer, 1965; Watson, 1965*a*; Sjöstrand, 1965, 1966). The appearance of glia cannot therefore be considered a necessary accompaniment of changes in neuronal nucleic acid

metabolism, which are similar in the two circumstances. It is possible that the regional accumulation of labelled glia found after axotomy is a response to an associated gross defect in axoplasmic transport: if axonal transport is bi-directional (Lubińska, 1964; Kerkut *et al.* 1967; Watson, 1968*b*) and dependent upon an organized system of tubules and fibrils (Droz, 1967) this transport will be grossly disrupted by axonal interruption, but not necessarily by preventing the release of acetylcholine from the nerve ending with botulinum toxin: strong support for this possibility comes from the finding (unpublished observations) that injection of colchicine into the tongue, a drug which impairs the transport of organelles (de Groot, 1957) by its influence upon fibrillary systems (Taylor, 1965), is associated with considerable accumulation of labelled glial cells around affected neurones.

Changes in muscle after botulinum toxin. After botulinum toxin (Thesleff, 1960; Josefsson & Thesleff, 1961), as after denervation (Axelsson & Thesleff, 1959; Miledi, 1960) the area of muscle membrane sensitive to acetylcholine increases. This change is accompanied by marked swelling of sarcolemmal nuclei, which become round with enlarged nucleoli: these nuclear changes when found in other cell types are associated with an increased rate of synthesis of RNA by the nucleus and of protein by the cell as a whole (Gurdon & Woodland, 1968). The autoradiographic changes reported here show that synthesis of DNA is occurring in a few of these nuclei, that the nucleolar enlargement is associated with a considerably increased rate of synthesis of RNA, and that these changes accompany an increased rate of protein synthesis.

It is suggested that the sarcolemmal nuclear reaction under these circumstances is precisely similar to that of the neurone, except that in the latter DNA synthesis is much less apparent (Watson, 1965*b*), and that the plasma membrane changes seen in each type of cell are also closely similar: the membrane extension in the neurone appears as sprouting, while that of the muscle appears as an extension of the surface area of specific chemosensitivity. The apparent differences in response reflect only differences in the morphology of the cell affected and not differences in the cell mechanisms involved. If this is so, then the chromatolytic response of the neurone to injury represents a striking example of a more general cell reaction, seen in both the nerve cell and muscle under these circumstances.

This work was supported by grants from the National Fund for Research into Poliomyelitis and other Crippling Diseases, and from the Medical Research Council. I wish to thank Mrs K. Grant for expert technical assistance in histology.

REFERENCES

ADAIR, G. S. & ROBINSON, M. E. (1930). CXIII. The specific refraction increments of serum albumin and serum globulin. *Biochem. J.* **24**, 993–1010.

AITKEN, J. T. & BRIDGER, J. E. (1961). Neurone size and mean population density in the lumbosacral region of the cat spinal cord. *J. Anat.* **95**, 38–53.

AITKEN, J. T., SHARMAN, M. & YOUNG, J. Z. (1947). Maturation of regenerating nerve fibres with various peripheral connexions. *J. Anat.* **81**, 1–22.

AMBACHE, N. (1948). Peripheral action of botulinum toxin. *Nature, Lond.* **161**, 482–483.

AMBACHE, N. (1949). The peripheral action of cl. botulinum toxin. *J. Physiol.* **108**, 127–141.

AMBACHE, N. (1951). A further survey of the action of *Clostridium botulinum* toxin upon different types of autonomic nerve fibre. *J. Physiol.* **113**, 1–17.

ARMSTRONG, S. H., BUDKA, M. J. E., MORRISON, K. C. & HASSON, M. (1947). Preparation and properties of serum and plasma protein XII. The refractive properties of the proteins of human plasma and certain purified fractions. *J. Am. chem. Soc.* **69**, 1747–1753.

AXELSSON, J. & THESLEFF, S. (1959). A study of supersensitivity in denervated mammalian skeletal muscle. *J. Physiol.* **147**, 178–191.

BARER, R. (1956). Phase contrast and interference microscopy in cytology. In *Physical Techniques in Biological Research*, ed. OSTER, G. & POLLISTER, A. W., pp. 29–90. New York: Academic Press.

BARER, R. & TKACZYK, S. (1954). Refractive index of concentrated protein solutions. *Nature, Lond.* **173**, 821–822.

