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Changes In Dorsal Root Ganglia Of The Rat Following Peripheral Nerve Section

Shiu Hong Kung

CHANGES IN DORSAL ROOT

GANGLIA OF THE RAT FOLLOWING PERIPHERAL

NERVE SECTION

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Shiu Hong Kung

Department of Anatomy

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ABSTRACT

In 1892, Nissl described the 'chromatolysis' or 'axon reaction' that occurs in neurons in certain experimental and pathological conditions. With the use of technics such as staining with aniline dyes (Nissl stain), the morphological changes in neurons undergoing axon reaction were described in detail. It was believed that axon reaction is a regressive phenomenon. In studies using cytochemical technics, Hydén (1934) concluded that hyperfunction of the synthetic mechanisms involving nucleoproteins was an essential feature of the chromatolysis that follows injury to a cell process. By using technics of quantitative cytology, autoradiography and electron microscopy, it was hoped to better understand certain of the basic mechanisms involved in the response of nerve cells to injury, as it occurs in primary sensory neurons of Sprague-Dawley rats after cutting the sciatic nerve.

In normal neurons with a single nucleolus or with double nucleoli, the volume of the single nucleolus was larger than the combined volumes of double nucleoli, while the surface areas were about the same. The involvement of nuclear synthetic activity in the process of axon reaction was indicated by an increase in volume and surface area of nucleoli and a decrease in the level of nuclear RNA synthesis. The number of nucleoli in a neuron did not influence the level of radioactivity in the cytoplasm of either normal or reacting cells, after administration of an appropriate RNA or protein precursor.

The level of radioactivity in the nucleus (nucleoplasm and

nucleoli) and cytoplasm was lower in reacting cells than in their normal counterparts at early time intervals, but higher at later time intervals, after injection of tritiated arginine. In other words, the nuclear and cytoplasmic proteins changed in both quantity (a lower level of radioactivity at the time of initial synthesis) and quality during axon reaction. The qualitative change, as suggested by the increase of radioactivity at the longer time intervals after injection, may consist of an increased synthesis of slow turnover proteins and/or a synthesis of new protein species of slow turnover. Concurrently, the level of radioactivity due to RNA in the reacting cytoplasm was lower than in control cells.

There was a proliferation of fibroblasts in the ganglia during the period of axon reaction of its neurons.

Two populations of neurons were identified on the basis of cell size and the number of nucleoli per cell. The level of radioactivity in the cytoplasm of small neurons (diameters $<$ 30μ) exceeded that of large neurons (diameters $>$ 30μ). Using the radioactivity of the cytoplasm of normal and reacting large neurons as the baseline, the elevation of radioactivity in normal and reacting small neurons was 70% and 41%, respectively following injection of cytidine-H^3 and 21% and 24% following arginine-H^3.

The number of nucleoli or the number of nucleoli and extra-nucleolar bodies per cell was always four or less. Three types of nucleoli, cortical, compact and loose types, were identified according to the spatial arrangement of the two basic structural components, i.e. the fibrillar and particulate components. Two types of extranucleolar bodies, granular and fibrillar, were identified.

I. INTRODUCTION

When a process of a neuron is traumatized, there is a sequence of changes in the parent cell body, known as axon reaction or retrograde degeneration. The main morphological characteristics of axon reaction are chromatolysis or dispersal of the Nissl material, swelling of the cell body with a lessening of the angularity of its contours, eccentricity of the nucleus and enlargement of the nucleolus. These are reflections of underlying chemical changes involving nucleic acids, proteins, certain enzymes and other cytochemical systems. The details of axon reaction vary from one type of neuron to another, depending on the size of the cell body, the length of the major cell process and where it is traumatized, the normal pattern of the Nissl material, the location of the neuron in the nervous system and other variables. The Nissl material of large motor neurons, in particular primary or lower motor neurons, is especially abundant. For this reason, and because their axons can be sectioned with ease in peripheral nerves, primary motor neurons have been a favourite object of study in connection with axon reaction.

Changes in the cell bodies of primary sensory neurons, as a result of trauma to their processes, have been less thoroughly investigated. These neurons have certain features that are not shared with other neurons. The cell bodies are in dorsal root ganglia of spinal nerves or in sensory ganglia of cranial nerves. Primary sensory neurons

are therefore included under the general heading of the peripheral

nervous system. The cell bodies are spherical and each is closely

enveloped by a capsule of flattened satellite cells. The nerve cells

vary in diameter, depending on the modality of general sensation with

which they are concerned. The pattern of Nissl material differs from

that of motor neurons, partly in being more diffuse under normal

conditions. Primary sensory neurons are unipolar, while other neurons

are bipolar or multipolar. The single process soon divides into two;

the peripheral branch, although it has the morphology of an axon, is

a dendrite in the physiological sense.

The foregoing characteristics suggested that it would be

worth while to study the changes in the cell bodies of primary sensory

neurons, after section of their peripheral processes, in greater

detail than has been done hitherto. The experiments to be reported

are based on technics of quantitative cytology, autoradiography and

electron microscopy.

A difficulty arises in the selection of a term to denote the

changes in dorsal root ganglion cells that follow section of their

peripheral processes. 'Retrograde degeneration' is not appropriate

because the reaction is not necessarily degenerative in nature.

'Chromatolysis' refers to only one aspect of the multiple changes in

the parent cell body that result from section of the peripheral process.

'Axon reaction' is not strictly correct because the peripheral process

conducts impulses toward the cell body and is therefore a dendrite

physiologically. However, the peripheral process has all the

morphological features of an axon, and the term 'axon reaction' will

be used in this thesis, with the mental reservation noted above.

II. HISTORICAL REVIEW

Today is yesterday's tomorrow;
It is also tomorrow's yesterday.
We cannot move ahead without knowing where we are,
We cannot understand where we are without knowing
where we were.

(anonymous)

This philosophical quotation portrays what generations of

investigators have been doing since Nissl's original description of

chromatolysis or axon reaction in 1892.

A. Definition of Axon Reaction

Nissl (1892) observed that the cytoplasm of neurons contains

granules of variable size that combine with basic dyes. In certain

experimental and pathological conditions, a series of morphological

changes occurs so that the chromatophilic or Nissl substance becomes

dispersed and depleted (chromatolysis). When an axon is injured, for

example by cutting or crushing, the Nissl substance assumes a dust-

like distribution. This form of chromatolysis, a reaction to axon

injury, together with concurrent cellular changes, came to be known

as 'axon reaction'.

B. Chronology of the Study of Axon Reaction

The experiments reported in this thesis are concerned with the

phenomenon of axon reaction as it occurs in primary sensory neurons in

dorsal root ganglia of the rat. It is impossible to review in detail

the massive literature on axon reaction in general that has accumulated

since the time of Nissl. The following summary records the contributions

that are especially pertinent to the present experiments.

1. Nissl stain and the neuron theory

Waller (1856) found that after section of a peripheral nerve,

the distal portion of the fiber degenerates, while the proximal portion

remains intact. Chromatolysis in the parent cell body was not known

until 1892, when Nissl used methylene blue as a stain to reveal

cytological details of the neuron. In the years following, axon

reaction was studied extensively, mainly in large motor neurons

(e.g. Lugaro, 1896; Marinesco, 1895, 1906; Flateau, 1897; Ballet and

Dutil, 1897; van Gehuchten, 1897; Spatz, 1920). The demonstration of

an intimate relationship between axon injury and cytological changes

in the cell body provided evidence in support of the neuron theory

as proposed by Waldeyer (1891). By the time of Cajal's publication

on "Degeneration and Regeneration of the Nervous System" in 1920, the

fact that nerve cells are independent trophic units had won wide-

spread acceptance and conversely the hypothesis of a neurosyncitium had

been definitely set aside (for review see Nonidez, 1944).

Nissl believed that chromatolysis was a regressive phenomenon.

This view was shared in principle by most of the early workers, who

limited their studies to the use of technics such as staining with

aniline dyes.

2. Neuron metabolism in relation to axon reaction

With the introduction of a selective gallocyanin-chromalum

staining technic by Einarson (1932), it was possible to formulate an

explanation of cytoplasmic nucleoprotein production. Einarson (1933)

proposed that the essential constituents of the Nissl substance

originates around the nucleolus and then diffuses through the nuclear membrane, to assume their usual location in the cytoplasm. Einarson (1935) listed four main constituents of Nissl substance as nucleoproteins, nucleic acids, basic proteins and other nuclein compounds. His findings were corroborated by Landström, Caspersson and Wohlfart (1941). Hydén (1943) used ultraviolet absorption technics and obtained results which indicated that hyperfunction of synthetic mechanisms involving nucleoproteins was an essential feature of the chromatolysis that follows injury to a cell process. This view was corroborated by studies using radioisotopes (Brattgård, Hydén and Sjöstrand, 1958; Fischer, Lodin and Kolousĕk, 1958; Miani, Rizzoli and Buccinate, 1960). Concurrently with Hydén's early work on nucleoproteins, Mellors and his associates demonstrated an increase of acid phosphatase activity and a decrease of cytochrome oxidase activity in chromatolytic motor neurons of the rhesus monkey (Bodian and Mellors, 1945; Howe and Mellors, 1945).

3.   Current advances

Modern technics have added new parameters to the study of the normal structure and composition of the neuron and of axon reaction.

A now classical study at the ultrastructural level by Palay and Palade (1955) showed that the Nissl substance corresponds to that region of the cytoplasm where the layers of endoplasmic reticulum are more thickly packed together and more thickly encrusted with ribosomes, than elsewhere. Electron microscopic studies also showed that the dust-like appearance of the cytoplasm of chromatolytic neurons is caused by the 'swollen' endoplasmic reticulum and the dispersion of ribonucleo-protein particles (Hartman, 1954; Yamato, 1958; Smith, 1961; Andres, 1961; Pannese, 1963; Mackey, Spiro and Weiner, 1964; Peach and Dixon, 1968). The ultrastructure of the nucleolus of normal spinal ganglion

cells has been described in some detail (Hiraoka and Breemen, 1964).

Autoradiography of neurons in hypoglossal nuclei of the rat showed that there is a change in the rate of uptake of RNA and protein in the cytoplasm of chromatolytic neurons, as compared with normal neurons (Watson, 1965a).

Enzyme histochemistry of chromatolysis has been investigated in more detail. The reports are in part contradictory, which may be a result of differences in the experimental animals used and the types of neurons that were studied. As noted above, acid phosphatase was the earliest phosphatase enzyme to be studied (Bodian and Mellors, 1945). Several authors subsequently reported an increase in acid phosphatase activity in motor neurons of the spinal cord of several species after section of the sciatic nerve (Lassek and Bueker, 1947; Smith, 1948; Barron and Tuncbay, 1962; Söderholm, 1965). However, Samorajski and Fitz (1961) could not detect any change in the acid phosphatase reaction of motor neurons in the rabbit's spinal cord after section of the sciatic nerve. An increase of acid phosphatase activity after axon section was observed in the facial motor nucleus of the rabbit (Coimbra and Tavares, 1964), in the hypoglossal nucleus of the cat (Barron and Sklar, 1961) and in the dorsal vagal nucleus of the monkey (Fisher and Sutherland, 1965). Similar changes were demonstrated in cells of the stellate and nodose ganglia of the cat (Smith and Luttrel, 1947; Smith, 1948) and the ciliary ganglion of the rat (Huihuri, 1966).

A lowered alkaline phosphatase activity was established biochemically in the spinal cord of the guinea pig and rabbit after sciatic nerve section (Fieschi and Soriani, 1959; Samorajski and Fitz, 1961). However, histochemical tests for alkaline phosphatase gave

normal results in cells of the spinal cord of the rabbit and rat, and ciliary ganglion cells of the rat after nerve section (Samorajski and Fitz, 1961; Söderholm, 1965; Huikuri, 1966).

A reduction in adenosine triphosphatase concentration was reported in biochemical studies of the guinea pig's spinal cord after bilateral sciatic nerve division (Fieschi and Soriani, 1959) and in histochemical studies of the rat's ciliary ganglion after postganglionic denervation (Huikuri, 1966). However, following unilateral sciatic nerve section, the ventral horn cells of the rat's spinal cord showed a strongly increased reaction at the cell membrane (Söderholm, 1965).

Turning to the oxidative enzymes, a decrease in cytochrome oxidase activity was found, by biochemical tests, in reacting cells at various sites, e.g. in ventral horn cells of the cat and monkey, in sympathetic ganglia and the ciliary ganglion of the rat and the spinal cord of the guinea pig (Howe and Mellors, 1945; Kumanoto and Bourne, 1963; Härkönen, 1964; Huikuri, 1966). Monoamine oxidase activity decreased considerably in reacting cells of the superior cervical ganglion and ciliary ganglion of the rat (Härkönen, 1964, Huikuri, 1966).

An increase in activity of all the dehydrogenases studied was seen in sympathetic ganglion cells of the rat after axon section, the enhanced activity being concentrated in a region of the cytoplasm around the nucleus (Härkönen, 1964). In motor neurons of the rat's spinal cord, succinate - and reduced nicotinamide adenine dinucleotide phosphate (NADPH) - tetrazolium reductase activities diminished after axon section (Friede, 1959), while a slight increase in diphosphopyridine nucleotide (DPN) - diaphorase activity and a decrease in succinate dehydrogenase activity was found in efferent cells of the spinal cord and

in dorsal root ganglia of the guinea pig (Kumanoto and Bourne, 1963).
Söderholm (1965) found an increase in the activity of dehydrogenases
in reacting ventral horn cells of the rat after axotomy, with the
exception of α-glycerophosphate-menadione and succinate tetrazolium
reductase, which remained at normal levels. Huikuri (1966) reported
an increase in NADP- and NADPH-tetrazolium reductases and succinate
dehydrogenase in the ciliary ganglion of rats after section of
efferent fibers. TPN-diaphorase and several dehydrogenases exhibited
increased activity in neurons of the facial nucleus of the guinea pig
and rabbit, after division of the ipsilateral facial nerve (Krentzberg,
1963; Fisher and Malik, 1964).

The carboxylic esterase, acetylcholinesterase (AChE), has been
studied extensively in this context. A marked decrease in histo-
chemically demonstrable AChE activity in the neuronal cytoplasm of
reacting sympathetic ganglion cells was observed in the rat and the
cat (Brown, 1958; Taxi, 1961; Härkönen, 1964; Eränkö and Härkönen,
1965; Fredricsson and Sjöqvist, 1962; Gromadzki and Koelle, 1965).
Similar changes were also found in motor neurons of the rat and toad
(Schwarzacher, 1958; Söderholm, 1965; Chucho and Cerf, 1960). However,
Szentagothai, Donhoffer and Rajkovitz (1955) did not detect any change
in AChE activity in reacting cells of the hen, while Taxi (1961) and
Huikuri (1966) reported a distinct decrease of the enzyme in reacting
ciliary ganglion cells of the cat, chicken and rat. A loss of non-
specific cholinesterase was demonstrated in chromatolytic cells of
the superior cervical and ciliary ganglia of the rat (Härkönen, 1964;
Huikuri, 1966), following a failure of earlier studies to detect any
changes in non-specific cholinesterase in chromatolytic sympathetic

ganglion cells of the rat and cat (Taxi, 1961; Fredriesson and
Sjöqvist, 1962). E 600-sensitive non-specific esterase (E-s ns. E)
activity was shown to be decreased in chromatolytic sympathetic
ganglion cells of the rat, while no change in E 600-resistant esterase
(E-r ns. E) activity was detected (Härkönen, 1964). However, both
non-specific esterases were found to be less active in reacting ciliary
ganglion cells of the rat (Huikuri, 1966). Both enzymes showed a
marked decrease of activity in the rat's ventral horn cells after
peripheral nerve section (Söderholm, 1965), while there was only a
slight decrease of ns. E activity in the guinea pig's spinal cord
under similar conditions (Kumanoto and Bourne, 1963). After an
initial increase in the nodose ganglion and vagal nucleus following
cervical vagotomy in the monkey, a fall in organophosphate-resistant
indoxyl esterase activity to below the normal level was described
by Fisher and Sutherland (1965).

It is thought that acid phosphatase is synthesized in the
endoplasmic reticulum and then transported via the Golgi apparatus
to the lysosomes (Sobel and Avrin, 1963; Goldfischer, Essner and
Novikoff, 1964). The distribution of histochemically demonstrable
AChE activity corresponds to that of the Nissl substance (Fukuda,
1959; Fukuda and Koelle, 1959). The Nissl material represents granular
endoplasmic reticulum, in which AChE may be synthesized and then
transported to the surface of the cell body and to its processes
(Fukuda and Koelle, 1959). Oxidative enzymes, such as the dehydro-
genases and monoamine oxidase, exhibit axonal flow (Klein, 1960;
Härkönen, 1964). It is therefore apparent that axon reaction is a
complicated change in the structural and chemical constituents of the
neuron, involving many enzyme systems and eventually the quantity and

types of proteins synthesized.

The experiments to be reported do not involve enzyme systems directly. The reports on enzymatic changes in axon reaction have been recorded in some detail because these biochemical changes are basic to all aspects of the phenomenon of axon reaction. (With respect to certain conflicting results, it should be pointed out that reaction to axon injury varies from one species to another. For example, there is no detectable response to axon section in ventral horn cells of certain rodents, in sections stained by the Nissl method (Bucy, 1928; Geist, 1933)).

C. Histology and Cytology of Dorsal Root Ganglia

The spinal ganglia are part of the peripheral nervous system and they contribute fibers to the peripheral nerves. Each ganglion is surrounded by a connective tissue capsule that is continuous with the epineurium of peripheral nerves. Trabeculae extend from the capsule into the ganglion to form a connective tissue framework containing blood vessels and surrounding the nerve cell bodies and their processes. The nerve cells themselves are separated into irregular groups by bundles of nerve fibers, which are central and peripheral processes of the ganglion cells. Each ganglion cell is surrounded by a thin capsule, derived from the ectoderm of the neural crest and consisting of flattened satellite or capsule cells. The perineuronal capsule extends over the emerging process and becomes continuous with the neurilemma sheath of that fiber. The single process of these unipolar neurons has the structure of an axon and, if it is myelinated, it acquires the myelin sheath soon after leaving the cell. The process may coil about the cell body in an intricate manner, then straighten, pass toward the center of the ganglion, and bifurcate into a central

and a peripheral process. The central process enters the spinal cord through the dorsal root; the peripheral one is the afferent fiber or functional dendrite of the peripheral nervous system. Therefore, a dorsal root ganglion contains three main types of cells: neurons, satellite or capsule cells (and the neurilemma or Schwann cells with which they are continuous) and the mesenchymal fibroblasts. The main characteristics of the three types of cells will now be summarized.

1. Neurons

On the basis of differences in size, distribution of Nissl substance and other characteristics, several types of neurons have been recognized (Warrington and Griffith, 1904; Dogeil, 1908; Ranson, 1912; Truex, 1940, 1941). The simplest classification includes only two cell types, larger 'clear cells' and smaller 'dark cells' (Scharf, 1958). Those with larger perikarya are concerned with such sensory modalities as discriminative touch and proprioception, while those with small perikarya are concerned with pain and temperature (Scharf, 1958; Crosby, Humphery and Lauer, 1962; Swanson, Buchman and Alvord, 1965).

The vesicular nucleus of primary sensory neurons of the rat occupies about one-third to one-half of the neuronal diameter. The large nucleoli, one to three in number, are clearly resolved against the lighter nucleoplasm and occupy about one-fourth of the diameter of the nucleus (Andres, 1961; Bunge et al. 1967). The sex chromatin, as found in nerve cell nuclei of many mammals, is not easy to detect in nerve cells of the rat and other rodents because of the similar appearance, with most stains, of small nucleoli and non-specific clumps of chromatin (Moore, 1966). With the electron microscope, the small neurons contain

endoplasmic reticulum (Nissl substance) dispersed throughout the perikaryon, while the large neurons have a perinuclear zone in which mitochondria, Golgi complexes and 'dense bodies' are concentrated. The Nissl substance of the large neurons is more concentrated at the periphery of the perikaryon (Bunge et al., 1967).

2. Satellite (capsule) cells

In addition to its characteristic size and location, the nucleus of satellite cells can be identified because the chromatin is more concentrated than it is in either neurons or fibroblasts (Hess, 1955; Kotani and Kawashima, 1961; Bunge et al., 1967). Pannese (1964) reported that, in dorsal root ganglia of the lizard, the number of satellite cells surrounding a nerve cell varies from two to twelve, the number being determined by the size of the nerve cell body. Bunge et al. (1967) reported that no more than two satellite cell nuclei are adjacent to nerve cells of the rat in culture, as counted in sections 2μ in thickness. Some nuclei are elliptical and lie against a flattened portion of the neuronal soma. Others are nearly triangular and perch on the neuron like a 'dunce cap'. Satellite cell nuclei contain a nucleolus and the density of the chromatin is comparable to that of Schwann cells. With the electron microscope, the arrangement of components of nuclei of satellite cells and Schwann cells appears identical and contrasts with the more dispersed chromatin pattern of fibroblasts and neurons.

3. Fibroblasts

These connective tissue cells are of mesenchymal origin and are scattered between the neurons with their surrounding satellite cells. The vesicular nucleus of the fibroblast is readily distinguished from the intensely stained satellite cell nucleus. With electron

microscopy, the fibroblast is characterized by the lack of a basement
membrane, lighter cytoplasm compared with Schwann cells, a prominent
granular endoplasmic reticulum, large vacuoles bounded by granular
membranes, and bundles of filaments in the cytoplasm (Pannese, 1964;
Bunge et al., 1967).

D. Axon Reaction in Dorsal Root Ganglion Cells

Lugaro (1896) cut the sciatic nerve near its exit from the
pelvis in dogs and found that not all cells of the affected ganglia
showed an equal degree of chromatolysis. Van Gehuchten (1897), after
vagus nerve section in rabbits, observed that the majority of the nerve
cells in the ganglion nodosum underwent complete degeneration and
disappeared. He believed that this result could be considered typical
of spinal, as well as cranial, ganglia. However, Marinesco (1898)
found that following vagal section in the dog, cells in the ganglion
nodosum passed through a phase of reaction to a phase of restoration
and did not undergo complete degeneration.

In contrast to the marked changes which occur when the
peripheral process of a primary sensory neuron is severed, sectioning
of the central process has little or no effect on the parent cell body.
Lugaro (1896, 1897) and Marinesco (1897) found either no change or
very minor alterations following dorsal root section. Anderson (1902)
sectioned lower lumbar and upper sacral dorsal roots proximal to the
ganglia, and the vagus nerve central to the nodose ganglion, in young
rabbits and in kittens. After intervals of from 26 to 119 days,
comparisons made between the cells of the experimental and control
sides showed no difference in the microscopic appearance or size of
the cells. The curious difference between the consequences of sectioning
central or peripheral processes has never been satisfactorily explained.

While the mechanism of induction of axon reaction in primary sensory neurons is at present unknown in any detail, the following reports have a bearing on this important question.

Leonowa (1894) and Sherrington (1894) reported two cases of amyelia in man, in which the posterior root ganglia and the sensory components of peripheral nerves developed in an apparently normal fashion, independent of a connection with a spinal cord.