BRATTGÅRD, S.-O., EDSTRÖM, J.-E. & HYDÉN, H. (1957). The chemical changes in regenerating neurones. *J. Neurochem.* **1**, 316–325.

BRATTGÅRD, S.-O. & HYDÉN, H. (1952). Mass, lipids, pentose nucleoproteins and proteins determined in nerve cells by X-ray microradiography. *Acta radiol.* suppl. **94**, 1–48.

BROOKS, V. B. (1954). The action of botulinum toxin on motor nerve filaments. *J. Physiol.* **123**, 501–515.

BROOKS, V. B. (1956). An intracellular study of the action of repetitive nerve volleys and botulinum toxin on miniature end-plate potentials. *J. Physiol.* **134**, 264–277.

BROWN, G. L., MCEWEN, M. B. & PRATT, M. I. (1955). Macromolecular weight and size of deoxypentose nucleic acids. *Nature, Lond.* **176**, 161–162.

BURGEN, A. S. V., DICKENS, F. & ZATMAN, L. J. (1949). The action of botulinum toxin on the neuromuscular junction. *J. Physiol.* **109**, 10–24.

CAMMERMEYER, J. (1965). Juxtavascular karyokinesis and microglia cell proliferation during retrograde changes in the mouse facial nucleus. *Ergebn. Anat. EntwGesch.* **38**, 23–45.

CERF, JEAN A. & CHACKO, L. W. (1958). Retrograde reaction in motoneurone dendrites following ventral root section in the frog. *J. comp. Neurol.* **109**, 205–216.

CROSSMON, G. C. (1949). Mounting media for phase microscope specimens. *Stain Technol.* **24**, 244–247.

DAVIES, H. G. (1958). The determination of mass and concentration by microscope interferometry. In *General Cytochemical Methods*, ed. DANIELLI, J. F., pp. 55–161. New York: Academic Press.

DAVIES, H. G. (1959). Cell interferometry and the specific refraction increment of crystalline proteins. *Biochim. biophys. Acta* **31**, 228–232.

DAVIES, H. G. & DEELEY, E. M. (1956). An integrator for measuring the 'dry mass' of cells and isolated components. *Expl Cell Res.* **11**, 169–185.

DAVIES, H. G., DEELEY, E. M. & DENBY, E. F. (1957). Attempts at measurement of lipid, nucleic acid and protein content of cell nuclei by microscope-interference. *Expl Cell Res.* suppl. **4**, 136–159.

DAVIES, H. G. & THORNBURG, J. (1959). Personal communication to Ross, K. F. A. The immersion refractometry of living cells by phase contrast and interference microscopy. In *General Cytochemical Methods*, ed. DANIELLI, J. E., pp. 1–59. New York: Academic Press.

DE GROOT, J. (1957). Neurosecretion in experimental conditions. *Anat. Rec.* **127**, 201–217.

DICKENSON, E. C. & SHEVKY, R. (1923). Botulism. Studies on the manner in which the toxin of *Clostridium botulinum* acts upon the body. II. The effect upon the voluntary nervous system. *J. exp. Med.* **38**, 327–346.

DROZ, B. (1965). Accumulation de protéines nouvellement synthétisées dans l'appareil de Golgi du neurone: étude radioautographique en microscopie éléctronique. *C. r. hebd. Séanc. Acad. Sci., Paris* **260**, 320–322.

DROZ, B. (1967). Perikaryal protein synthesis. *Neurosciences Res. Prog. Bull.* **5** (4), 347–351.

DROZ, B. & LEBLOND, C. P. (1962). Migration of protein along the axon of the sciatic nerve. *Science, N.Y.* **137**, 1047–1048.

DROZ, B. & LEBLOND, C. P. (1963). Axonal migration of proteins in the central nervous system and peripheral nerves as shown by radioautography. *J. comp. Neurol.* **121**, 325–345.

DROZ, B. & LEBLOND, C. P. (1964). État dynamique des protéines de l'axone. *C. r. Ass. Anat.* **122**, 106–114.

DUCHEN, L. W. & STRICH, S. J. (1968). The effects of botulinum toxin on the pattern of innervation of skeletal muscle in the mouse. *Q. Jl exp. Physiol.* **53**, 84–89.

ENGSTRÖM, E. (1956). Historadiography. In *Physical Techniques in Biological Research*, ed. OSTER, G. & POLLISTER, A., pp. 489–544. New York: Academic Press.