Bucy (1928) showed that sectioning of the sciatic nerve of the rabbit was followed by chromatolysis of the parent ganglion cells. However, only minor chromatolytic changes could be detected in ventral horn cells, indicating that the laboratory animal used, as well as the type of nerve cell, is pertinent to such studies.

Following amputation of the limbs of amphibian larvae, Detwiler (1921) found that dorsal root ganglia failed to develop, although ventral horn neurons matured normally. The dependence of sensory neurons on their peripheral field was also shown by Howe (1935) who found that the number of cells in the cochlear ganglion was decreased in deaf cats with a congenital lesion in the organ of Corti.

The studies mentioned thus far were done with technics such as staining with basic dyes (the Nissl method), which limits attempts to evaluate the functional significance of morphological changes. An important step in clarifying the fundamental nature of axon reaction in sensory neurons followed the application of cytochemical methods.

Cytochemical and related studies throw some light on the significance of axon reaction, in terms of cellular physiology. By means of ultraviolet microspectroscopy, Hydén (1943) found that 19 hours, 3, 15 and 33 days after sectioning of the rabbit's sciatic nerve, the cytoplasm of spinal ganglion cells show a markedly lower

absorption capacity for ultraviolet light at pertinent wave lengths

for nucleoproteins. That is to say, the concentration of ribonucleic

acids and proteins in the cytoplasm was decreased, compared with that

of normal cells. The author emphasized that the observed decrease of

nucleotides and proteins in a given volume of cytoplasm might be in

part attributable to an increase in size of the cell, owing to the

absorption of fluids. However, Hydén found an increase in concentration

of nucleotides around the nucleus of reacting cells. Differing from the

earlier morphologists, who believed that axon reaction is a degenerative

phenomenon, Hydén (1943) stated:

> "The production and development of nuclear-membrane nucleotides
> can be observed in the ganglion cells immediately after the nerve section,
> when the content of the protein substance and nucleotides in the cell
> is rapidly diminished, as well as during the recovery stage when, after
> the lapse of some time, those substances increase so that the original
> content is eventually restored. It has been pointed out above, in
> regard to other ganglion cell material, that these nucleotides
> participate in the formation of cytoplasmic protein, and that their
> function must be viewed as an indication of an intense activity of the
> protein metabolism system in the nerve cell."

In the same review, Hydén reported an increase of cellular

nucleotides and protein concentration in 'uninjured' cells irritated

by electric current, and commented as follows:

> "....On the other hand, it has been found that the increase
> of cellular substance, resulting from the irritation of 'uninjured'
> cells, is accompanied by all signs of an intense activity of the
> nucleolar and protein-producing system.
>
> The above mentioned changes in the chemical composition of
> the ganglion cell after nerve excision showed that retrograde reaction
> has the characteristic of a transitory inhibition of the protein-
> producing system in the ganglion cell."

With the advent of the electron microscope, chromatolytic

changes in dorsal root ganglion cells at the ultrastructural level

were the subject of several investigations (Smith, 1961; Andres, 1961;

Pannese, 1963; Mackey, Spiro and Weiner, 1964; Peach and Dixon, 1968).

The results were perhaps what one would expect from previous work with the light microscope. Nissl aggregates lose their compact arrangement and cisternae of the rough surface endoplasmic reticulum show swelling and may undergo fragmentation. Mitochondria are swollen, nucleoli enlarged and nuclei became eccentric. The various authors felt that the ribonucleoprotein particles of chromatolytic neurons, although altered in distribution, showed no apparent change in number per cell. They also interpreted the altered distribution of ribonucleoprotein particles and the nucleolar enlargement as reflections of enhanced protein synthesis, during which the cytoplasmic synthetic system is transformed from a less active form to a more active form, in preparation for regeneration of the nerve fiber.

With the foregoing historical background introduced, it is appropriate at this point to state the purposes of the experiments that are recorded in this thesis.

III. AIMS OF THE RESEARCH

From the study of plant material, and more recently animal tissues, it was concluded that nucleolar organizers are specific chromosomal loci associated with the formation of nucleoli (Hertz, 1931; McClintock, 1934; Stewart and Bramford, 1942; Brown, 1949; Lin, 1955; Longwell and Svihla, 1960; Mirsky and Ssawa, 1961; Sirlin, Tandler and Jacob, 1963). Even though there are variations in the number of nucleoli, there is a maximum number per nucleus, which is determined by the number of nucleolar organizers, which in turn is constant for each species.

As a result of experiments using gallocyanin-chromalum staining, Einarson (1933) believed that the essential constituent of the Nissl substance originates around the nucleolus and then diffuses gradually through the nuclear membrane, to assume its usual location in the cytoplasm. This interpretation was supported by Landström, Caspersson and Wohefart (1941) and Hydén (1943).

Weiss and Hiscoe (1948) formulated the axon flow concept, implying a flow of cytoplasm from the cell body, peripherally within the axon. If that is so, and the concept seems now to be well established, the nucleus would influence the cytoplasm, which in turn would influence the peripheral nerve axon.

Nucleolar enlargement in chromatolytic neurons has been described by many authors. In discussing a study of chromatolysis in the hypoglossal nucleus of rabbit, Brattgård, Edström and Hydén (1958)

proposed the following hypothesis of the metabolism of axon reaction:

"The primary problem, however, lies at the level of gene action on the cytoplasm and the action of external or cell milieu factors back on the genes of the nucleus. As has been frequently pointed out, the nucleus and the genes are surrounded by the cytoplasm from which raw material is provided to the genes. Since external conditions have been shown to affect gene action, a change in the external cell milieu must be expected to give a detectable effect as a consequence of gene action, i.e. the equilibrium in the expression of the genes must be changed."

Studies of axon reaction in motor neurons indicated that there were quantitative cytometric changes in the cell body and nucleolus (Barr and Hamilton, 1948; Barr and Bertram, 1949, 1951; Lindsay and Barr, 1955). Therefore, a foundation has been laid for the present investigation on dorsal root ganglia. Three technics, quantitative cytology, autoradiography and electron microscopy, were used for the following reasons.

Since quantitative cytometric studies on axon reaction in primary sensory neurons have not been reported previously, it was felt that such a study should be undertaken to compare certain changes in sensory cells with those established previously for motor cells. It was noted in preliminary observations that some dorsal root neurons have more than one nucleolus. According to classical cytogenetic interpretations, a nucleolus represents at least one set of nucleolar organizer genes in a functional state. As noted above, the nucleolar organizers are specific chromosomal loci associated with the formation of nucleoli, and the intrinsic or maximum number of nucleoli is a constant for each species (Hertz, 1931; McClintock, 1934; Stewart and Bramford, 1942; Brown, 1949; Lin, 1955; Longwell and Svihla, 1960; Mirsky and Ssawa, 1961; Sirlin, Tandler and Jacob, 1963).

Variations in nucleolar number have frequently been related to the phenomenon of "competition" or conversely, of nucleolar fusion.

Competition implies that nucleolar organizers within a chromosomal complement compete with one another for nucleolar material or its precursors (Navashin, 1934; McClintock, 1934; Swanson, 1957; Swift, 1959; H.J. Barr and Esper, 1963). As a result, there is either a decrease or an increase in the size of nucleoli arising at different organizer loci. When Navashin (1934) presented evidence of competition between organizers of different 'strength' in hybrid species, for instance in Crepis, the broad outlines of RNA and protein synthesis were not known. However, the advances of cytochemical technics at the ultrastructural level showed that chromatin material penetrates from the nucleolar surface deeply into the nucleolar body, in the form of irregular strips of regularly-spaced lamellae (Altmann, Stocker and Thoene, 1963; Smetana and Busch, 1964; Granboulan and Granboulan, 1964a). It was assumed that DNA material located at the periphery and inside the nucleolus represents ribosomal cistrons or the nucleolar organizer. Indeed, the nucleolus is well-known to be a site of RNA synthesis (c.f. Goldstein and Plaut, 1955; Woods and Taylor, 1959; Prescott, 1959; Perry, 1960, Leblond and Amano, 1962; Shimada and Nakamura, 1966). With the technic of artificial RNA-DNA hybridization, clear evidence was presented that ribosomal cistrons are responsible for the synthesis of ribosomal RNA (Ritossa and Spirgelman, 1965; Wallace and Birnstiel, 1966). In the light of current knowledge, the competition theory could be interpretated in terms of the control of the activity of ribosomal RNA cistrons, rather than the ability of the nucleolar organizers to collect nucleolar material competitively (Waddington, 1966). Despite the apparent necessity of modifying the competition theory, it remains of value in suggesting generalizations and in predicting the behaviour of nucleoli under various experimental

conditions (c.f. McClintock, 1934; Swanson, 1957; Swift, 1959; H.J. Barr and Esper, 1963).

Axon reaction is known to involve an altered state of nucleic acid metabolism and is believed to be the cytological expression of restoration of an equilibrium through the expression of genes (Brattgård, Edström and Hydén, 1958). The number of nucleolar organizers that are responsible for each nucleolus of primary sensory neurons of the rat is not known. Nevertheless, it was considered worthwhile to compare normal neurons and neurons undergoing axon reaction, taking the comparative volume and surface area of nucleoli into consideration. Therefore, in neurons with one, or more than one, nucleolus (presumably representing single or multiple units of nucleolar organizer genes), a system was offered to test the involvement of the nucleolar organizer genes of chromosomes in the phenomenon of axon reaction.

Previous studies of axon reaction with the autoradiographic technic have shown that there is an increased incorporation of radioactive precursors into cytoplasmic RNA and proteins of chromatolytic neurons (Brattgård, Hydén and Sjöstrand, 1958; Fischer, Lodin and Kolousek, 1958; Watson, 1965a). Although changes in nucleolar size during axon reaction are well documented, data on the synthetic activities of the nucleus and nucleolus during axon reaction are still lacking. Previous studies were done mainly on motor neurons (c.f. Brattgård, Hydén and Sjöstrand, 1958; Watson, 1965a). The sensory neuron has special characteristics which might extend to metabolic changes during axon reaction that can be shown by isotope labelling and autoradiography.

Biochemical geneticists classified the proteins in the cell nucleus into two types, histone (basic proteins) and non-histone (non-

basic proteins or acidic proteins). Histone has been shown to be a
suppressor of chromosomal RNA synthesis (Haung and Bonner, 1962;
Allfrey, Littau and Mirsky, 1963; Bonner, 1968). However, others
believe that detectable changes in quantities of acidic proteins in
the course of differentiation might be a more important factor in the
regulation of DNA-dependent RNA synthesis (Ürsprung, 1964; Sporn and
Dingman, 1966). In short, current theories emphasize the role of
nuclear basic proteins and non-basic proteins as the biological
material responsible for regulating the template activities of the
chromatin, which would in turn influence nucleic acid and protein
metabolism in the cytoplasm. Therefore, in addition to an expected
change of uptake of nucleic acid and protein precursors in the
cytoplasm during chromatolysis, of a direction and magnitude to be
determined, it was hoped that the autoradiographic studies might
detect changes in uptake of radioactivity of RNA and protein precursors
in the nucleus and nucleolus.

The main constituents of cytoplasm, RNA (Penman, 1967) and
proteins, are heterogeneous. For example, the protein enzyme acid
phosphatase has been shown to increase, and the protein enzyme
acetylcholinesterase to diminish, in neurons undergoing axon reaction
(Huikuri, 1966). Early autoradiographic studies on the uptake of
precursors of RNA and proteins in normal and chromatolytic neurons
were done at time intervals such as hours after the introduction of
radioactive precursors (Brattgård, Hydén and Sjöstrand, 1958; Fischer,
Lodin and Kolousěk, 1958). In view of the heterogeniety of RNA and
proteins, which might have different rates of turnover, there is an
inherent weakness in such an experimental design. One of the factors
determining the intensity of the autoradiographic reaction is the

turnover rate of the particular radioactive product examined. While

the initial amount of material synthesized might be the same, a fast

turnover product, when examined at a later time interval, would

demonstrate a lower 'radioactive uptake' than a slow turnover product.

It was therefore thought advisable to examine the uptake of radioacti-

vity at short time intervals (minutes) after the administration of

cytidine-H^3 and arginine-H^3. The metabolism of RNA and proteins in

normal and chromatolytic neurons was then followed at longer time

intervals.

The classical concept of nucleolar structure has recognized

two intranucleolar phases, a nucleolar matrix and intranucleolar

vacuoles or nucleolini, composed of ribonucleoproteins (Montgomery,

1898; Gates, 1942; Vincent, 1955; Stich, 1956; Hertl, 1957; Swift,

1959; Sirlin, 1962; Busch, Byoret and Smetana, 1963). Recent

electron microscopic studies of nucleoli suggest a segregation of the

nucleolar matrix into two phases, characterized by structural

components distinguishable by difference in density (Bernhard et al.,

1955; Hortman and Knoop, 1957; Swift, 1962; Lafontaine and Chouinard,

1963; Jones, 1965; Brinkley, 1965; Narayan et al., 1966). Further

observations with the electron microscope provide evidence that the

more dense component consists of granules of the order of 100 Å

that resemble cytoplasmic ribosomes and occasional groups of fibrils

dispersed among the granules. It is assumed that this structural

component corresponds to the nucleolonema described by Estable and

Sotelo (1951). The less dense component is non-granular and is called

'pars amorpha' (Estable and Sotelo, 1951; Bernhard, 1959; Swift, 1962b).

The ultrastructure of normal nucleoli of dorsal root ganglia, fixed in

buffered osmium tetroxide, has been described (Hiraoka and Breemen, 1964). Osmium tetraoxide, once considered as a 'universal fixative', has revealed many new aspects of the cytoplasm. However, it is a poor fixative for nuclear cytology because of partial extraction of molecular complexes, in comparision with materials fixed in aldehydes (Marinozzi, 1963). Among the aldehydes, glutaraldehyde was found by Sabatini, Bensch and Barrett (1963) to give the best general preservation of fine structure of enzymatic proteins. However, osmium tetraoxide is also a stain, while glutaraldehyde is 'colorless'. Since Huxley and Zubay (1961) first combined uranyl acetate and lead hydroxide in a double staining technic, the method has been adopted routinely in many laboratories including our own. In addition, the technic provides a substantially higher contrast at the ultrastructural level (Frasca and Parks, 1965). It was therefore considered worthwhile to examine the ultrastructural morphology of nucleoli of normal and reacting neurons with a superior technic.

In summary, by using technics of quantitative cytology or cytometry, autoradiography and electron microscopy, it was hoped to add to our knowledge of axon reaction as it occurs in primary sensory neurons, and perhaps to gain a better understanding of the basic mechanisms involved in the response of nerve cells to injury.

IV. MATERIALS AND METHODS

A. Nerve Section

The female Sprague-Dawley rat was the experimental animal

throughout. The rats weighed 50 gm on being received from C.B.L.

Breeding Laboratory, Montreal. The sciatic nerve was chosen for section

because of its accessability and large size. The following anatomical

description and Fig. 1 will establish the relation between the sciatic

nerve and the 5th and 6th lumbar ganglia, whose cells were the object

of study.

The sciatic nerve is a branch of the lumbosacral trunk, which

is formed in turn by parts of the 4th, 5th and 6th lumbar spinal nerves.

The trunk runs parallel with the remainder of the 6th nerve over the

ventral aspect of the sacrum and becomes the sciatic nerve in the pelvis

minor, where it is separated from the pudendal nerve by the superior

gluteal artery. The sciatic and pudendal nerves run together through

the groove between the dorsal border of the ischium and the root of the

tail as far as the caudal extent of the sciatic notch, where the sciatic

nerve enters the thigh. The nerve gives off several small branches, such

as the superior and inferior gluteal nerves. It then divides into its

terminal branches, the common peroneal and tibial nerves, which course

between the biceps femoris and obturator externus muscles, and the

quadratus femoris and adductor magnus muscles of the thigh. The

terminal branches lie side-by-side in the thigh and separate on

Figure 1. Schematic drawing of sciatic nerve trunk of the
rat, to illustrate origin at the sciatic nerve and the level at which
the nerve was sectioned.
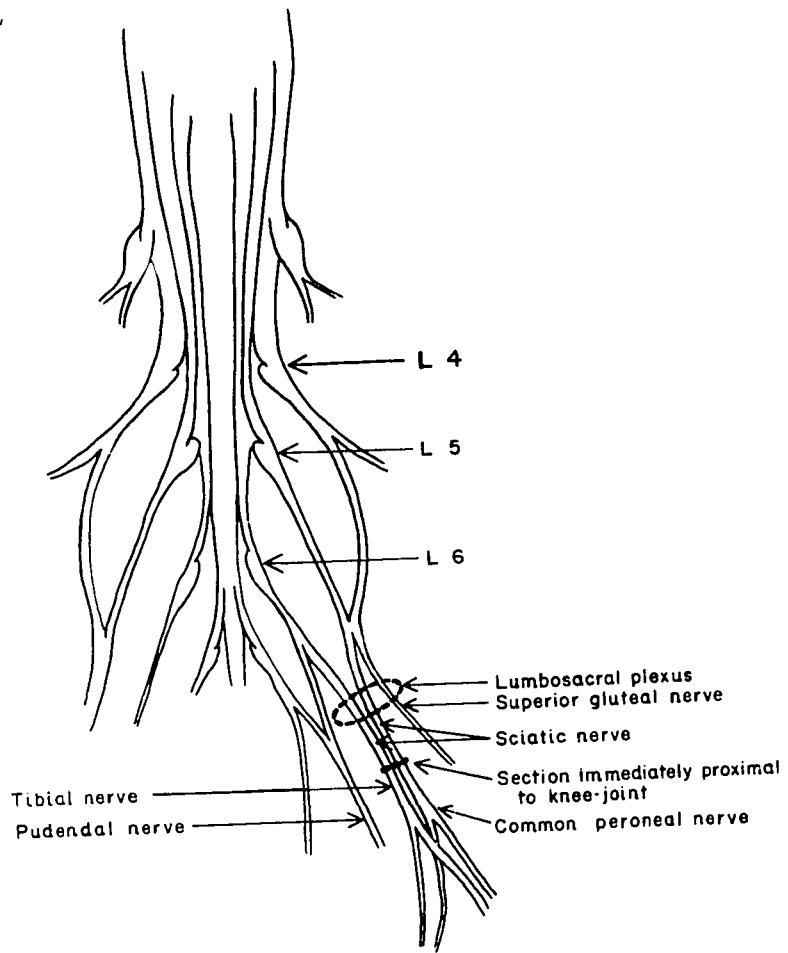
SCHEMATIC DRAWING OF SCIATIC NERVE TRUNK

Figure 1

reaching the popliteal fossa.

Under ether anesthesia, and using sterile precautions, the right sciatic nerve was sectioned cleanly with scissors exactly 1 cm above the knee joint in all animals.

The animals were maintained in an environmental temperature of $75 \pm 2^\circ F$. Food, in the form of Canadian Fox Chow pellets, and tap water were provided ad libitum. A daily weight gain record was kept and rats with a regular daily gain were considered to be in good health and were used in the subsequent experiments.

B. Quantitative Cytology

Ten rats were used in this aspect of the study. The thoracic cavity was opened under ether anesthesia and a perfusion-fixation technic was employed, as follows. The needle of a syringe was inserted into the left ventricle, ligated in the aortic arch, and the arterial system was perfused with 50 ml of 10% formalin. The perfusions were done at 4 day intervals between 4 and 40 days after section of the nerve. The 5th and 6th lumbar ganglia were dissected out immediately after perfusion and post-fixed in 10% formalin for 48 hr. Paraffin sections were cut at 7μ and stained with cresyl violet.

Quantitative data were collected by making cell counts, using the two methods described below, and by measuring cell bodies, nuclei and nucleoli.

1. Cell counts

(a) Total number of normal and chromatolytic neurons in the ganglia. The purpose of this enumeration was to gain familiarity with the particular cell population under investigation and to determine the proportion of cells in the ganglia whose processes had been interrupted by nerve section at the level selected. With a microscope

projector apparatus, Nissl-stained sections were projected on to a screen.
About 180 serial sections for each L6 ganglion and about 130 for each L5
ganglion were examined. Only neurons containing a nucleolus were counted,
to minimize the chances of counting the same cell more than once. One
control and seven experimentally altered ganglia were studied in this
way, from animals sacrificed at 4, 8, 12 and 16 days after nerve section.
Eccentricity of the nucleus was the main criterion of axon reaction
because other morphological changes were difficult to detect at the low
magnification employed. The total number of neurons in L5 and L6 ganglia
was established in this manner. In addition, an estimate of the pro-
portion of chromatolytic neurons on control and experimental sides, at
various times after nerve section, was obtained.

(b) Proportions of chromatolytic neurons with single or
multiple nucleoli. The study of sections with a 45X objective revealed
two types of neurons with respect to the number of nucleoli, those with
a single nucleolus and those with more than one nucleolus. Fifty* cells
with one nucleolus and an equal number with more than one nucleolus were
recorded as normal or reacting in the right L6 ganglion at 4, 8, 12, 16,
20, 24, 28, 32, 36 and 40 days following nerve section. The purpose of
this aspect of the study was to determine whether different numbers of
nucleoli might have something to do with the rate of recovery of cells
after nerve section.

2. Measurements of cell body, nucleus and nucleolus

Because of the rather wide spectrum of cell size in the ganglia,
measurement of a relatively large sample of cells was required.

The measurements were made with an oil immersion objective and

* Trials showed that the classification of 50 cells yielded reproducible
results.

a Leitz 12.5X filar micrometer eye-piece, only on cells containing a nucleolus. An average of the least and the greatest diameters was designated as the diameter of the cell body, nucleus and nucleolus, respectively.

For every 50 randomly selected cells measured, the percentage distribution of cell sizes in micrometer scale units was calculated (1 unit=0.31 μ). When 250 normal cells, and an equal number of reacting cells, were measured and the size distribution calculated, it was found that the change in distribution was only $\pm$ 1%, compared with the distribution of cell sizes when 200 cells were measured. It was decided, therefore, that measurement of 250 neurons was sufficient to give a reasonably accurate estimate of the size distribution of cellular components. Data on the diameter of the nucleolus were used for calculation of its volume and surface area.

The measurements on neurons undergoing axon reaction were made on ganglia in which the reaction was of 12 days duration. This interval was chosen because of the high proportion of chromatolytic neurons and the maximal chromatolytic changes, compared with other time intervals.

3. Size of fibroblast and satellite cell nuclei and the number of fibroblasts in control and experimental ganglia

The least and the greatest diameters of 50 fibroblast and 50 satellite cell nuclei in control and experimental ganglia (12 days) were measured with an oil immersion objective and a filar micrometer eye-piece.

Since study of the sections of experimental ganglia suggested that there was an increase in the number of fibroblasts, counts of fibroblast nuclei were made in randomly selected fields in areas between

the encapsulated neurons. The size of these areas varied to some extent
and preliminary counts indicated that reproducible figures were
obtained when 50 fields were selected randomly. The counts were made
on normal and experimentally altered ganglia for each of the 10 time
intervals from 4 days to 40 days after nerve section. Similar counts
of capsular cell nuclei were omitted because visual inspection did not
suggest any change in the number of these cells in relation to the cell
bodies of reacting neurons.

C. Quantitative Autoradiography

The day before the animals were sacrificed, rats of the same
or closely similar weights were divided into groups of two or four and
housed in small cages. Two series of experiments were then performed
(Table 1).