GIACOBINI, E. & HOLMSTEDT, B. (1958). Cholinesterase content of certain regions of the spinal cord as judged by histochemical and Cartesian diver technique. *Acta physiol. scand.* **42**, 12–27.

GOLDBERG, I. H. & RABINOWITZ, M. (1962). Actinomycin D inhibition of deoxyribonucleic acid-dependent synthesis of ribonucleic acid. *Science, N.Y.* **136**, 315–316.

GURDON, J. B. & WOODLAND, H. R. (1968). The cytoplasmic control of nuclear activity in animal development. *Biol. Rev.* **43**, 233–267.

HYDÉN, H. (1960). The neuron. In *The Cell*, ed. BRACHET, J. & MIRSKY, A. E., pp. 214–234. New York: Academic Press.

HYDÉN, H. & PIGÒN, A. (1960). A cytophysiological study of the functional relationship between oligodendroglial cells and nerve cells of Deiter's nucleus. *J. Neurochem.* **6**, 57–72.

JOSEFSSON, J. O. & THESLEFF, S. (1961). Electromyographic findings in experimental botulinum intoxication. *Acta physiol. scand.* **51**, 163–168.

KERKUT, G. A., SHAPIRA, A. & WALKER, R. J. (1967). The transport of labelled material between muscle and brain along a nerve trunk. *Comp. Biochem. Physiol.* **23**, 729–748.

KOENIG, E. (1965*a*). Synthetic mechanisms in the axon II. RNA in myelin-free axons of the cat. *J. Neurochem.* **12**, 357–361.

KOENIG, E. (1965*b*). Synthetic mechanisms in the axon-I. Local axonal synthesis of acetyl cholinesterase. *J. Neurochem.* **12**, 343–355.

LUBIŃSKA, L. (1964). Axoplasmic streaming in regenerating and in normal nerve fibres. In *Mechanisms of Neural Regeneration: Progress in Brain Research*, ed. SINGER, M. & SCHADE, J. P., pp. 1–66. Amsterdam: Elsevier.

LUBIŃSKA, L., NIEMIERKO, S., ODERFELT, B., SZWARC, L. & ZELENA, J. (1963). Bidirectional movements of axoplasm in peripheral nerve fibres. *Acta Biol. exp., Vars.* **23**, 239–247.

MCILVAINE, T. C. (1921). A buffer solution for colorimetric comparison. *J. biol. Chem.* **49**, 183–186.

MIANI, N. (1962). Evidence of a proximo-distal movement along the axon of phospholipid synthesised in the nerve cell body. *Nature, Lond.* **193**, 887–888.

MIANI, N. (1963). Analysis of the somato-axonal movement of phospholipids in the vagus and hypoglossal nerves. *J. Neurochem.* **10**, 859–874.

MIANI, N., DI GIROLAMO, A. & DI GIROLAMO, M. (1966). Sedimentation characteristics of axonal RNA in the rabbit. *J. Neurochem.* **13**, 255–259.

MILEDI, R. (1960). The acetylcholine sensitivity of frog muscle fibres after complete or partial denervation. *J. Physiol.* **151**, 1–23.

MILLONIG, G. (1961). A modified procedure for lead staining of thin sections. *J. biophys. biochem. Cytol.* **11**, 736–739.

MITCHELL, R. A., LOESCHKE, H. H., MASSION, W. H. & SEVERINGHAUS, J. W. (1963). Respiratory responses mediated through superficial chemosensitive areas on the medulla. *J. appl. Physiol.* **18**, 523–533.

MITCHISON, J. M., PASSANO, L. M. & SMITH, F. H. (1956). An integration method for the interference microscope. *Q. Jl microsc. Sci.* **97**, 287–302.

NAKAI, J. (1956). Dissociated dorsal root ganglia in tissue culture. *Am. J. Anat.* **99**, 81–129.

NATHANS, D. (1964). Inhibition of protein synthesis by puromycin. *Fedn Proc.* **23**, 984–989.

NORTHROP, T. G., NUTTER, R. L. & SINSHEIMER, R. L. (1953). Refractive increment of thymus nucleic acid. *J. Am. chem. Soc.* **75**, 5134–5135.

NORTHROP, T. G. & SINSHEIMER, R. L. (1954). Light scattering by tobacco mosaic virus nucleic acid. *J. chem. Phys.* **22**, 703–707.