1. Main experiment

The purpose of this series of experiments was to examine
the synthesis and metabolism of RNA and proteins at 6, 14 and 28 days
after nerve section. The three time intervals probably represent the
early and intermediate periods of intense axon reaction and the recovery
period, respectively. For each of the time intervals, two experiments,
namely the RNA experiment with tritiated cytidine as a precursor and
the protein experiment with tritiated arginine as a precursor, were
performed. For each experiment, a certain number of animals was
sacrificed at different times after injection of the tritiated
precursor, as shown in Table 1.

2. Subsidiary experiment

To study the uptake of RNA and protein precursors at various
stages of axon reaction, another experiment was designed to see whether
there is a pattern of uptake of radioactivity which might be different

Table 1

Summary of Autoradiograph Experiments

Experiments	Days of axon reaction	Time of sacrifice after injection of precursors	Number of animals in each group	Number of animals used	Precursor
Main Experiment					
RNA	6	20 min, 3 hr, 6 hr, 4 days	4	16	Cytidine-H^3
Protein		10 min, 2 hr, 4 hr, 4 days	2	10	Arginine-H^3
RNA	14	20 min, 3 hr, 6 hr, 4 days	4	16	Cytidine-H^3
Protein		5 min, 4 hr, 16 hr, 4 days	2	8	Arginine-H^3
RNA	28	20 min, 3 hr, 6 hr, 4 days	4	16	Cytidine-H^3
Protein		5 min, 4 hr, 16 hr, 4 days	2	8	Arginine-H^3
Subsidiary Experiment					
RNA	3, 11, 20, 40	3 hr	2	8	Cytidine-H^3
Protein	3, 11, 20, 40	3 hr	2	8	Arginine-H^3

from what would be expected from the results of the main experiment. Therefore, animals whose sciatic nerve had been sectioned 3, 11, 20 and 40 days previously were sacrificed 3 hr after injection of a radioactive precursor.

The isotopes were used under good chemical laboratory conditions, with the recommended precautions to avoid contamination. The tritium-labelled nucleotide cytidine (specific activity 2000 mc/mM) was obtained from New England Nuclear Corporation, Boston. The tritium-labelled amino acid arginine (specific activity 210 mc/mM) was obtained from Radiochemical Center, Amersham, England. Each animal received 2 μc of tritiated cytidine per gm body weight in the RNA experiments or 2.5 μc tritiated arginine per gm body weight in the protein experiments. The radioactive material was administered intraperitoneally. The ganglia were fixed, after predetermined time intervals, by the perfusion method as described above. Paraffin sections were cut at 7 μ. A coating or dipping emulsion technic was preferred to the stripping film method, in view of the large number of slides that had to be prepared. Samples of the coated slides were exposed in the dark at 4°C for 7, 15, 21, 29, 50 days and 5 months to establish the best exposure time for counting silver grains. Coated slides exposed for 15 days for RNA experiments and 21 days for protein experiments were finally used for grain counting.

Because of the heterogeniety in the size of neurons, a time-consuming but accurate method of grain counting had to be used. Silver grains were counted over nucleoli, nucleoplasm and cytoplasm, using an oil immersion objective. The greatest and the least diameters of nucleoli, nuclei and cell bodies were measured with a filar micrometer eye-piece. The area for each cellular component was then calculated,

using the formula for an ellipse (area=abx3.14, where a and b represent one-half of the greatest and the least diameters). The nuclear area was subtracted from the cell area to obtain the area of the cytoplasm and the nucleolar area was subtracted from the nuclear area to obtain the area of nucleoplasm. The level of radioactivity was designated as the number of silver grains per 100 μ^2 for each component of the cell.

Normal and chromatolytic neurons were selected at random for measurement and grain counting. In the subsidiary experiment and the 6-day RNA and protein experiments, grain counts were done on 15 large and 15 small neurons and the same number of reacting neurons for each time interval. In the 14-day and 28-day RNA and protein experiments, grain counts were done on 15 large neurons from each of the control and experimental ganglia.

In order to examine the bearing of the number of nucleoli on nucleolar and cytoplasmic synthetic activities, data on neurons of approximately the same size with a single nucleolus and with two nucleoli, in normal and reacting ganglia, were compared. The number of silver grains was counted over the nucleolus and cytoplasm of ten pairs of neurons (single nucleolus and two nucleoli) from the 6-day axon reaction experiment at various time intervals after injection of cytidine-H^3 (20 min, 3 and 96 hr) or arginine-H^3 (10 min, 2 and 96 hr). Since nucleoli are closely related functionally to the Nissl substance, the level of radioactivity in the cytoplasm at each time interval was also measured.

D. Electron Microscopy

Ten rats whose right sciatic nerve had been sectioned 10 days

previously were used. The rats were perfused with 4% glutaraldehyde

in 0.2 M Sorensen's solution at pH 7.4 (Sjöstrand, 1967) by the perfusion

technic previously described. Sorensen's buffer solution was used

throughout. Right and left dorsal root ganglia (L5 and L6) were fixed,

immediately after removal, in chilled 4% buffered glutaraldehyde for 5

to 7 hr. Following immersion in 5% buffered sucrose solution overnight,

the tissue was fixed for 2 hr in 2% buffered osmium tetroxide solution.

The ganglia were dehydrated in alcohol, transferred to acetone, embedded

in vestopal W and sectioned with a Porter-Blum ultramicrotome. The

sections were first stained with uranyl acetate (Kellenberger, Ryter

and Sechand, 1958) and then Reynold's lead citrate (Reynold, 1963) and

examined with a Phillip's 100 C electron microscope.

V. RESULTS.

After cresyl violet staining, the ganglia showed four cell types, viz., neurons, satellite or capsule cells, fibroblasts and Schwann cells.

Neurons whose processes had been severed showed the chromatolytic changes of axon reaction (Figs. 2-5). Nissl aggregates had lost their compact arrangement and were dispersed throughout the cytoplasm, and the nucleus was eccentric. The temporal sequence of cytological changes followed the same pattern in each experimental series. There was a peak period of intense reaction between 4 and 20 days after nerve section, when a maximum proportion of chromatolytic neurons was present, followed by a recovery period, represented by 24 and 36 day intervals, during which the proportion of chromatolytic neurons declined. By 40 days after nerve section, cells of the experimental ganglia had regained, with few exceptions, a normal morphology.

The nucleoli stood out clearly against the lightly stained nucleoplasm. In neurons with a single nucleolus, the organelle was situated near the center of the nucleus. When there were multiple nucleoli, they were located either free in the nucleoplasm or adjacent to the nuclear membrane. The darkly stained 'small nucleoli' or 'extra-nucleolar bodies' were located free in the nucleoplasm or adjacent to the nucleoli. The number of nucleoli or combination of nucleoli and 'extranucleolar bodies' per cell was four or less (Figs. 6-22). The nature of the 'extranucleolar bodies' is not clear. They may be

Figures 2-5. Large and small neurons, normal and reacting, stained with cresyl violet (X1,000).

2. Large normal (47.9 μ).

3. Large reacting (46.4 μ).

4. Small normal (28.0 μ).

5. Small reacting (26.6 μ).
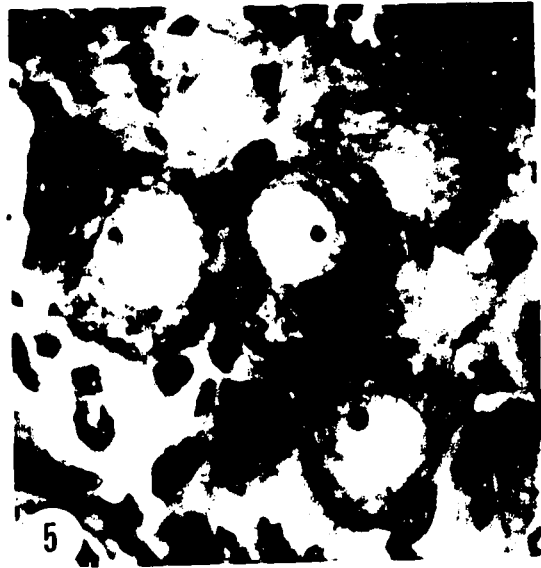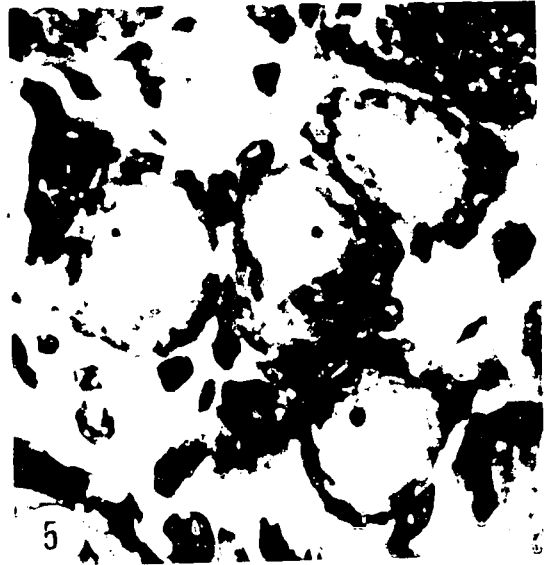
Figures 6-22.   Types of neurons according to number, size and

location of typical nucleoli and 'small nucleoli or

extranucleolar bodies' (X1100).

6. One nucleolus (40.4 μ).

7. Two nucleoli (29.5 μ).

8. Two nucleoli (30.4 μ).

9. Two nucleoli (15.3 μ).

10. Two nucleoli (23.6 μ).

11. Two nucleoli (26.2 μ).

12. One nucleolus and one extranucleolar body (31.1 μ).

13. One nucleolus and one extranucleolar body (44.2 μ).

14. Three nucleoli (16.1 μ).

15. Three nucleoli (26.4 μ).

16. Three nucleoli (29.5 μ).

17. Three nucleoli (24.2 μ).

18. Three nucleoli (18.3 μ).

19. Two nucleoli and one extranucleolar body (34.2 μ).

20. One nucleolus and two extranucleolar bodies (40.7 μ).

21. One nucleolus and two extranucleolar bodies (40.4 μ).

22. Four nucleoli (31.2 μ).

Figure 23. A group of fibroblasts (X1100).

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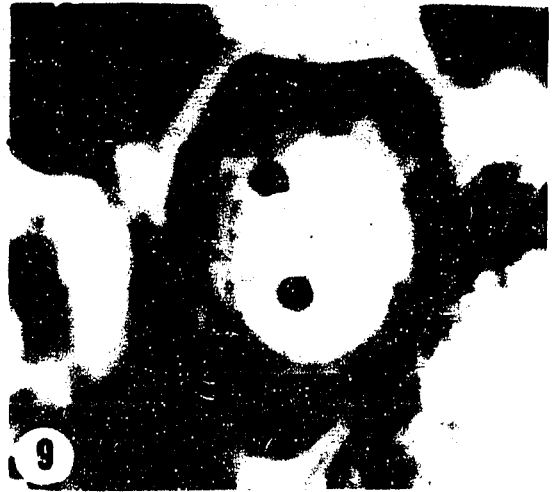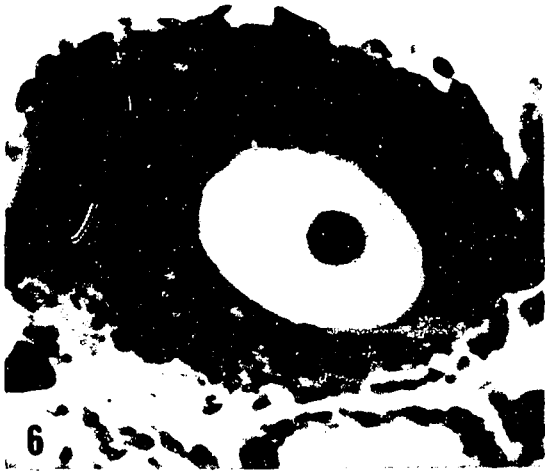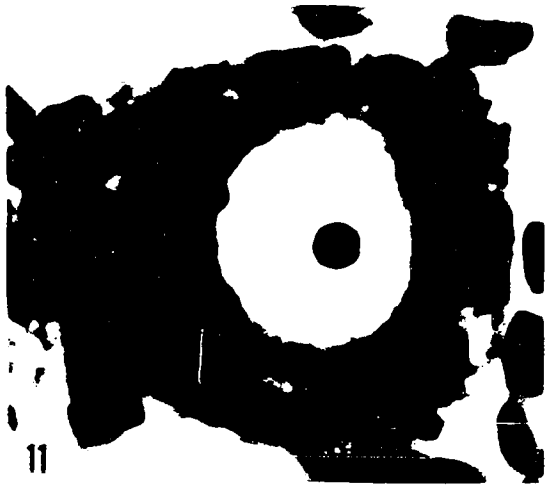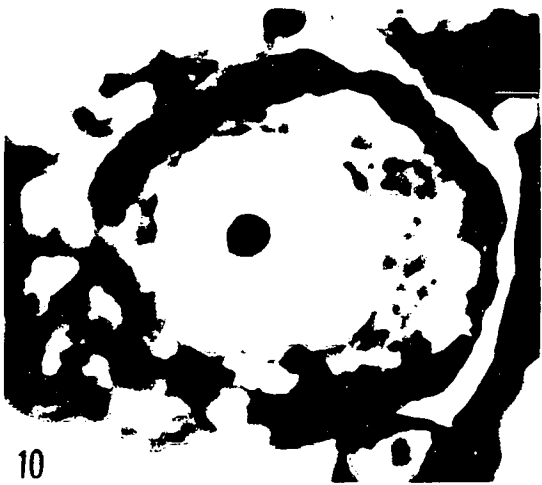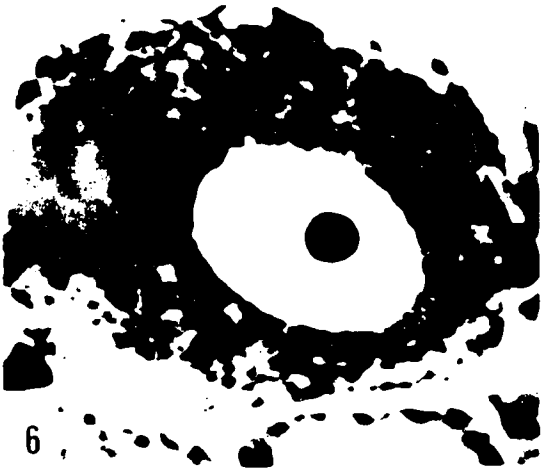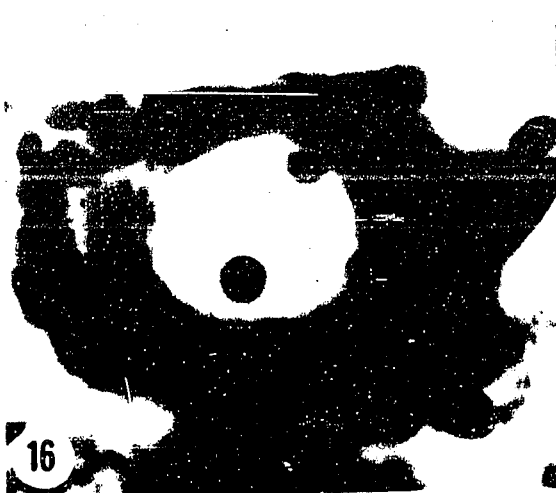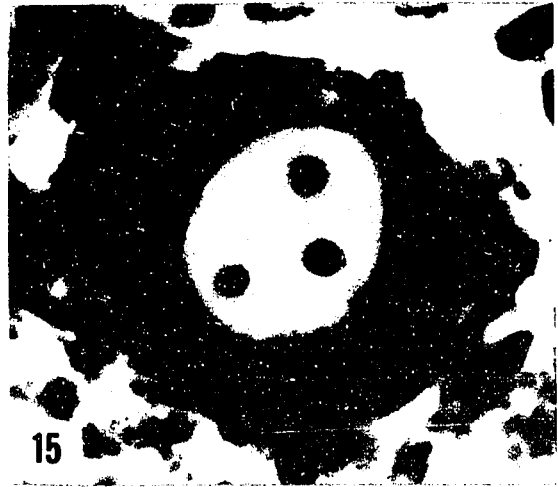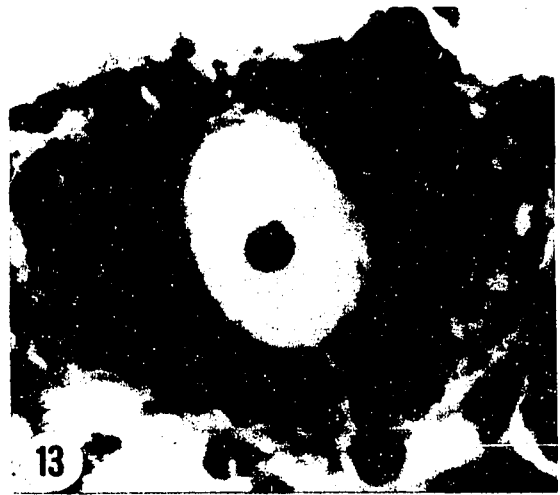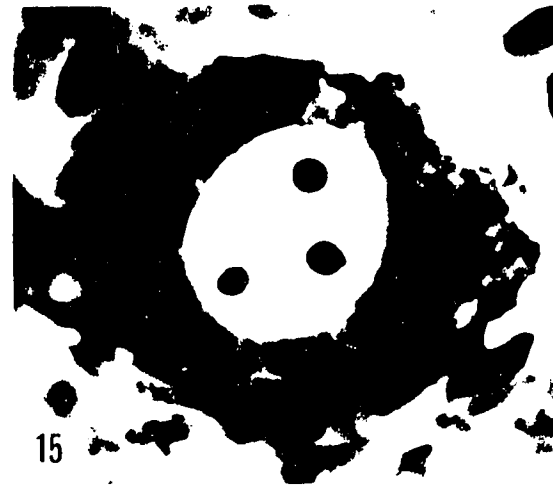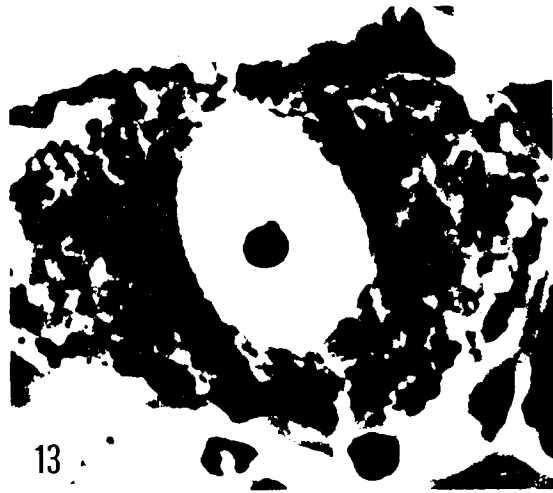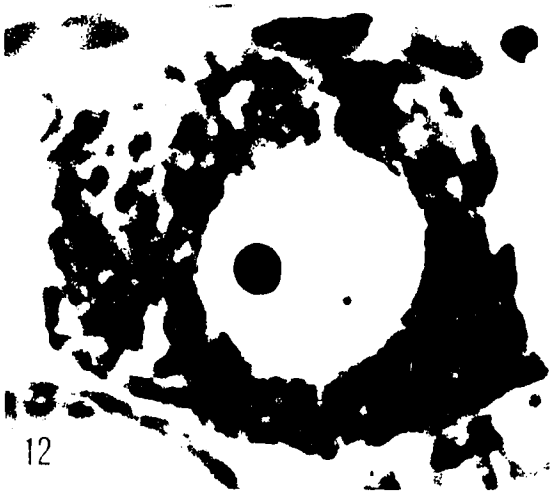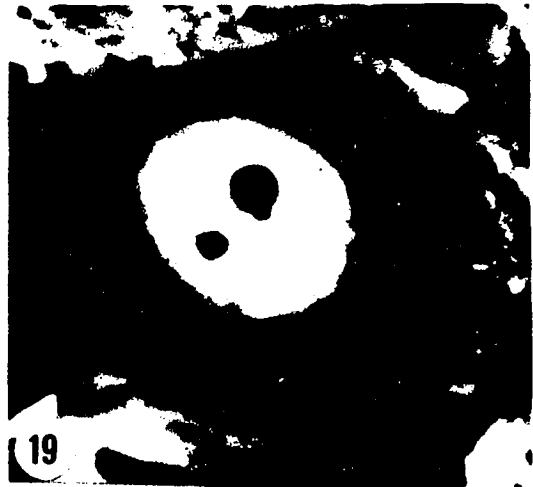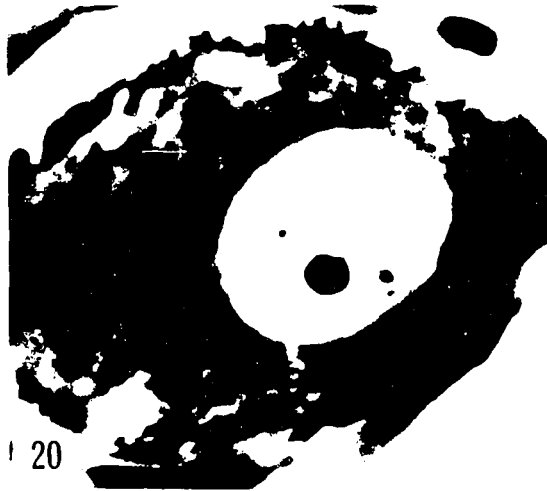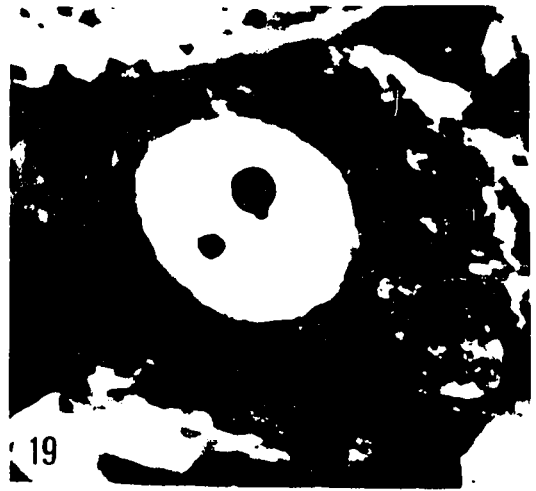
similar to nucleoli or they may be chromocenters derived from large
heterochromatic regions of chromosomes.

A. Quantitative Cytology

    1. Number of neurons and proportion of reacting neurons

The total number of nerve cells in L5 and L6 ganglia was 10,162
and 8,542, respectively, these figures being average values for four
ganglia. A larger proportion of cells was undergoing axon reaction in
the L5 ganglion than in the L6 ganglion (Fig. 24), indicating that a
higher proportion of cells from L5, compared with L6, contribute fibers
to the sciatic nerve at the level of section.

    2. Size of cell bodies, nuclei and nucleoli

A sufficiently large sample of neurons was measured 12 days
after nerve section so that the whole population of cells was adequately
represented. The size distributions of cell body, nucleus and nucleolus
in the control and experimental ganglia were compared statistically
(Table 2). The mean diameters of the cell bodies and nucleoli increased
by 6.4% and 7.8% in reacting neurons, the change being highly significant
statistically. The mean nuclear diameter of neurons undergoing axon
reaction was 4.3% larger than in their normal counterparts, a difference
which proved to be barely significant statistically.

    3. Volume and surface area of nucleoli

Data were obtained for four categories of cells: (a) normal neurons
with one nucleolus, (b) normal neurons with two nucleoli, (c) chromatolytic
neurons with one nucleolus, and (d) chromatolytic neurons with two
nucleoli. (Neurons with more than two nucleoli were too infrequent to
be incorporated in the study.) The two categories of cells among normal
and chromatolytic neurons were compared (b/a and d/c) as to volume and

Figure 24. Histogram to record the proportion of nerve cells

having the appearance of reacting cells in control

ganglia and in experimental ganglia at various time

intervals after nerve section. The control figures

combine data for L5 and L6 ganglia.
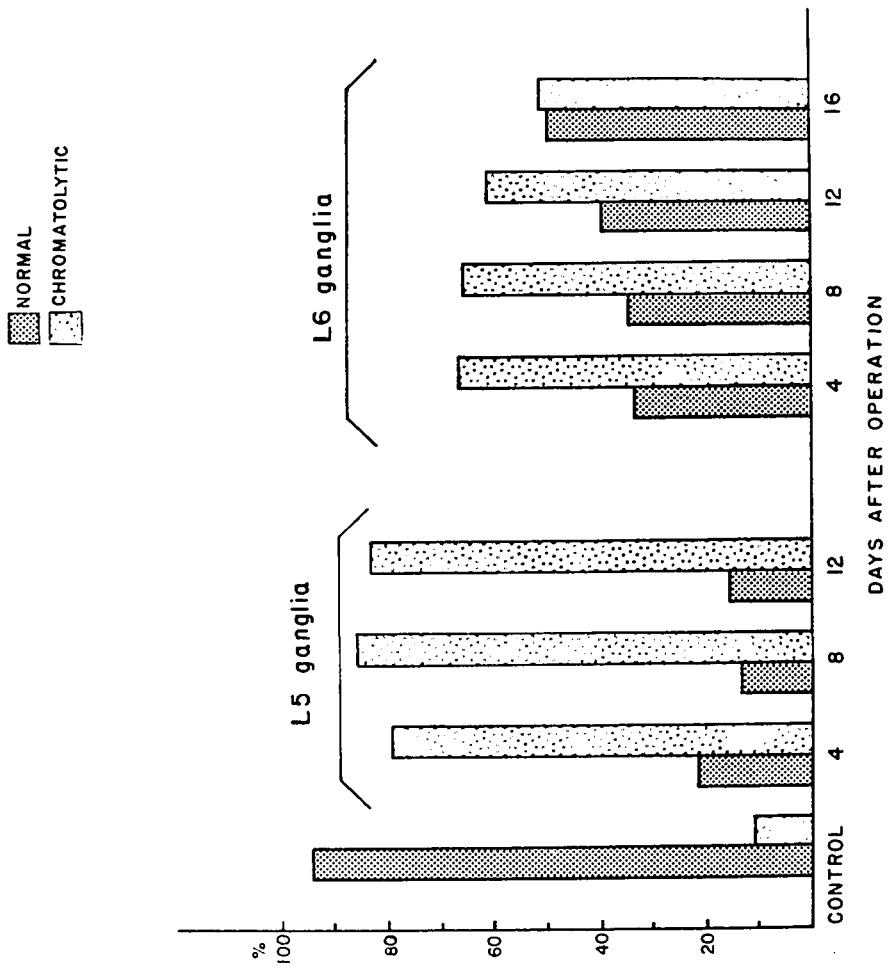
Figure 24

Table 2

Changes in Diameters of Cell Body, Nucleus and

Nucleolus during Axon Reaction

(a)   Cell Body

	N	$\bar{X}$	% of Difference	± Value	Significance
Normal	250	28.5 μ			
			6.5	4.60	P < 0.001
Axon Reaction	250	30.3 μ			

(b)   Nucleus

	N	$\bar{X}$	% of Difference	± Value	Significance
Normal	250	16.2 μ			P < 0.01
			4.3	2.90	> 0.001
Axon Reaction	250	16.9 μ			

(c)   Nucleolus

	N	$\bar{X}$	% of Difference	± Value	Significance
Normal	250	3.9 μ			
			7.7	4.41	P < 0.001
Axon Reaction	250	4.2 μ			

surface area of nucleoli and the ratio was expressed as D/S. Animals whose sciatic nerve had been sectioned 12 days before sacrifice were used for these measurements because this time interval appeared to be at or near the peak of axon reaction. Since there is a relationship between cell size and nucleolar size, 30 pairs of cells of approximately the same diameter in normal and experimental ganglia were used for each comparison.

The results presented in Table 3 show that the single nucleolus in the normal neuron has a larger volume than that of two nucleoli combined; i.e. the D/S ratio is less than one. On the other hand, the surface areas of the two categories of nucleoli are approximately the same; therefore, the D/S ratio is close to one. During axon reaction, the D/S ratio for both volume and surface area of nucleoli increased. It appears, therefore, that the combined volume and surface area of double nucleoli increased, during axon reaction, faster than the volume and surface area of a single nucleolus.

4. Comparative rate of recovery in neurons with one nucleolus and in those with multiple nucleoli

At various time intervals after nerve section, the difference in proportion of chromatolytic neurons with one or multiple nucleoli did not follow a definite pattern, indicating that the number of nucleoli is not related, in any obvious way, to recovery from chromatolysis (Fig. 25). (Cells with a single nucleolus and cells with more than one nucleolus occur in about equal proportions. Counts of these two classes of neurons in control and experimental ganglia, 4, 16 and 40 days after nerve section, gave no clear evidence of either fusion or fragmentation of nucleoli in reacting cells.)

Table 3

Relative Volumes and Surface Areas of

Nucleoli in Neurons with Double Nucleoli

and with a Single Nucleolus

D/S ratios *

	Normal	Axon Reaction
Volume (μ^3)	0.70 ± 0.10	1.28 ± 0.16
Surface Area (μ^2)	0.97 ± 0.10	1.32 ± 0.14

*
D/S = Σ of volume (μ^3) or surface area (μ^2) of nucleoli
in neurons containing double nucleoli / volume (μ^3) or
surface area (μ^2) of nucleolus in neurons containing
only one nucleolus.

Figure 25. Relative rates of recovery from axon reaction in

neurons with one nucleolus and in those with more

than one nucleolus (L6 ganglia).
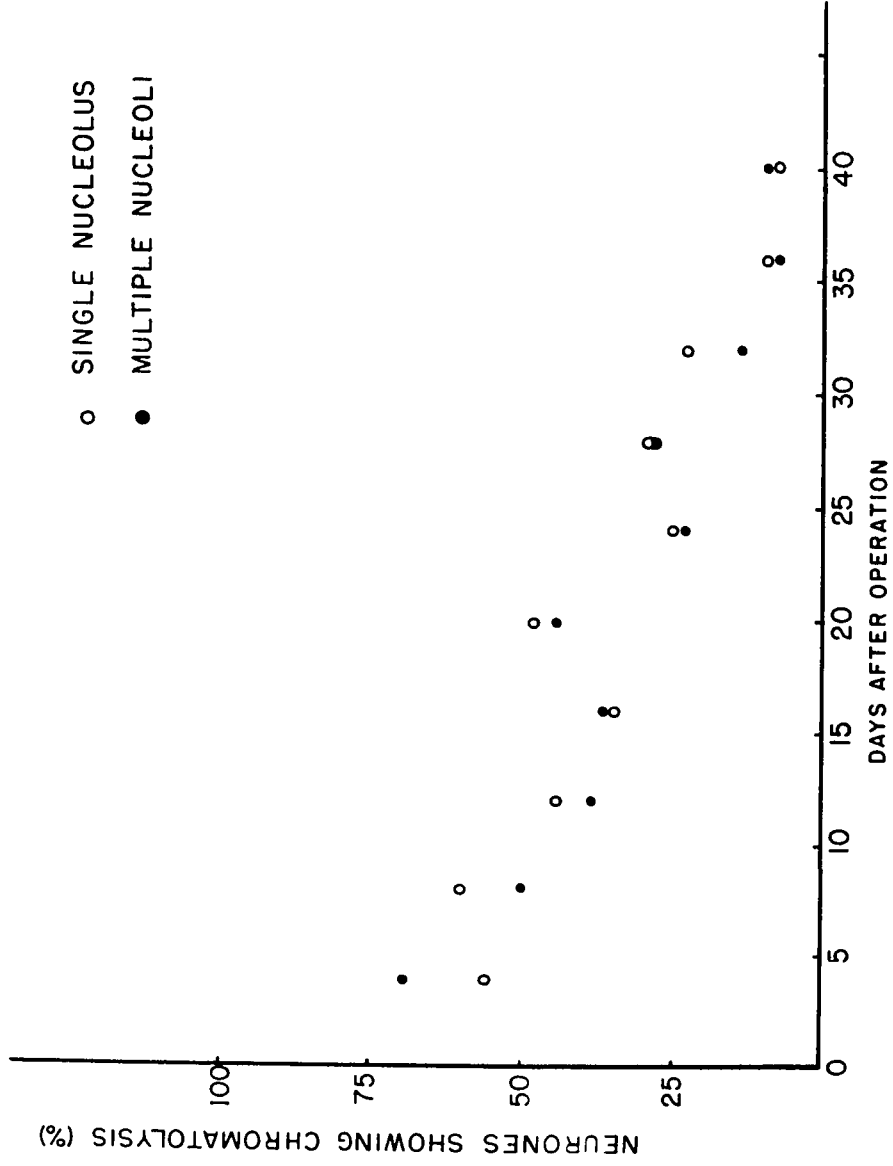
Figure 25

5. Size and number of fibroblast nuclei

The fibroblast nuclei (Fig. 23) varied considerably in size in both experimental and control ganglia. Statistical treatment of the measurements of width and length (Table 4) was done with 2x2 tables, with the correction of Yates (1934). The corrected chi-square showed 90% > P > 50%. Therefore, the length and width (size and shape) of fibroblast nuclei are not statistically different in normal and experimental ganglia.

The number of fibroblast nuclei appeared to be increased, as a visual impression, in the experimental ganglia and actual counts showed that the number was in fact significantly greater than in the control ganglia of animals sacrificed 4, 8, 12, 16, 20, 24, 28 and 32 days after operation (Fig. 26). The population of fibroblast nuclei in the experimental ganglia returned to normal 36 days after nerve section.

6. Size of satellite cell nuclei

In general, the nerve cells swell slightly as a result of axon reaction (Table 2). Since the neurons are closely encapsulated by satellite cells, there should be a slight increase in the area of the satellite cell capsule. It was not possible to obtain data on the size of the capsular cells because the borders between them could not be identified.

When the length and width of the satellite cell nuclei in control and experimental ganglia were compared, no statistically significant change in the size or shape of the nuclei could be demonstrated (Table 4).

Inspection of many sections gave no impression of an increase in the number of satellite cells around reacting neurons and therefore, no actual counts were made. In the course of studying large numbers

Table 4

Sizes of Nuclei of Fibroblasts and Satellite Cells

a. Fibroblasts

	length (μ)	width (μ)
Normal	6.75 ± 0.16	4.55 ± 0.13
Axon Reaction	6.95 ± 0.12	4.81 ± 0.11

b. Satellite cells

	length (μ)	width (μ)
Normal	6.82 ± 0.93	4.24 ± 0.84
Axon Reaction	6.91 ± 0.91	4.51 ± 0.5

Using the correction of Yates (1934) for 2 x 2 tables, the chi-square showed 90% > P > 50%; therefore, the dimensions of normal and axon reaction categories were not statistically different.

Figure 26. Numbers of fibroblast nuclei per unit area in
normal and experimental ganglia, L5 and L6.
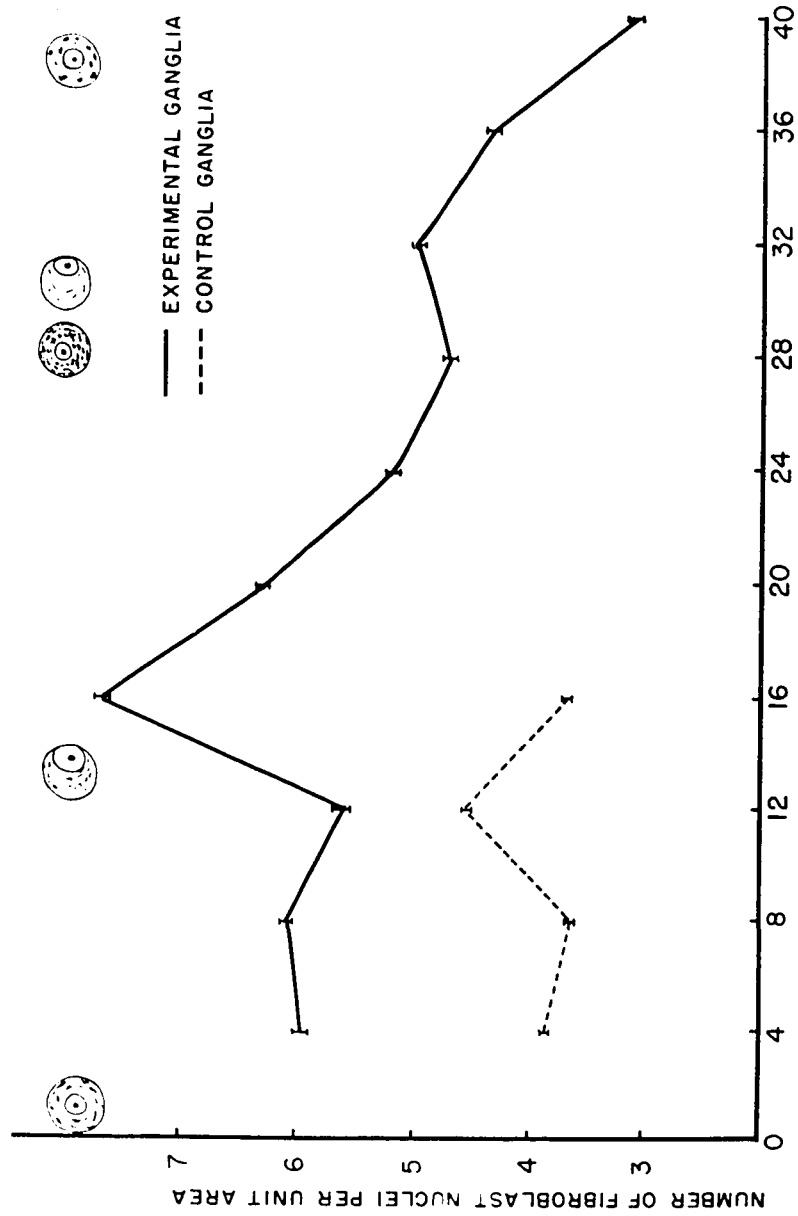
Figure 26

of sections, mitotic figures were seen among capsule cells only three times, twice in control ganglia and once in experimental ganglia. It is appreciated, however, that because of the shortness of the mitotic phase, the incidence of mitotic figures is low, in the absence of treatment with colchicine, even when there is fairly frequent cell division.

B. Quantitative Autoradiography

Autoradiographs prepared from ganglia at several time intervals after the injection of tritiated arginine or tritiated cytidine showed various patterns of silver grain distribution over the different regions of the neurons, i.e., nucleoli, nuclei and cytoplasm. The intensity of reaction was similar in all normal or, conversely, chromatolytic neurons at any given time after injection of either the protein precursor, tritiated arginine, or the RNA precursor, tritiated cytidine.

1. General distribution of silver grains (comparisons between normal and reacting cells are made in a subsequent section)

(a) Protein experiments. At all time intervals, silver grains were found located in the photographic emulsion at sites corresponding to the neuronal cytoplasm, nucleoplasm and nucleoli. At early time intervals, i.e. 5 and 10 min (Figs. 27, 28) after the injection of arginine-H^3, the radioactivity in the cytoplasm, nucleoplasm, and nucleoli was weak. It reached a peak at 2, 3 and 4 hr (Figs. 29, 30, 39, 40) time intervals and was less marked 16 hr (Figs. 31, 32) after injection of arginine-H^3.

(b) RNA experiments. At all time intervals, silver grains were found located in the photographic emulsion overlying the cytoplasm, nucleoplasm and nucleoli. Twenty minutes after injection of tritiated

Figures 27-32. Autoradiographs of normal and reacting neurons

after administration of arginine-H^3. Figs. 27

and 28 cresyl violet stain, Figs. 29-32 hematoxylin

and eosin stain (X1100).

27. Normal neurons 10 min after injection. The reaction is

weak in all components of the cells.

28. Reacting neurons 10 min after injection.

29. Normal neurons 4 hr after injection. The reaction is

strong over the nucleoli, nucleoplasm and cytoplasm.

30. Reacting neurons 4 hr after injection.

31. Normal neurons 16 hr after injection. The pattern of

silver grain distribution is similar to that of 10 min

and 4 hr time intervals except that the overall reaction

is weaker than at the 4 hr interval.

32. Reacting neuron 16 hr after injection.
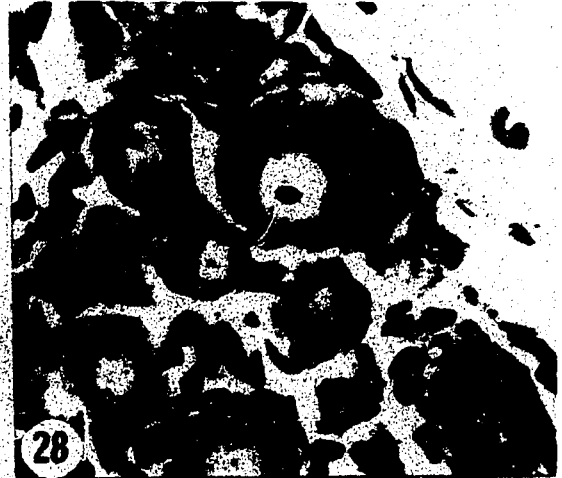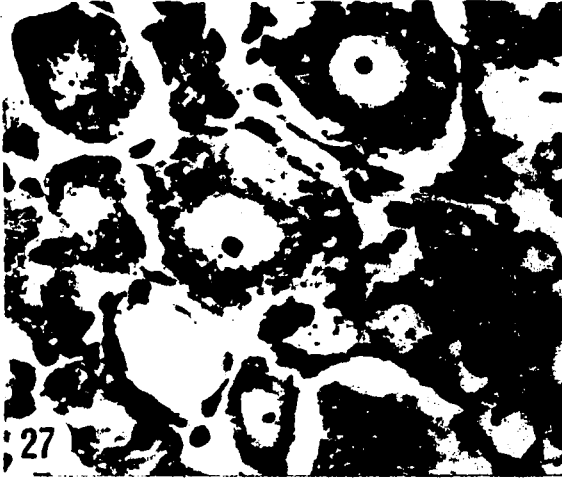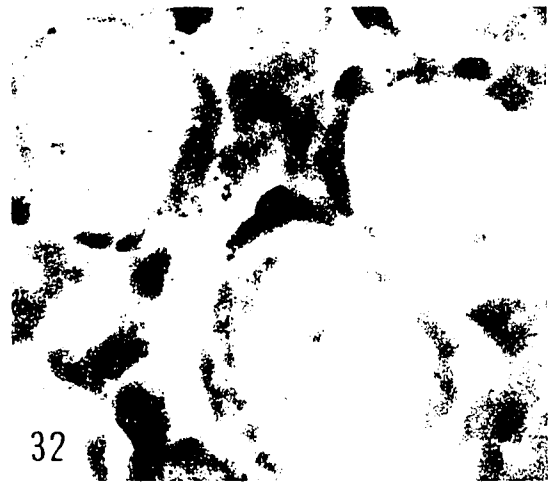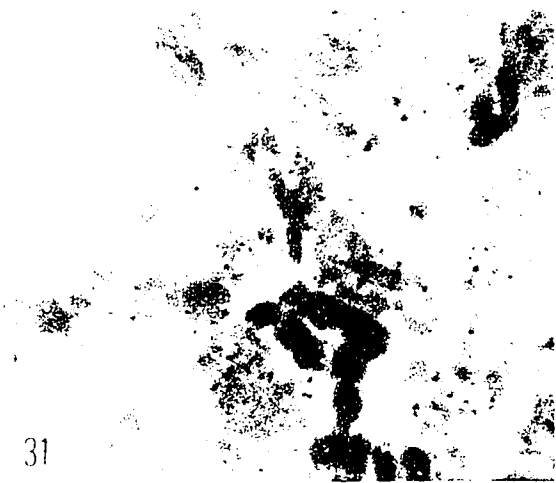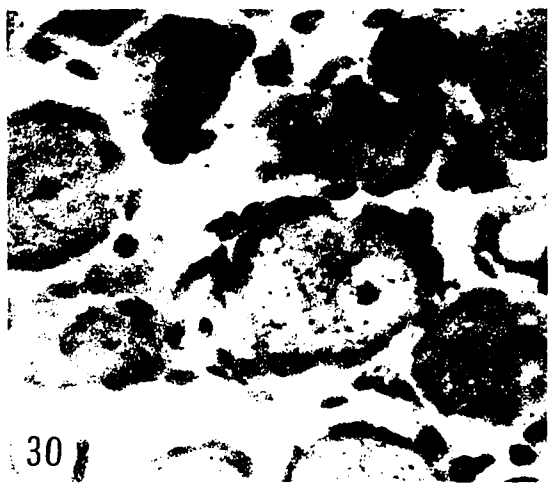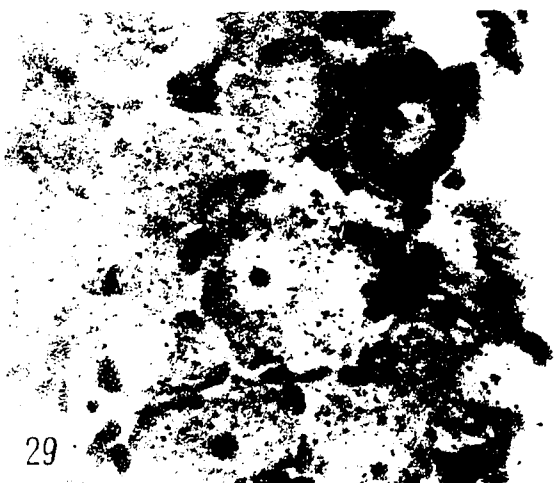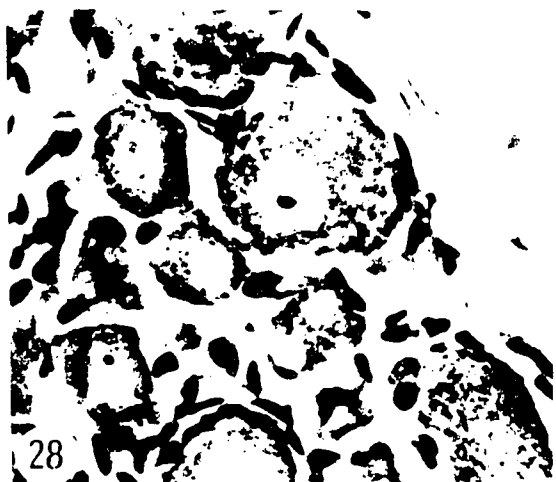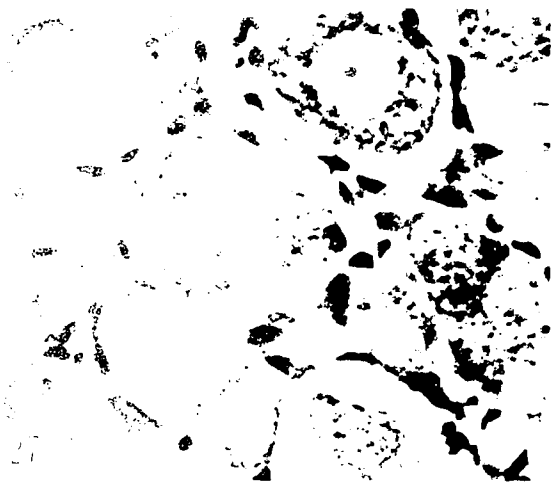
cytidine (Figs. 33-35), there was a fairly strong reaction over the nucleoplasm and nucleoli in normal and chromatolytic neurons, but few silver grains were present over the cytoplasm. At 3 and 6 hr after injection (Figs. 36, 39, 40), there was strong radioactivity in the nucleoli, nucleoplasm and cytoplasm. The pattern of silver grain distribution was similar to that seen after a 20 min time interval, but there was a stronger autoradiographic reaction. After 96 hr, the radioactivity in the nucleoli and nucleoplasm was weak and most of the silver grains were over the cytoplasm (Figs. 37, 38).