OCHS, S., JOHNSON, J. & NG, M.-H. (1967). Protein incorporation and axoplasmic flow in motoneuron fibres following intra-cord injection of labelled leucine. *J. Neurochem.* **14**, 317–331.

OETTLÉ, A. G. (1950). Experiments with a variable amplitude and phase microscope. *Jl R. microsc. Soc.* **70**, 232–254.

PERLMAN, G. E. & LONGSWORTH, L. G. (1948). The specific refractive increment of some purified proteins. *J. Am. chem. Soc.* **70**, 2719–2724.

POMERAT, C. M. (1960). Cinematography, indispensable tool for cytology. *Abstr. Xe Congr. int. Biol. cell. (Paris)*, **5**, 55–56.

REICH, E., FRANKLIN, R. M., SHATKIN, A. J. & TATUM, E. L. (1961). Effect of Actinomycin D on cellular nucleic acid synthesis and viral production. *Science, N.Y.* **134**, 556–557.

REISS, E. (1903). Eine neue Methode der quantitativen Eiweissbestimmung. *Arch. exp. Path. Pharmak.* **51**, 18–29.

ROODYN, D. B. (1965). Further study of factors affecting amino acid incorporation into protein by isolated mitochondria. *Biochem. J.* **97**, 782–793.

ROUSER, G., BAUMAN, A. J. & KRITCHEVSKY, G. (1961). New methods for the separation and quantitative isolation of lipids. Initial applications to the study of beef brain and spleen lipids in Gaucher's disease. *Am. J. clin. Nutr.* **9**, 112–123.

SCHADÉ, J. P. & VAN HARREVELD, A. (1961). Volume distribution of moto- and interneurons in the peroneus-tibialis neuron pool of the cat. *J. comp. Neurol.* **117**, 387–398.

SIRLIN, J. L., JACOB, J. & TANDLER, C. J. (1963). Transfer of the methyl group of methionine to nucleolar ribonucleic acid. *Biochem. J.* **89**, 447–452.

SJÖSTRAND, J. Z. (1965). Proliferative changes in glial cells during nerve regeneration. *Z. Zellforsch. mikrosk. Anat.* **68**, 481–493.

SJÖSTRAND, J. Z. (1966). Glial cells in the hypoglossal nucleus of the rabbit during nerve regeneration. *Acta physiol. scand.* **67**, suppl. 270, 1–17.

SWIFT, H. & RASCH, E. (1956). Microphotometry with visible light. In *Physical Techniques in Biological Research*, ed. OSTER, G. & POLLISTER, A. W., pp. 353–400. New York: Academic Press.

TAYLOR, E. W. (1965). The mechanism of colchicine inhibition of mitosis. I Kinetics of inhibition and the binding of H^3-colchine. *J. cell Biol.* **25**, 145–160.

THESLEFF, S. (1960). Supersensitivity of skeletal muscle produced by botulinum toxin. *J. Physiol.* **151**, 598–607.

UTAKOJII, T. & HSU, T. C. (1965). Nucleic acids and protein synthesis of isolated cells from chick embryonic spinal ganglia in culture. *J. exp. Zool.* **158**, 181–202.

VINCENT, W. S. (1952). cited by BARER, R. (1952). Interference microscopy and mass determination. *Nature, Lond.* **169**, 366.

WATSON, W. E. (1965*a*). An autoradiographic study of the incorporation of nucleic-acid precursors by neurones and glia during nerve regeneration. *J. Physiol.* **180**, 741–753.

WATSON, W. E. (1965*b*). DNA synthesis in injured neurons of adult mice. *J. Neurochem.* **12**, 907–908.

WATSON, W. E. (1966*a*). Some quantitative observations upon the oxidation of substrates of the tricarboxylic acid cycle in injured neurons. *J. Neurochem.* **13**, 849–856.

WATSON, W. E. (1966*b*). Quantitative observations upon acetyl choline hydrolase activity of nerve cells after axotomy. *J. Neurochem.* **13**, 1549–1550.

WATSON, W. E. (1968*a*). Observations on the nucleolar and total cell body nucleic acid of injured nerve cells. *J. Physiol.* **196**, 655–676.

WATSON, W. E. (1968*b*). Centripetal passage of labelled molecules along mammalian motor axons. *J. Physiol.* **196**, 122–123*P*.