The newly-synthesized RNA appeared first, therefore, in the nucleoli and nucleoplasm and then in the cytoplasm.

2. Definition of 'large neuron' and 'small neuron' and their relative cytoplasmic radioactivity

Size spectra of normal neurons with a single nucleolus or multiple nucleoli were prepared (Fig. 43). In some cells the multiple nucleoli included the poorly understood 'extranucleolar bodies'.

The diameters of neurons with one nucleolus varied from 15μ to 48μ (mean 28.5μ) with a normal distribution. Neurons with more than one nucleolus tended to have a smaller average diameter, although large neurons with multiple nucleoli could be found. A diameter of 30μ was selected arbitrarily as the dividing line between 'large' and 'small' neurons for the study of a possible difference in their synthetic activities.

In the RNA experiments, the cytoplasmic radioactivity per $100\mu^2$ of the normal and chromatolytic small neurons was 70% and 40% higher, respectively, than that of large neurons (Table 5).

In the protein experiments, the cytoplasmic radioactivity per

Figures 33-38. Autoradiographs of normal and reacting neurons

after administration of cytidine-H^3, stained

with cresyl violet and exposed for 15 days (except

Fig. 35) (X 1100 except Fig. 33).

33. Normal neurons 20 min after injection. There is a strong

autoradiographic reaction over nucleoli and nucleoplasm

(X 2800).

34. Reacting neurons 20 min after injection. There is a

depression of synthetic activity in nucleoli and nucleo-

plasm. Silver grains, as in the normal neuron, are mainly

localized over the nuclei. The cytoplasm shows an

extremely weak reaction.

35. Normal neurons 20 min after injection. Long exposure

(50 days) to emphasize the radioactivity of nucleoli

and nucleoplasm.

36. Reacting neurons 6 hr after injection. While some

reaction persists over the nuclei, there is moderate

radioactivity over the cytoplasm.

37. Normal neuron 4 days after injection. Some reaction

persists over the nuclei. However, most of the silver

grains are distributed over the cytoplasm.

38. Reacting neurons 4 days after injection. The number of

silver grains over the cytoplasm is reduced in these

reacting cells.
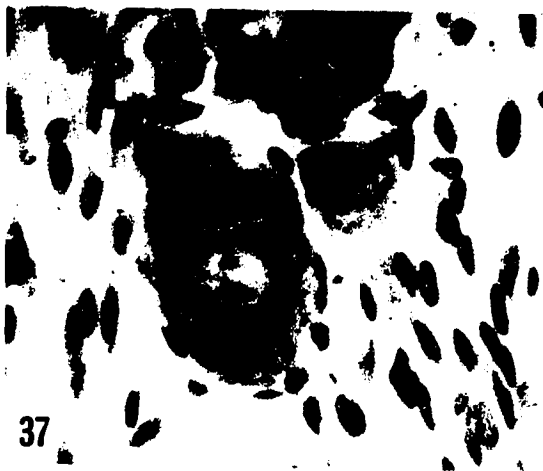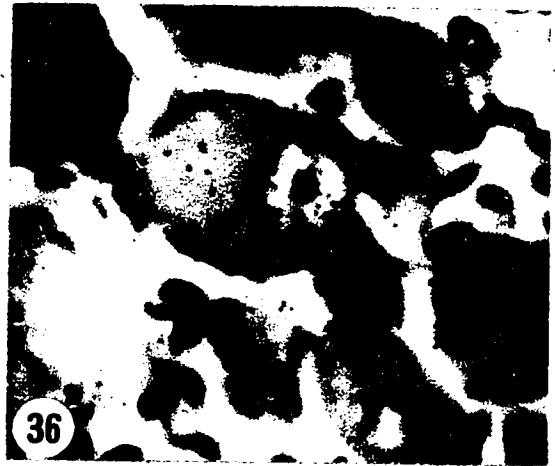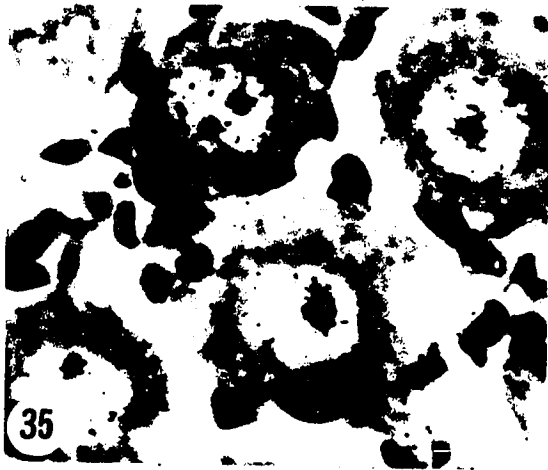
33
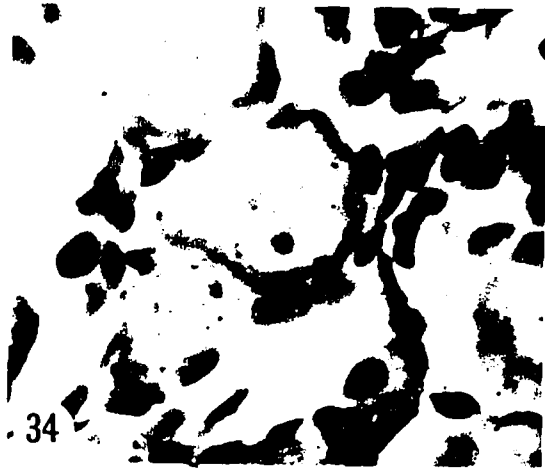
34
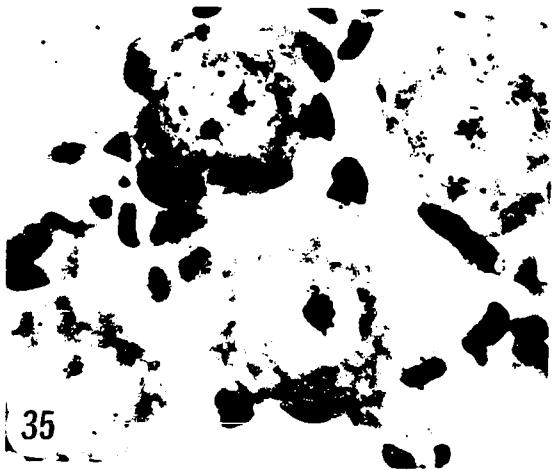
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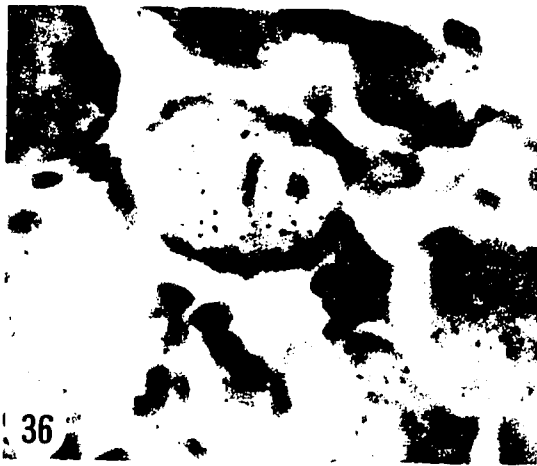
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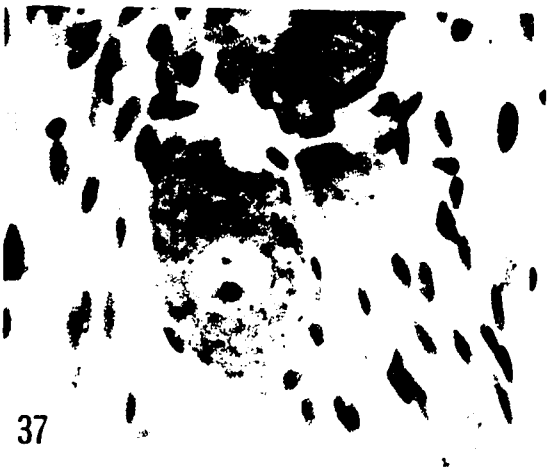
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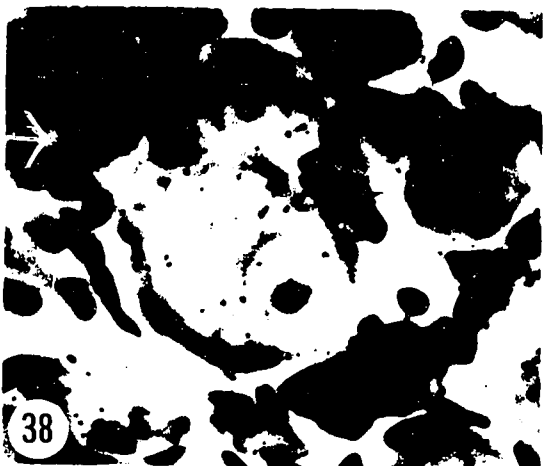
38

Figures 39-42. Autoradiographs of normal and reacting neurons
3 hr after injection of arginine-H^3 or cytidine-H^3
and stained with cresyl violet (X1100).

39. Normal neurons after injection of arginine-H^3. There is
moderate radioactivity over the nucleolus, nucleoplasm
and cytoplasm.

40. Reacting neurons after injection of tritiated arginine.

41. Normal neurons after injection of cytidine-H^3. The
silver grains are mainly localized over the nucleoli and
nucleoplasm, although there are a few silver grains over
the cytoplasm.

42. Reacting neuron after injection of cytidine-H^3. The
concentration of silver grains over the nucleolus and
nucleoplasm is lower than in normal cells.

39

40

41

42
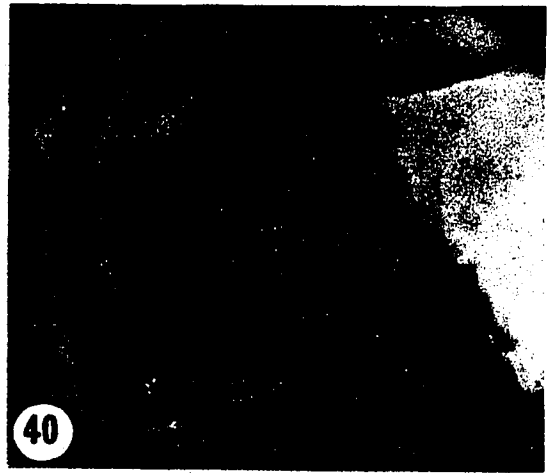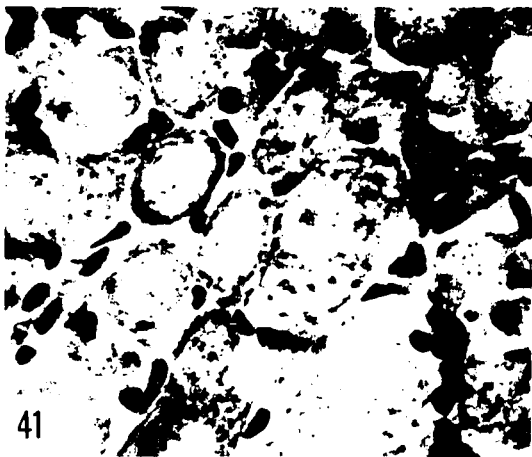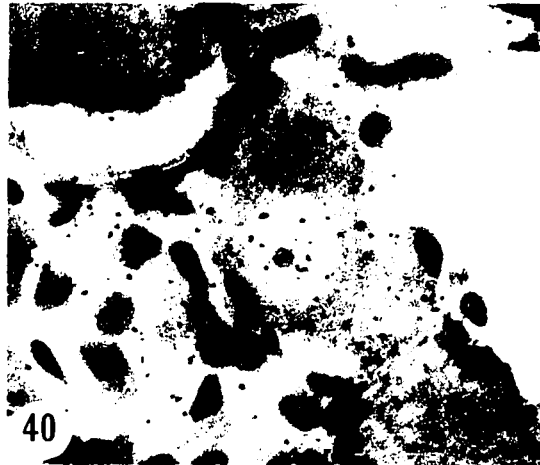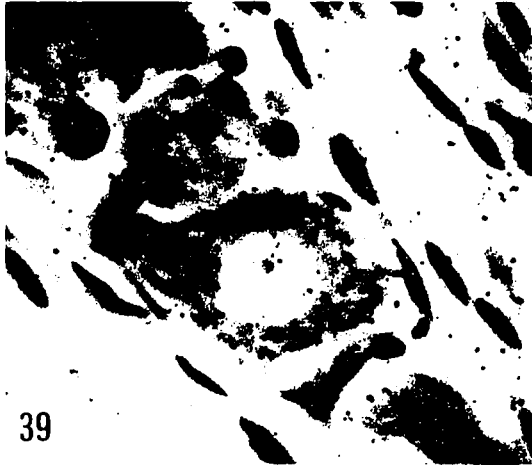
Figure 43. Histograms to indicate the size spectra of normal

neurons selected at random, with one nucleolus

(250 cells measured) and with multiple nucleoli

(50 cells measured). On the abscissae are shown

the size classes in micra after conversion from

units on the micrometer scale.
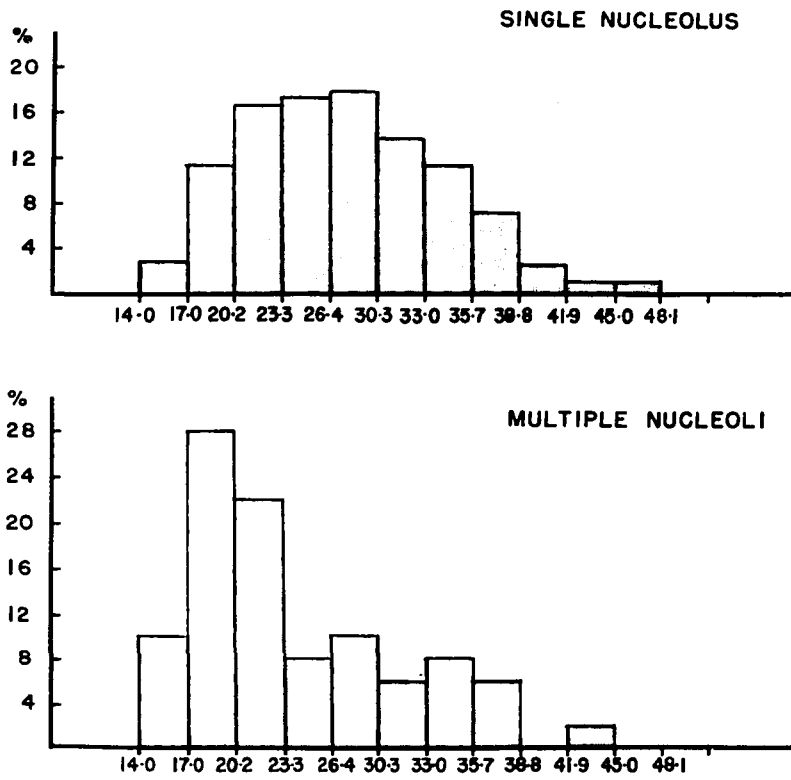
Figure 43

$100\mu^2$ of the normal and chromatolytic _small_ neurons was 21% and 24% higher, respectively, than that of _large_ neurons (Table 6).

In some cases, especially in chromatolytic neurons, the difference was not statistically significant, probably because of the wide variation in the radioactivity of individual cells (each value in Tables 5 and 6 expresses the mean $\pm$ S.E.M. for 15 cells).

3. Synthesis and metabolism of proteins

Synthesis and metabolism of proteins in the cytoplasm at early time intervals followed the same general pattern at 6, 14 and 28 days of axon reaction (Figs. 44-46). That is, 5 and 10 min after injection of tritiated arginine, cytoplasmic radioactivity was less intense in chromatolytic than in normal neurons. However, when the interval following injection of the isotope was in excess of 4 hr, the radio-activity of the cytoplasm of reacting cells was in general at a higher level than that of normal cells. As will be pointed out in the Discussion, the foregoing observations are regarded as particularly significant in relation to the basic nature of axon reaction.

A pattern of synthesis and metabolism of proteins similar to that noted above for the cytoplasm, was also found in the nucleoplasm and nucleoli of normal and chromatolytic neurons (Figs. 47-50). At early time intervals, e.g. 5 min after injection of tritiated arginine, radioactivity was lower in reacting neurons than in normal cells. The relation changed gradually until at later time intervals, for example at 96 hr, the nucleoplasm and nucleoli of chromatolytic neurons had a higher level of radioactivity than their normal counterparts.

4. Synthesis and metabolism of RNA

As in the protein experiments, the synthesis and metabolism of

Table 5

Relative Cytoplasmic Radioactivity of Small and Large

Neurons Following Injection of Cytidine-H^3

(a) 6-day axon reaction

		20 Min after Injection	Differ-ence(%)	3 Hours after Injection	Differ-ence(%)	6 Hours after Injection	Differ-ence(%)	96 Hours after Injection	Differ-ence(%)
Normal	Small	0.37 ± 0.05	23	2.06 ± 0.37	87	1.68 ± 0.16	34	2.49 ± 0.17	34
	Large	0.30 ± 0.0008		1.10 ± 0.10		1.25 ± 0.25		1.85 ± 0.12	
Reacting	Small	0.46 ± 0.09	84	1.37 ± 0.31	50	1.51 ± 0.16	67	1.27 ± 0.11	28
	Large	0.25 ± 0.04		0.91 ± 0.14		0.90 ± 0.14		0.99 ± 0.10	

(b) 3 hours after injection

		3-Day Axon Reaction	Differ-ence(%)	11-Day Axon Reaction	Differ-ence(%)	20-Day Axon Reaction	Differ-ence(%)	40-Day Axon Reaction	Differ-ence(%)
Normal	Small	1.56 ± 0.11	245	1.02 ± 0.17	29	1.40 ± 0.26	37	1.18 ± 0.18	63
	Large	0.44 ± 0.03		0.79 ± 0.15		1.02 ± 0.13		0.72 ± 0.10	
Reacting	Small	0.90 ± 0.11	95	0.49 ± 0.1	4 *	0.92 ± 0.17	6 *	0.91 ± 0.17	7 *
	Large	0.46 ± 0.01		0.47 ± 0.1		0.86 ± 0.15		0.90 ± 0.13	

* not significant (P > 0.01)

Average relative radioactivity of small normal neurons is 70% greater than that of large normal neurons.

Average relative radioactivity of small reacting neurons is 41% greater than that of large reacting neurons.

Table 6

Relative Cytoplasmic Radioactivity of Small and Large

Neurons Following Injection of Arginine-H^3

(a) 6-day axon reaction

		10 Min after Injection	Difference (%)	2 Hours after Injection	Difference (%)	4 Hours after Injection	Difference (%)	16 Hours after Injection	Difference (%)	96 Hours after Injection	Difference (%)
Normal	Small	2.26 ± 0.13	24	4.41 ± 0.20	7	3.97 ± 0.22	13	2.15 ± 0.26	5*	0.59 ± 0.06	5*
	Large	1.81 ± 0.11		4.09 ± 0.11		3.50 ± 0.21		2.03 ± 0.15		0.56 ± 0.08	
Reacting	Small	1.77 ± 0.10	45	4.06 ± 0.30	22	3.92 ± 0.31	5*	2.87 ± 0.21	2*	1.68 ± 0.09	27*
	Large	1.22 ± 0.11		3.32 ± 0.20		3.72 ± 0.21		2.81 ± 0.31		1.32 ± 0.46	

(b) 3 hours after injection

		3-Day Axon Reaction	Difference (%)	11-Day Axon Reaction	Difference (%)	20-Day Axon Reaction	Difference (%)	40-Day Axon Reaction	Difference (%)
Normal	Small	1.84 ± 0.16	26	2.08 ± 0.11	20	2.24 ± 0.18	73	2.64 ± 0.20	23
	Large	1.46 ± 0.21		1.72 ± 0.10		1.29 ± 0.65		2.13 ± 0.11	
Reacting	Small	1.30 ± 0.22	36	1.97 ± 0.35	34	2.74 ± 0.28	26	2.50 ± 0.17	27
	Large	0.95 ± 0.10		1.47 ± 0.14		2.17 ± 0.22		1.96 ± 0.10	

* not significant (P > 0.01)

Average relative radioactivity of small normal neurons is 21% greater than that of large neurons.

Average relative radioactivity of small reacting neurons is 24% greater than that of large neurons.

Figures 44-46. Relative radioactivity of the proteins synthesized

and metabolized in the cytoplasm of normal and

experimentally altered neurons. The data are

derived from grain counts over the entire

cytoplasm of 15 cells for each point shown on the

curves.

44. 6-day axon reaction, small neurons.

45. 6-day axon reaction, large neurons.

46. 14-day and 28-day axon reaction, large neurons.
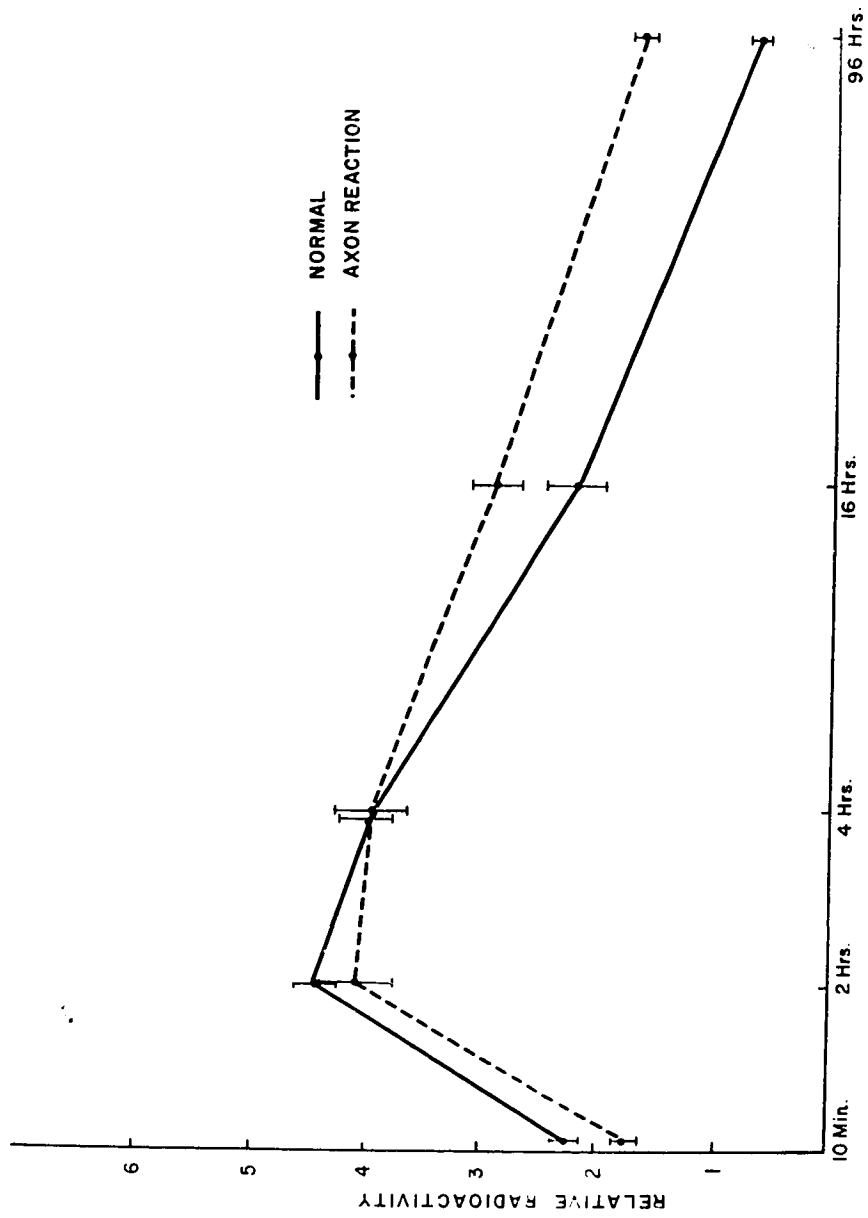
Figure 44
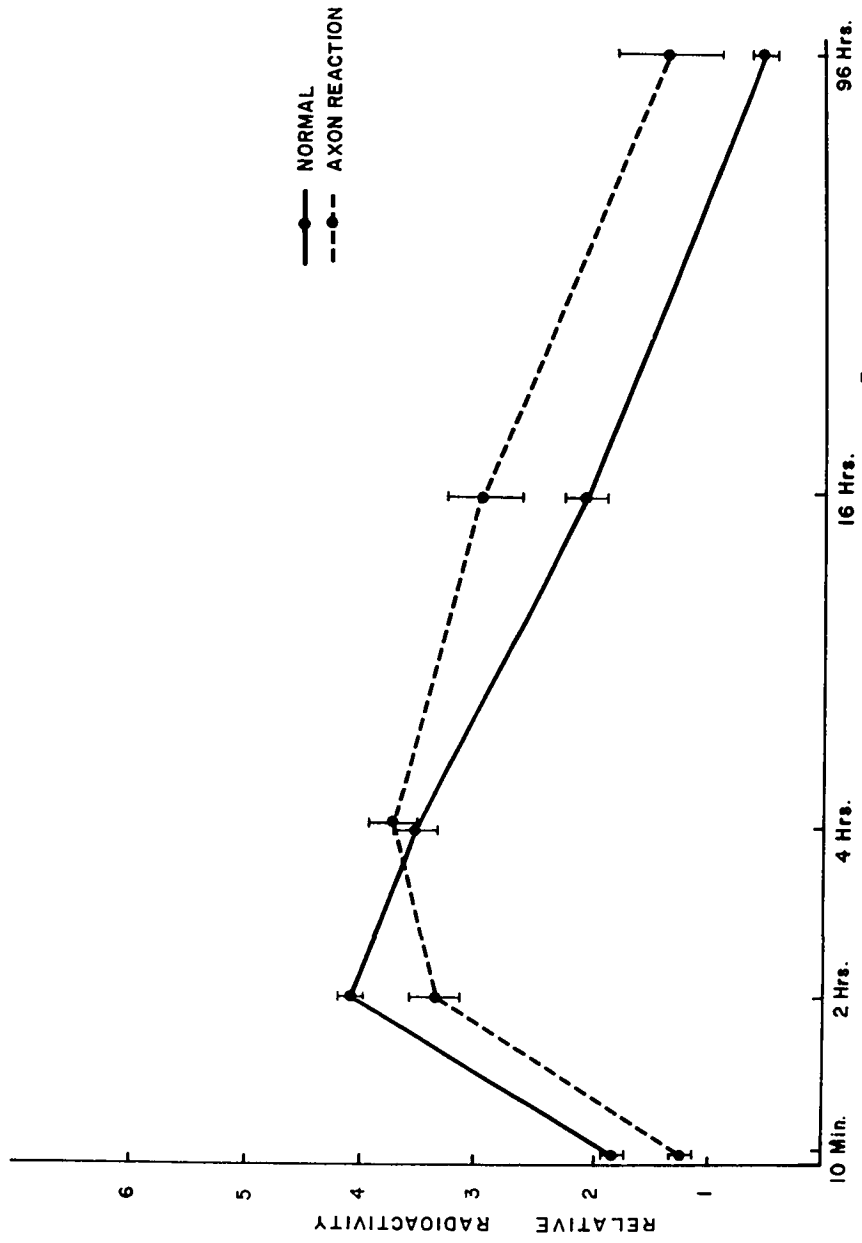
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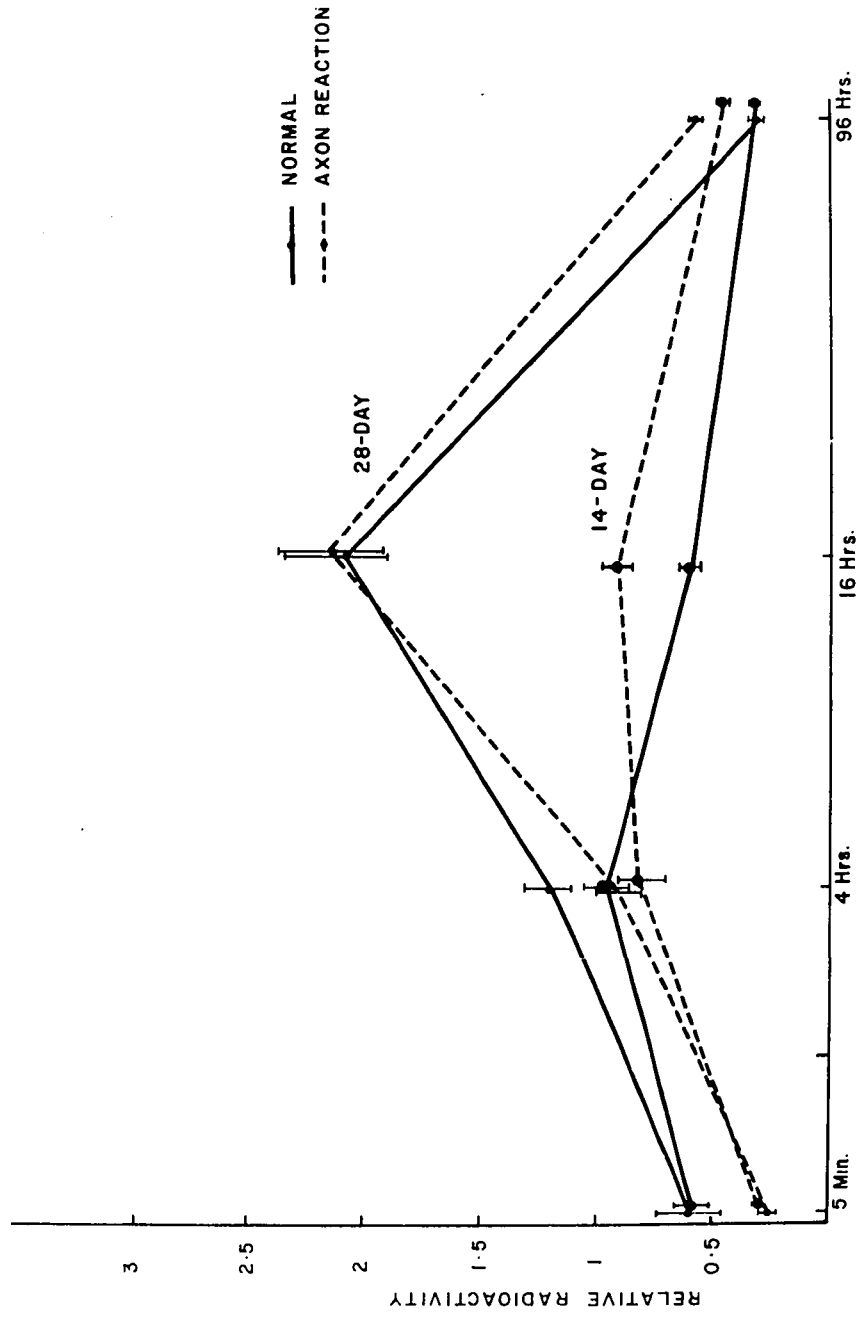
Figure 46

Figures 47-50. Histograms to illustrate the radioactivity of proteins synthesized and metabolized in nucleoli and nucleoplasm of normal and experimentally altered neurons. The data are derived from grain counts on 15 cells for each bar of the histograms.

47. 6-day axon reaction, small neurons.

48. 6-day axon reaction, large neurons.

49. 14-day axon reaction, large neurons.

50. 28-day axon reaction, large neurons.
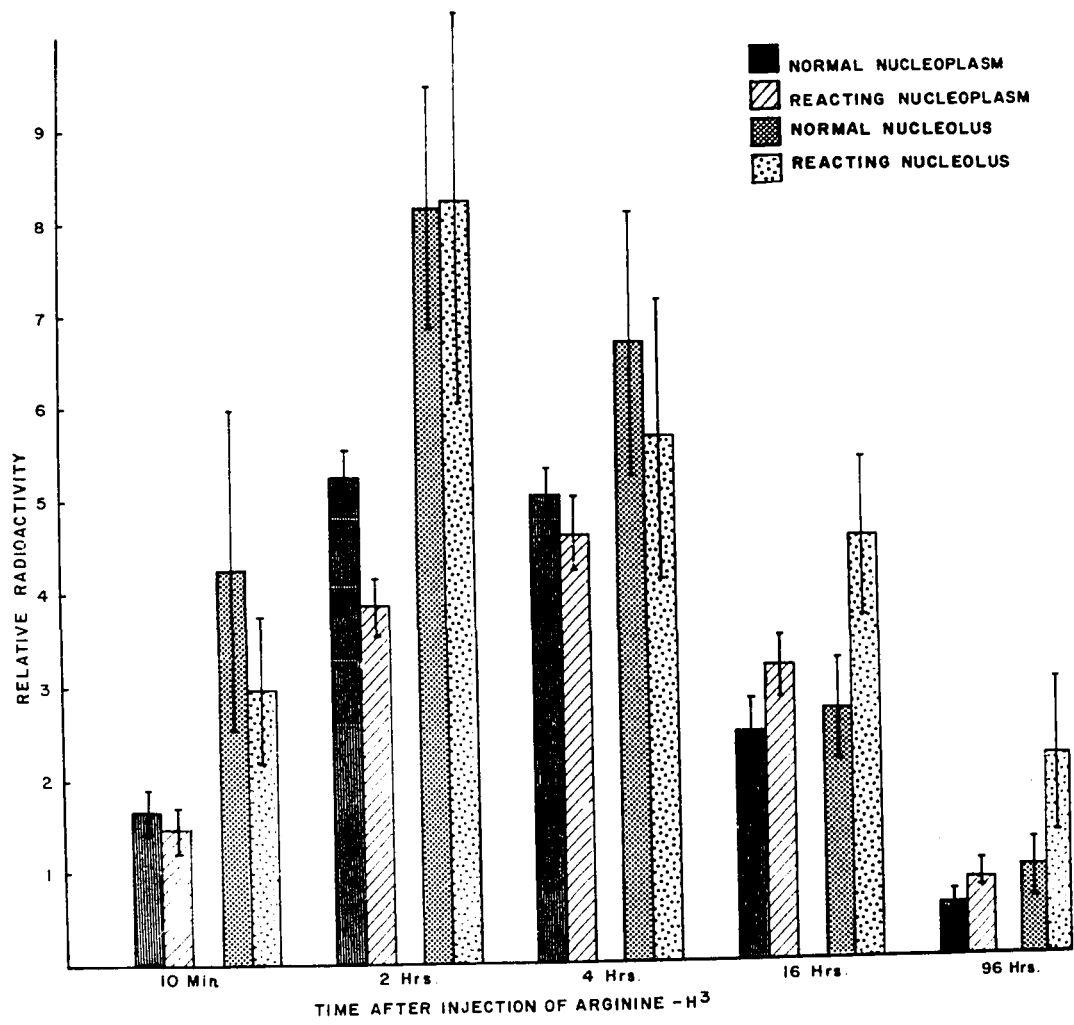
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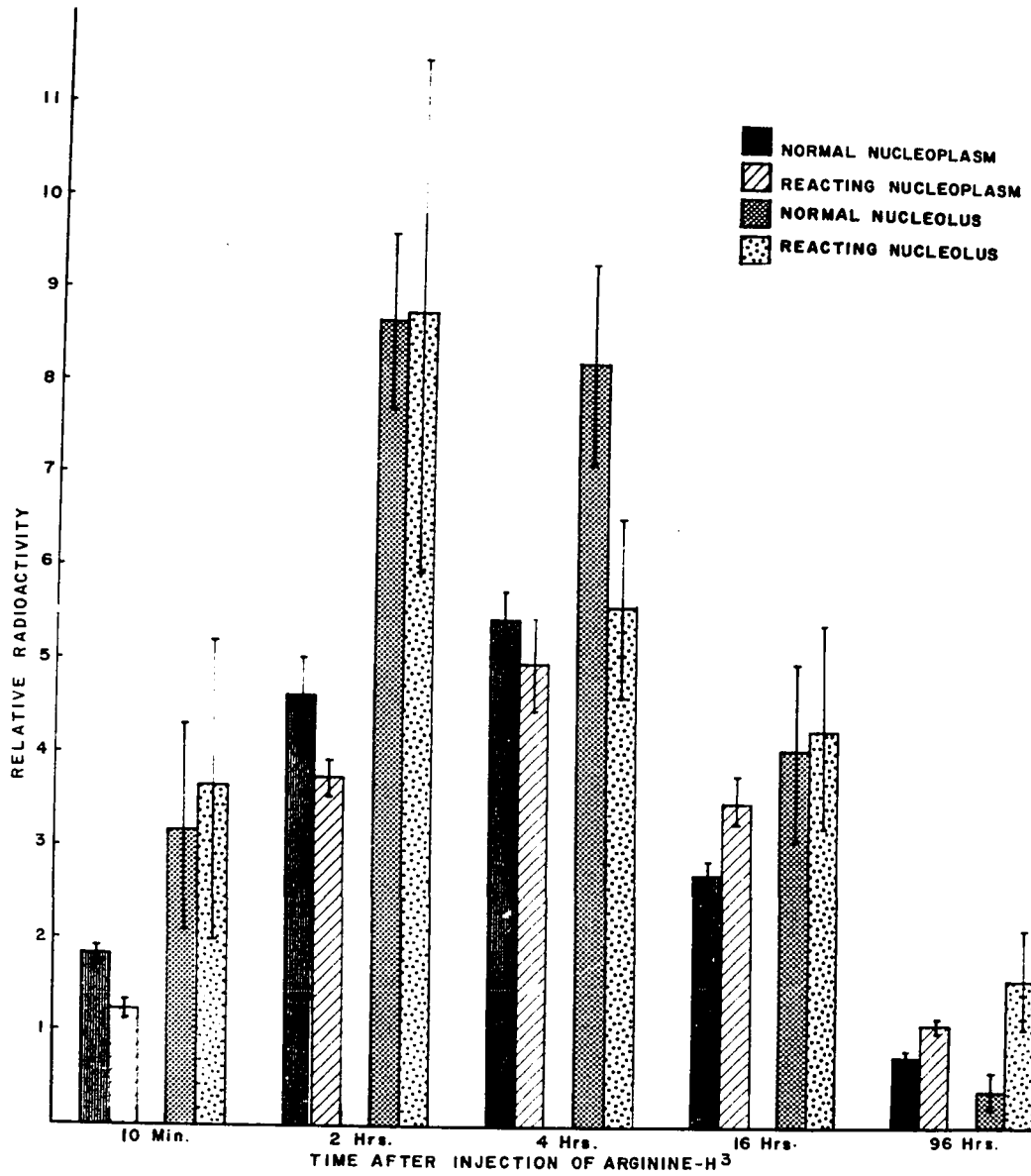
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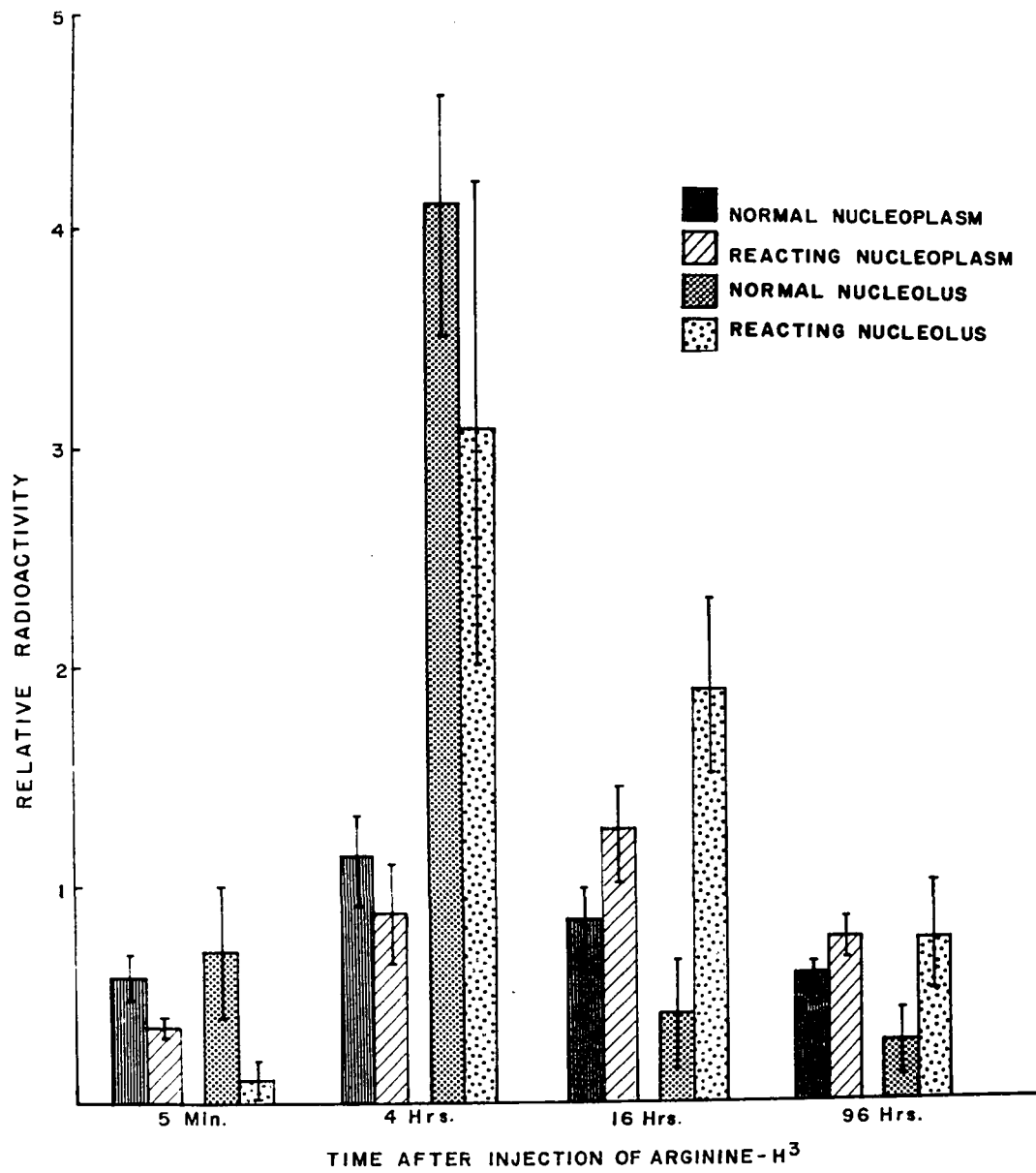
Figure 49
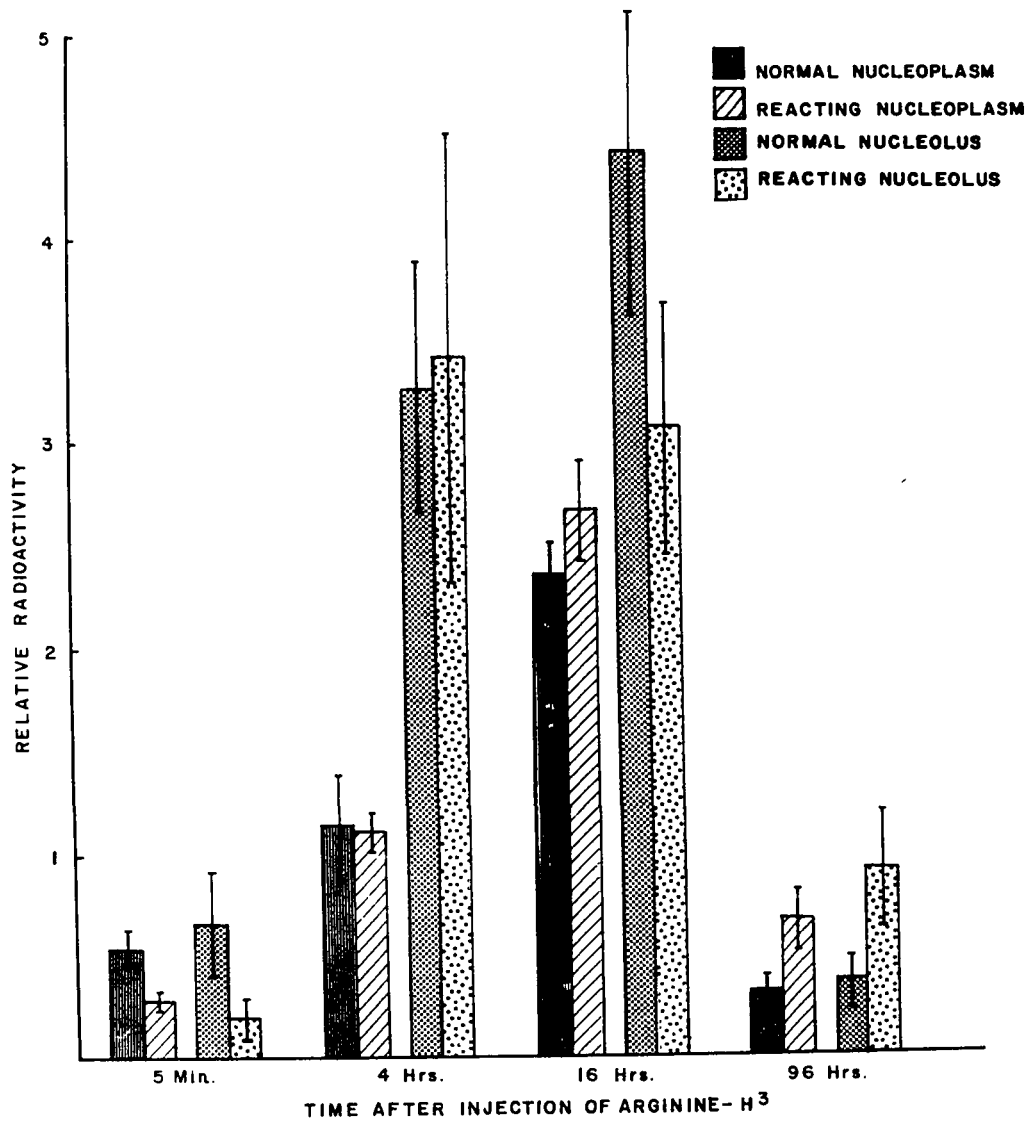
Figure 50

Figure 51.   Histogram illustrating the relative radioactivity of

RNA at sites of synthesis (nucleolus and nucleoplasm)

and metabolism (cytoplasm). The data are derived

from grain counts on 15 cells for each bar of the

histogram.