WATSON, W. E. (1969). The change in dry mass of hypoglossal neurones induced by puromycin, and the effects of nerve injury. *J. Physiol.* **201**, 80*P*.

WEISS, P. & HISCOE, H. B. (1948). Experiments on the mechanism of nerve growth. *J. exp. Zool.* **107**, 315–395.

YOUNG, J. Z. (1942). The functional repair of nervous tissue. *Physiol. Rev.* **22**, 318–378.

ZEUTHEN, E. (1953). Growth as related to the cell cycle in single-cell cultures of *Tetrahymena piriformis*. *J. Embryol. exp. Morph.* **1**, 239–249.

EXPLANATION OF PLATE

Fig. 1. Photomicrograph obtained with an interference microscope using banded field of an isolated hypoglossal neurone suspended in a sucrose solution of refractive index 1·472. Note that band displacement does not exceed 0·2 λ. Scale 100 μm.

Fig. 2. Photomicrograph of identical field obtained with infinite band separation. Note the absence of phase reversal at the densest part of the cell when background phase retardation is correctly selected. Scale 100 μm.

Fig. 3. Microdensitometric traces across a clear banded field (distorted sine curve), and a trace across the densest part of the cell shown in Fig. 2. Note that the background density lies close to one end of the 'linear' part of the sine curve, and that the optical density of the densest part of the cell also lies upon the same 'linear' part of the curve.

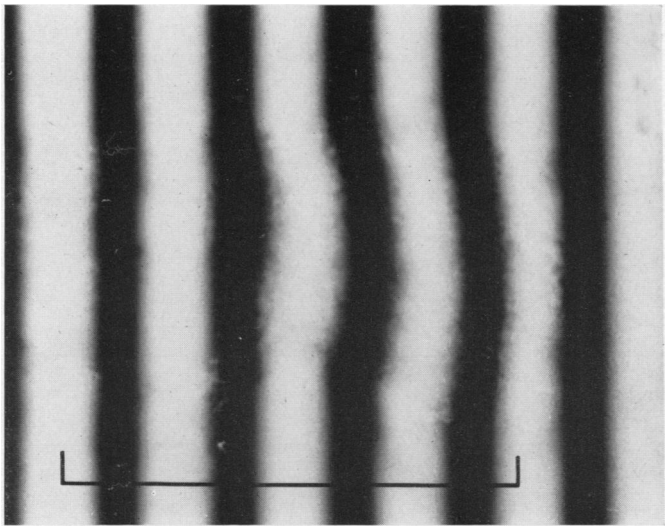


Fig. 1

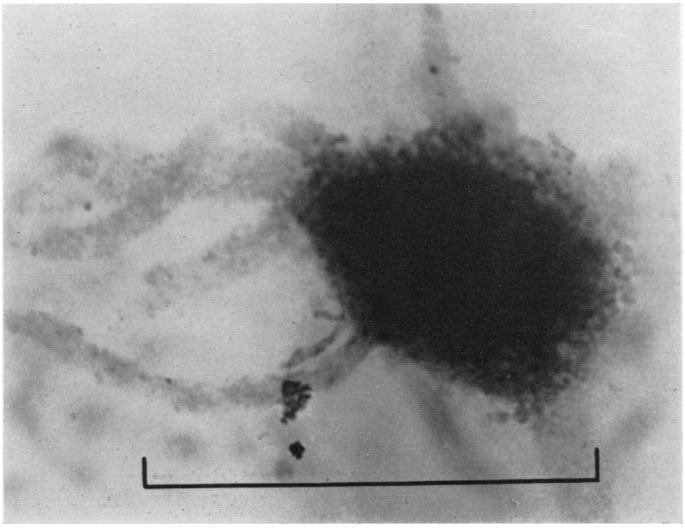


Fig. 2

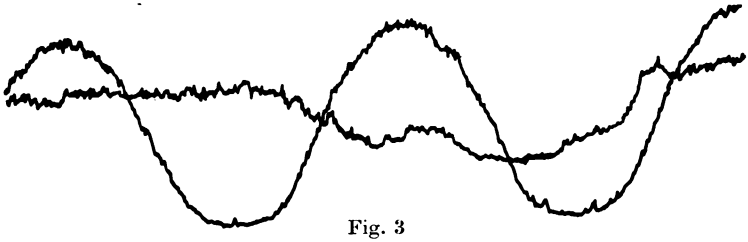


Fig. 3