64
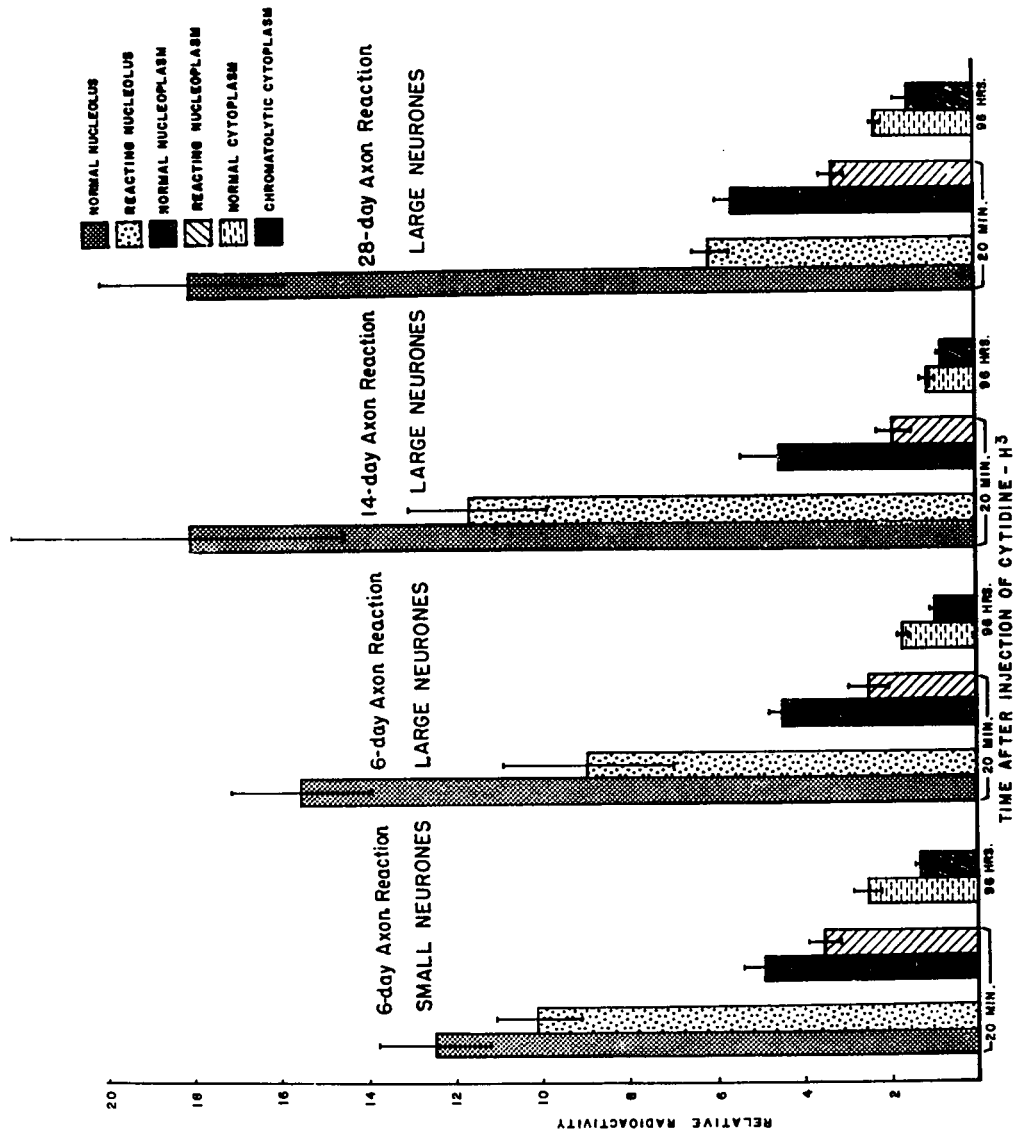
Figure 51

RNA in various cellular components followed the same general pattern at 6, 14 and 28 days of axon reaction (Fig. 51; Table 7). Twenty minutes after injection of tritiated cytidine, radioactivity over the nucleoplasm and nucleoli of reacting cells was less intense than in the normal neurons. However, radioactivity over the cytoplasm of reacting and normal cells showed no consistent differences. Ninety-six hours after injection of the isotope, radioactivity in the cytoplasm of reacting neurons was less than in control cells. Radioactivity in the nucleoplasm and nucleoli did not show consistent differences between experimental and control cells.

At 3 and 6 hr intervals after injection of cytidine-H^3, the radioactivity in nucleoli, nucleoplasm and cytoplasm was in general lower in reacting neurons than in control cells (Table 7). As was mentioned in connection with the protein synthesis experiments, the autoradiographic results (especially at short time intervals after injection of the isotope) are regarded as significant with respect to the nature of axon reaction (see Discussion).

5. Synthesis and metabolism of RNA and proteins in neurons with different numbers of nucleoli (Table 8)

At all time intervals, the level of radioactivity due to RNA or proteins in the cytoplasm of normal and reacting neurons was the same whether the cells contained one or two nucleoli. The radioactivity of nucleoli in single-nucleolus and double-nucleoli categories of cells, after injection of cytidine-H^3 and arginine-H^3, showed a high standard error when examined statistically. Because of the technical difficulties, as will be discussed later, in comparing nucleolar radioactivity in 'S' and 'D' types of cells and the high standard error, the data are

Table 7

Summary of RNA Experiments

(a) Main Experiment

Experiment			Grain Counts per $100\,\mu^2$ after Injection of Cytidine-H^3			
			20 Minutes	3 Hours	6 Hours	96 Hours
6-day axon reaction (small neurons)	normal reacting	Cytoplasm	0.37 ± 0.05 0.46 ± 0.09	2.06 ± 0.37 1.37 ± 0.31	1.68 ± 0.16 1.51 ± 0.16	2.49 ± 0.17 1.27 ± 0.11
	normal reacting	Nucleoplasm	4.91 ± 0.37 3.77 ± 0.38	9.62 ± 2.03 7.19 ± 1.2	5.44 ± 0.46 3.92 ± 0.6	0.79 ± 0.05 0.70 ± 0.14
	normal reacting	Nucleolus	12.45 ± 1.23 10.06 ± 1.90	27.13 ± 3.8 16.86 ± 5.32	11.78 ± 1.5 11.89 ± 1.7	1.81 ± 0.34 1.76 ± 0.65
6-day axon reaction (large neurons)	normal reacting	Cytoplasm	0.3 ± 0.0008 0.25 ± 0.04	1.10 ± 0.10 0.91 ± 0.14	1.25 ± 0.25 0.90 ± 0.14	1.85 ± 0.12 0.99 ± 0.10
	normal reacting	Nucleoplasm	4.40 ± 0.16 2.50 ± 0.5	9.21 ± 0.67 4.52 ± 0.91	8.65 ± 1.02 3.30 ± 0.37	1.02 ± 0.48 0.63 ± 0.14
	normal reacting	Nucleolus	15.72 ± 1.6 8.91 ± 1.9	25.26 ± 4.2 17.80 ± 2.3	23.87 ± 2.4 9.08 ± 1.31	1.04 ± 0.10 1.03 ± 0.95
14-day axon reaction (large neurons)	normal reacting	Cytoplasm	0.28 ± 0.10 0.17 ± 0.05	0.90 ± 0.06 0.60 ± 0.06	0.91 ± 0.1 0.67 ± 0.1	1.18 ± 0.09 0.95 ± 0.09
	normal reacting	Nucleoplasm	4.49 ± 0.75 1.88 ± 0.40	9.72 ± 4.1 4.32 ± 1.11	5.48 ± 0.48 2.31 ± 0.3	0.63 ± 0.22 0.63 ± 0.17
	normal reacting	Nucleolus	17.99 ± 4.2 11.56 ± 1.81	33.47 ± 5.91 15.22 ± 5.2	21.20 ± 2.31 14.02 ± 2.30	1.43 ± 0.16 1.28 ± 0.62

Table 7 (cont'd)

Summary of RNA Experiments

(a) Main Experiment

Experiment			Grain Counts per 100 μ^2 after Injection of Cytidine-H^3			
			20 Minutes	3 Hours	6 Hours	96 Hours
28-day axon reaction (large neurons)	Cytoplasm	normal	0.17 ± 0.07	0.68 ± 0.07	1.03 ± 0.10	2.25 ± 0.10
		reacting	0.20 ± 0.01	0.44 ± 0.07	0.64 ± 0.015	1.66 ± 0.4
	Nucleoplasm	normal	5.78 ± 0.38	9.57 ± 0.70	9.15 ± 1.01	1.03 ± 0.02
		reacting	3.27 ± 0.15	3.95 ± 0.80	4.54 ± 0.60	1.04 ± 0.13
	Nucleolus	normal	17.9 ± 2.02	30.34 ± 5.1	30.70 ± 3.9	3.66 ± 1.01
		reacting	6.10 ± 0.18	11.89 ± 2.1	13.58 ± 2.10	0.4 ± 0.30

(b) Subsidiary Experiment (3 hr Following Injection of Cytidine-H^3)

			Grain Counts per 100 μ^2 after Nerve Section			
			3 Days	11 Days	20 Days	40 Days
small neurons	Cytoplasm	normal	1.56 ± 0.11	1.02 ± 0.17	1.40 ± 0.26	1.18 ± 0.18
		reacting	0.90 ± 0.11	0.49 ± 0.10	0.92 ± 0.17	0.91 ± 0.17
	Nucleoplasm	normal	8.20 ± 0.52	6.21 ± 1.29	9.13 ± 1.6	7.00 ± 1.00
		reacting	4.05 ± 0.58	2.22 ± 0.45	3.56 ± 0.86	5.29 ± 0.81
	Nucleolus	normal	18.33 ± 5.35	19.10 ± 2.75	19.96 ± 3.92	22.74 ± 7.1
		reacting	13.54 ± 3.52	13.05 ± 2.45	10.45 ± 6.3	20.71 ± 4.2
large neurons	Cytoplasm	normal	0.44 ± 0.026	0.79 ± 0.15	1.02 ± 0.13	0.72 ± 0.10
		reacting	0.46 ± 0.01	0.47 ± 0.10	0.86 ± 0.15	0.90 ± 0.13
	Nucleoplasm	normal	4.83 ± 0.31	5.92 ± 0.48	11.58 ± 1.02	5.68 ± 1.12
		reacting	2.88 ± 0.13	1.77 ± 0.17	6.35 ± 2.15	7.02 ± 1.00
	Nucleolus	normal	18.8 ± 3.2	19.49 ± 0.98	31.71 ± 4.12	30.41 ± 5.9
		reacting	14.42 ± 2.75	7.61 ± 2.45	18.76 ± 7.52	22.99 ± 3.80

Table 8

Level of Radioactivity in Normal and Chromatolytic Neurons with

One (S) or Two Nucleoli (D)

a. Cytidine-H³ experiment

		20 Minutes		6 Hours		96 Hours	
		D	S	D	S	D	S
Normal	Cytoplasm	0.29 ± 0.18	0.30 ± 0.15	1.48 ± 0.20	2.00 ± 0.51	-	-
	Nucleoli	*14.64 ± 1.87	18.69 ± 1.82	15.88 ± 3.18	17.4 ± 2.72	*0.64 ± 0.35	1.32 ± 0.3
Axon Reaction	Cytoplasm	0.51 ± 0.15	0.51 ± 0.32	1.26 ± 0.26	1.15 ± 0.20	1.17 ± 0.10	1.43 ± 0.20
	Nucleoli	10.40 ± 3.25	11.86 ± 2.85	8.13 ± 1.98	11.35 ± 2.35	1.69 ± 0.73	1.59 ± 0.51

b. Arginine-H³ experiment

		10 Minutes		2 Hours		96 Hours	
		D	S	D	S	D	S
Normal	Cytoplasm	2.19 ± 0.23	1.91 ± 0.19	4.46 ± 0.20	4.25 ± 0.26	-	-
	Nucleoli	2.81 ± 0.60	2.59 ± 0.45	6.14 ± 1.24	8.78 ± 1.48	0.49 ± 0.26	0.87 ± 0.28
Axon Reaction	Cytoplasm	1.48 ± 0.18	1.32 ± 0.18	3.32 ± 0.36	3.91 ± 0.39	-	-
	Nucleoli	1.64 ± 0.56	2.37 ± 0.80	6.45 ± 1.58	6.91 ± 2.15	1.34 ± 0.64	1.91 ± 0.55

* $P < 0.001$

considered to be inconclusive. Conclusive results would have to be sought

by means of other technical methods, which could not be undertaken in this

particular project.

C. Electron Microscopy

1. Fine structure of the neurons (cytoplasm), satellite cells and

fibroblasts

The ultrastructure of normal and chromatolytic neurons, satellite

cells and fibroblasts, as recorded by Palay and Palade, 1955; Mackey,

Spiro and Wiener, 1964; Pannese, 1963; Bunge et al., 1967 and other

authors, was described in an earlier section. Some of the features

which were also seen in the present study will now be briefly described.

The Nissl substance as seen with the light microscope corresponds

to aggregates of rough surface endoplasmic reticulum (Fig. 52). In

neurons undergoing axon reaction, there was a decrease in the granular

endoplasmic reticulum and a disturbance of its compact arrangement.

Scattered between profiles of granular reticulum, free ribosomes were

found in greater abundance in reacting cells and several ribosomes often

aggregated to form a cluster (Fig. 53). The cytoplasm of the reacting

cells was relatively free of organelles in the perinuclear zone, although

there were clusters of free ribosomes, dense bodies and neurofilaments,

etc. However, the dispersed reticulum was frequently found to occupy

the periphery of the cytoplasm, a region which is relatively free of

Nissl substance in normal neurons. Indentations of the nuclear membrane

were conspicuous in some reacting neurons and the adjacent cytoplasm

contained free or membrane-bound ribosomes, dense bodies and mito-

chondria (Fig. 54).

The irregular contour of the neuronal surface was completely

Figure 52. Normal dorsal root ganglion cell, satellite cell and myelinated (A_1) and unmyelinated (A_2) nerve fibers. This low power view shows the Nissl aggregates consisting of elongated cisternae of rough surface endoplasmic reticulum and clusters of ribosomes. The vesicles and elongated sacs without ribosomes represent the smooth surface endoplasmic reticulum or Golgi complexes (G). Numerous mitochondria are present. Dense bodies (D) present throughout the cytoplasm of the neurons as well as that of the satellite cells, are sometimes different in density and internal structure. The peripheral portion of the cytoplasm of the neurons is relatively free of Nissl substance. The irregular surface of the nerve cell is surrounded by and apposed to the satellite cell (SC). The Schwann cell nucleus (SN) is more dense, and hence more electron opaque, than the neuronal nucleus (N). (X9,200).
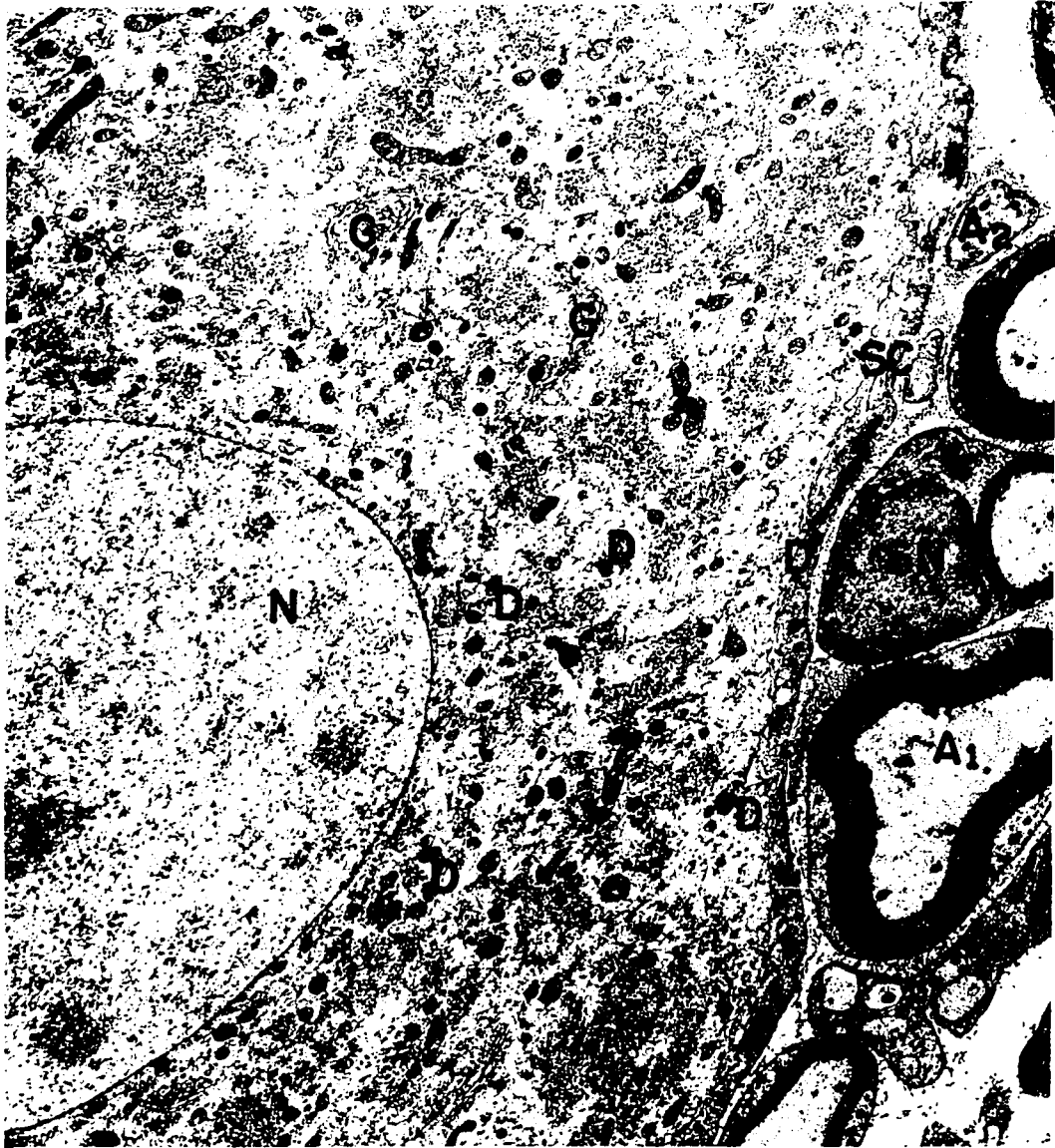
Figure 52
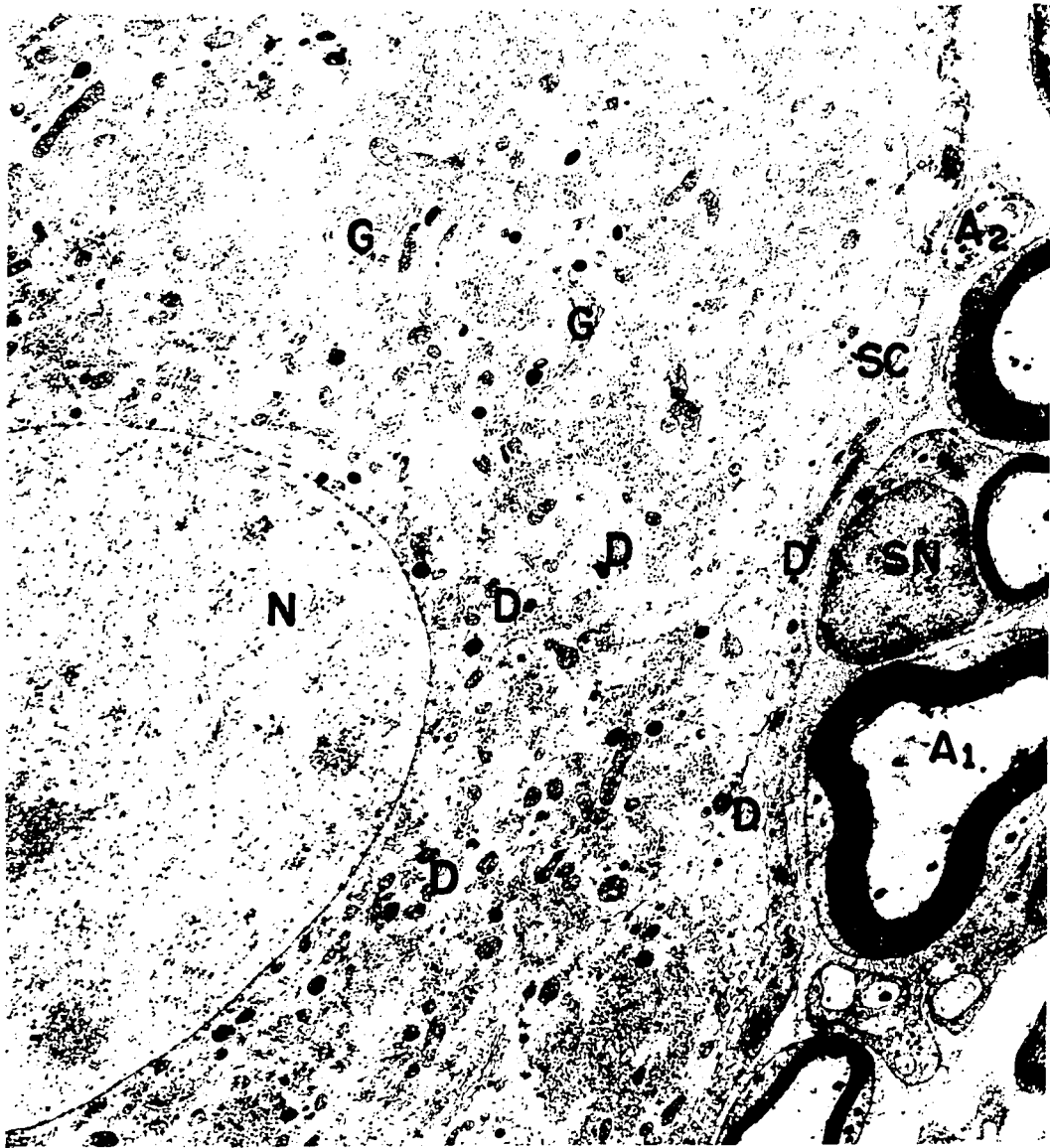
Figure 52

Figure 53. Reacting neuron showing cytoplasmic chromatolysis.

There is a peripheral location of the Nissl substance.

The perinuclear zone is free of endoplasmic reticulum

(X 32,480).

Note: All electronmicrographs of reacting neurons were

ten days after sciatic nerve section.

Figure 53
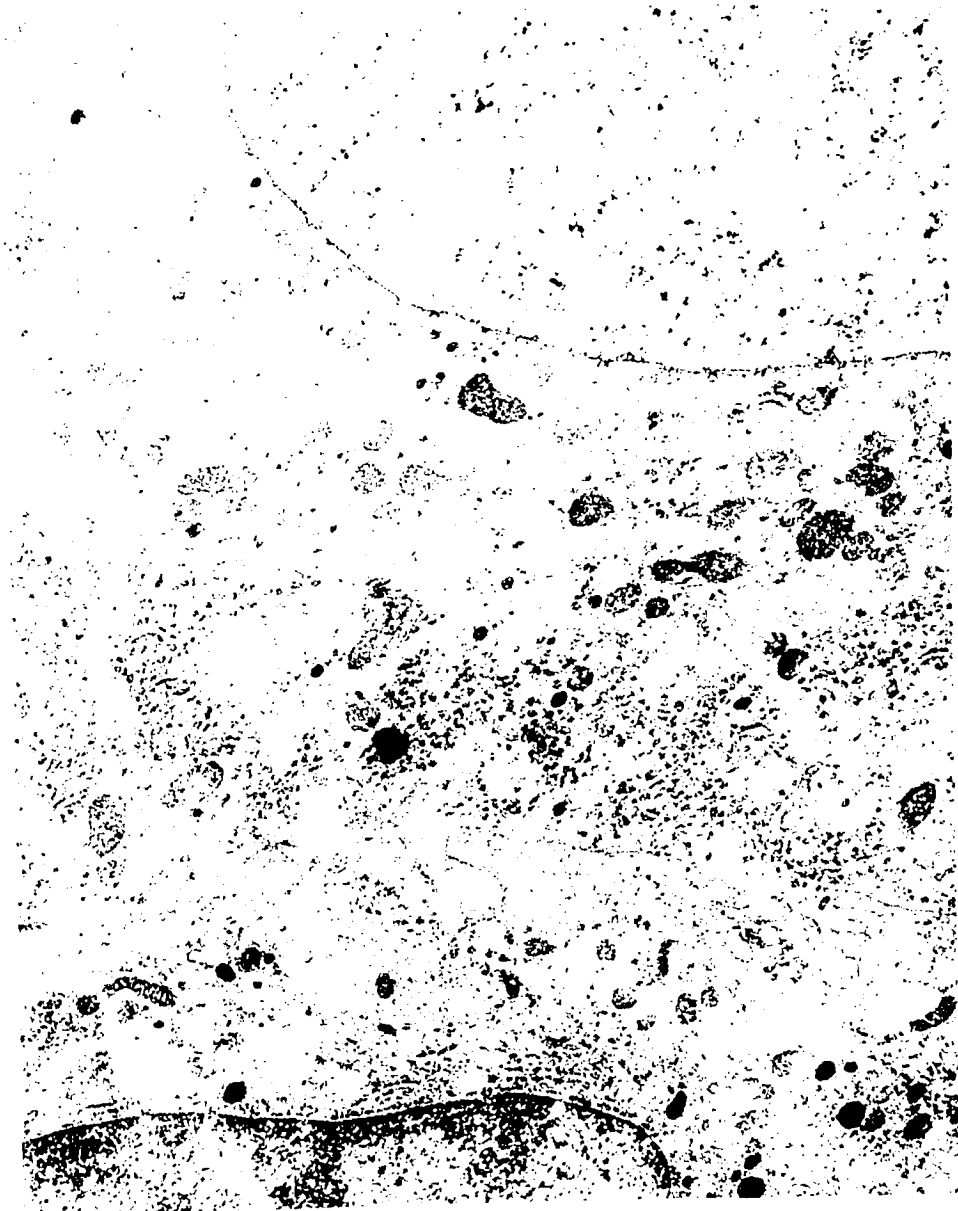
surrounded by, and apposed to, a thin layer of satellite cell cytoplasm (Figs. 52, 53, 56).

Fibroblasts were typical of those found in connective tissue elsewhere. The rough surface endoplasmic reticulum was very prominent and appeared as parallel tubular profiles. Ribosomes in moderate numbers were scattered between the profiles of endoplasmic reticulum and there were electron dense granules elsewhere in the cytoplasm. No ultra-structural differences were noted between the fibroblasts of control and experimental ganglia (Figs. 55, 56).

2. Fine structure of the nucleoli of neurons

The fine structure of normal nucleoli was as follows. Two macromolecular components, fibrillar and particulate, could be recognized in all nucleoli studied. Their spatial relationships were in the form of a cortical, a compact or a loose type. In the cortical type, a large centrally located fibrillar component was surrounded by a mosaic of fibrillar and particulate components, with the particulate component predominating (Fig. 57). In both compact and loose types, the macromolecular components interdigitated with one another in a compact (Figs. 58-60) or loose arrangement (Fig. 61), in such a manner that a clear demarcation of two components was difficult.

Nucleolar 'vacuoles', more or less spherical in shape, were present in all nucleoli examined. There were two types of vacuoles. The typical ones, present in all nucleoli studied, contained granules about 140 Å in diameter and a few fibrillar structures about 80 Å in thickness. The electron density and vacuolar inclusions resembled loose chromatin of euchromatin nature. Another type of vacuole, more or less uniform in size and containing a central dense granule, was found evenly

Figure 54. Infolding or indentation of the nuclear membrane of

a reacting neuron. There is an accumulation of

granular endoplasmic reticulum in the indented

area (X23,000).

Figure 54
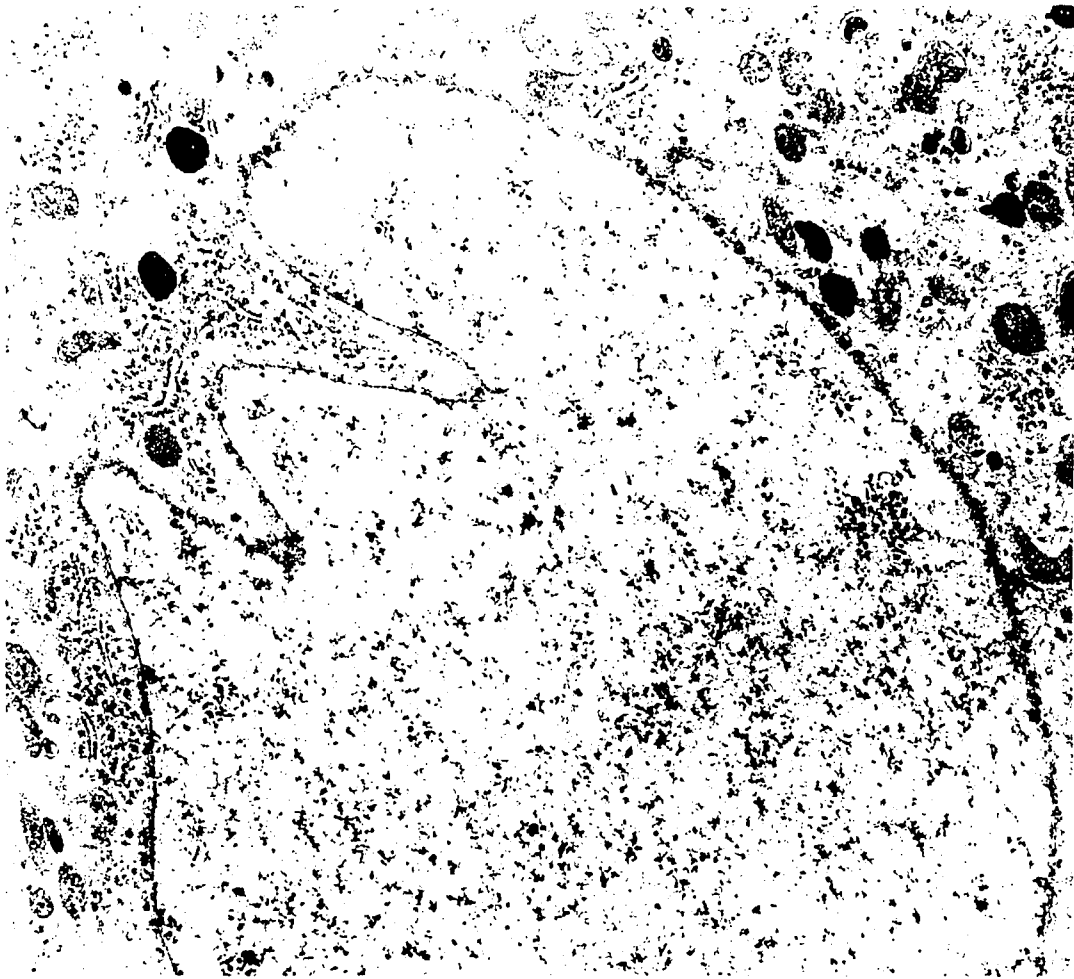
Figure 31

Figure 55. Normal neuron with an adjacent fibroblast. The

neuron is closely associated with a fibroblast

which displays parallel arrays of elongated cisternae

of rough surface endoplasmic reticulum. FN-nucleus

of fibroblast (X15,040).
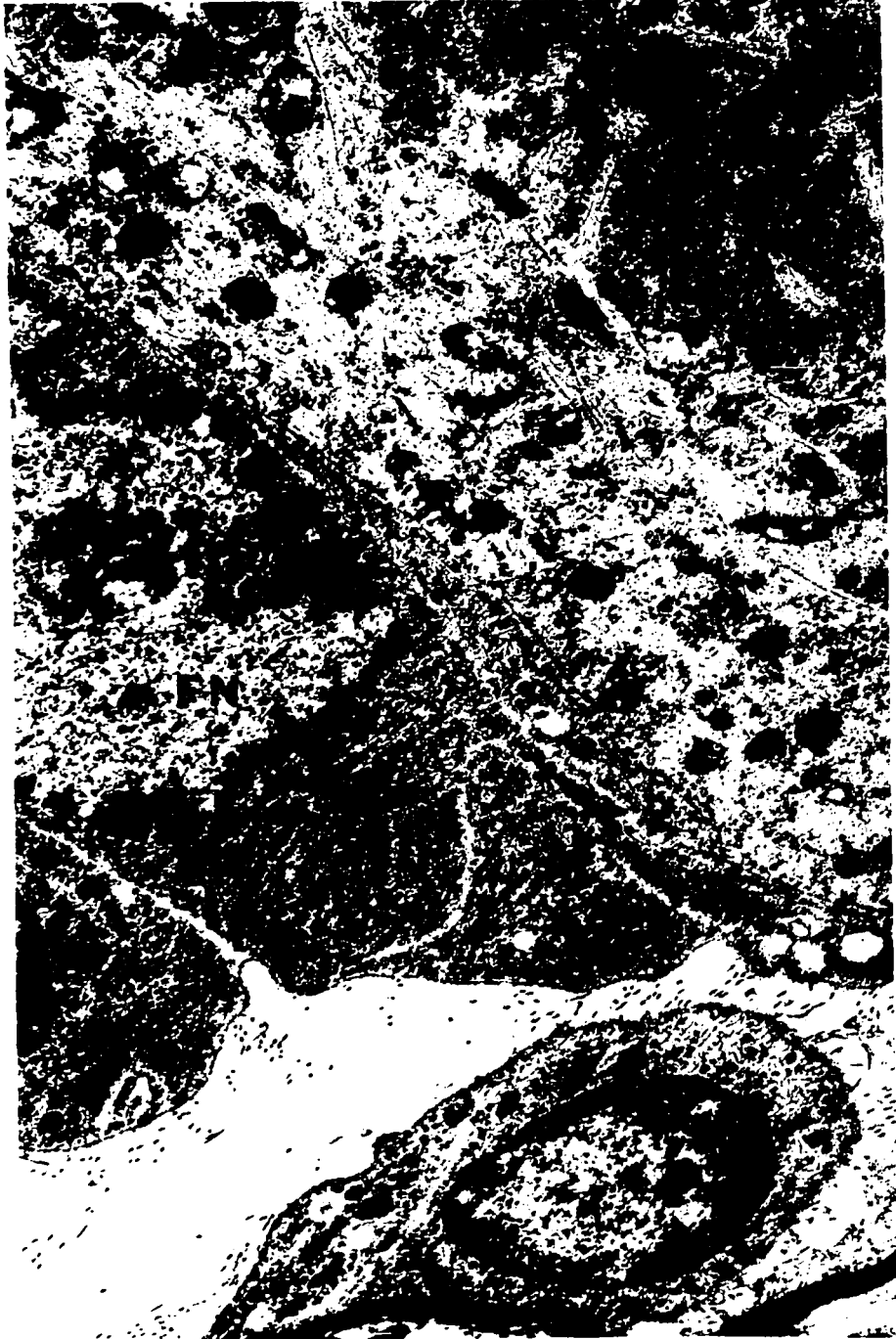
Figure 55
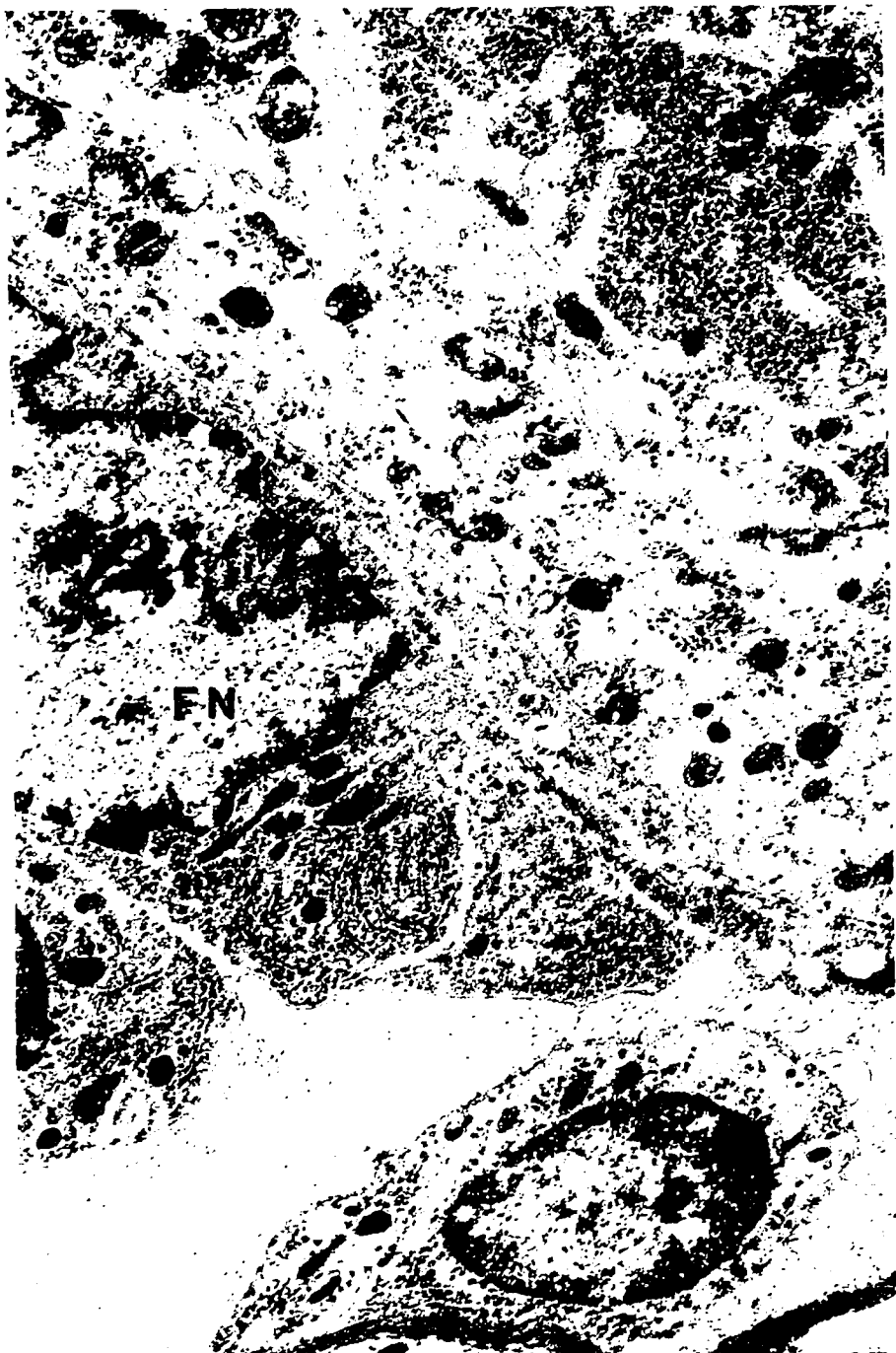
Figure 56. Reacting neuron, satellite cell and fibroblast.
The cytoplasm of the fibroblast displays parallel
elongated cisternal profiles of rough surface
endoplasmic reticulum (X16,240).
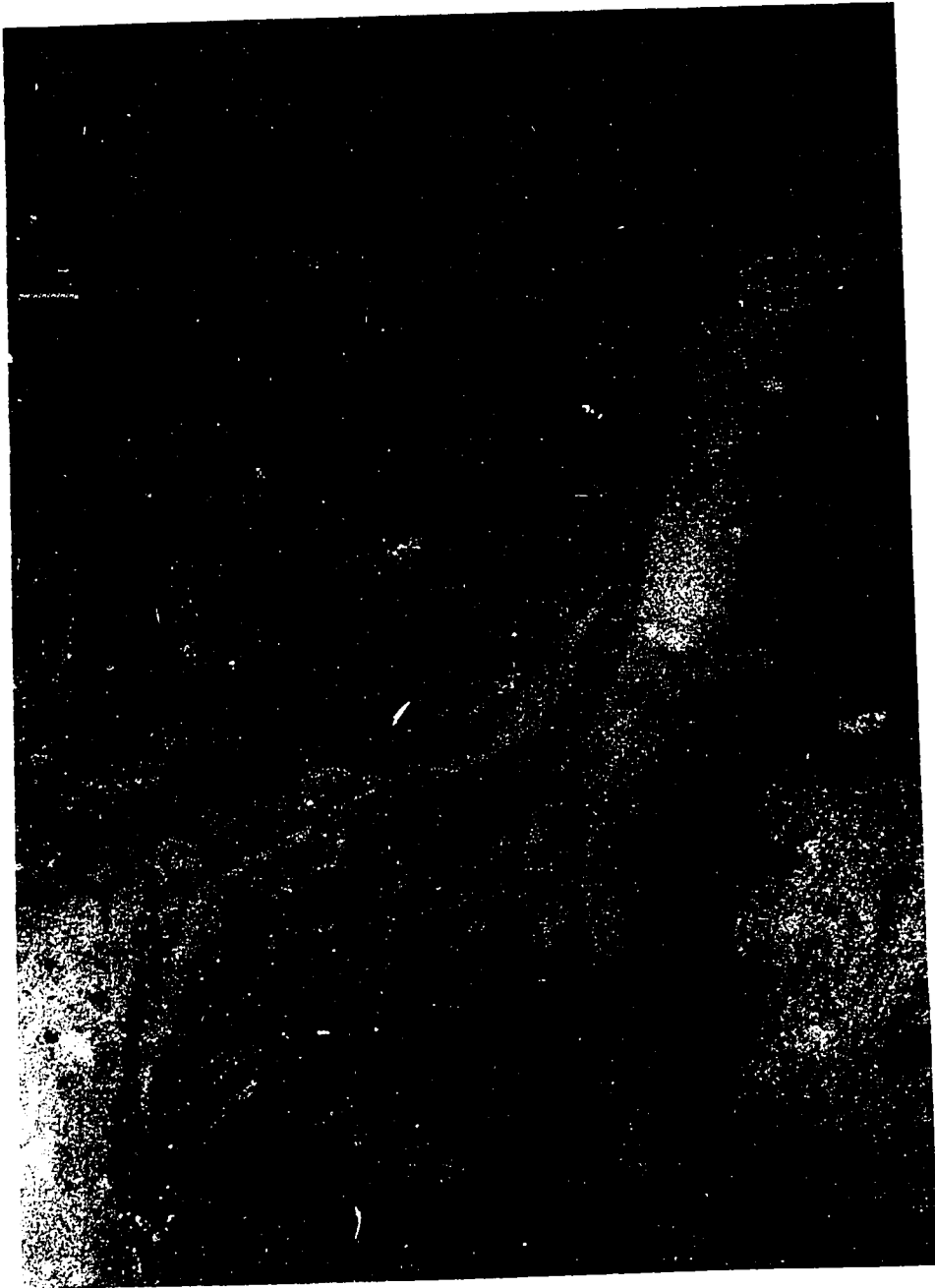
Figure 56

distributed throughout the compact nucleolus. The electron dense granule
that gave the vacuole a 'halo-like' structure was about 550 Å in
diameter (Figs. 58-60). In nucleoli of the cortical type, no vacuole-
like structures were present in the central fibrillar components.
However, in the cortical regions, vacuoles were surrounded by nucleolonema
which were predominantly particulate or fibrillar in nature (Fig. 57).
Occasionally, vacuoles located close to the periphery of the nucleolus
were continuous with the nucleoplasm (Fig. 61).

Rather dense aggregates of chromatin material, assumed to be
heterochromatin, were present in the nucleoplasm, attached to the
nuclear membrane or to the nucleolus. In addition, extranucleolar
bodies were present, which had either a fibrillar or a particulate
internal structure (Figs. 58, 59, 61). The extranucleolar bodies
were located adjacent to the compact or loose types of nucleoli or
free in the nucleoplasm. The fibrillar extranucleolar body adjacent
to the nucleolus (Fig. 60), resembled dense chromatin in possessing
granules in the size range of 250Å - 500Å in diameter. However, it
also resembled the fibrillar component of the nucleolus in having a
dense matrix. The particulate extranucleolar body was composed
predominantly of granules 550 Å in diameter. Similar granules were
responsible for the halo-like vacuolar appearance in the compact type
nucleolus. The granular or particulate extranucleolar body resembled
the particulate component of the nucleolus in having a dense matrix
(Figs. 58, 59, 61). When the granular extranucleolar body was adjacent
to the nucleolus, chromatin fibrils 80 Å in thickness tended to join
them together (Fig. 58). In general, chromatin filaments were in
close contact with the periphery of nucleoli and extranucleolar bodies.

Figure 57. A cortical type of nucleolus adjacent to the nuclear

membrane in a normal neuron. The nucleolus has two

structural components, one fibrillar (F) in the

central part of the nucleolus mainly, and the other

particulate (P), intermingling with the fibrillar

components at their junction. Vacuoles (V) are

confined to the cortex of the nucleolus. They

contain granules about 140 Å in diameter and filaments

about 80 Å in width, and their electron density

resembles that of loose chromatin or euchromatin

(X 45,600).
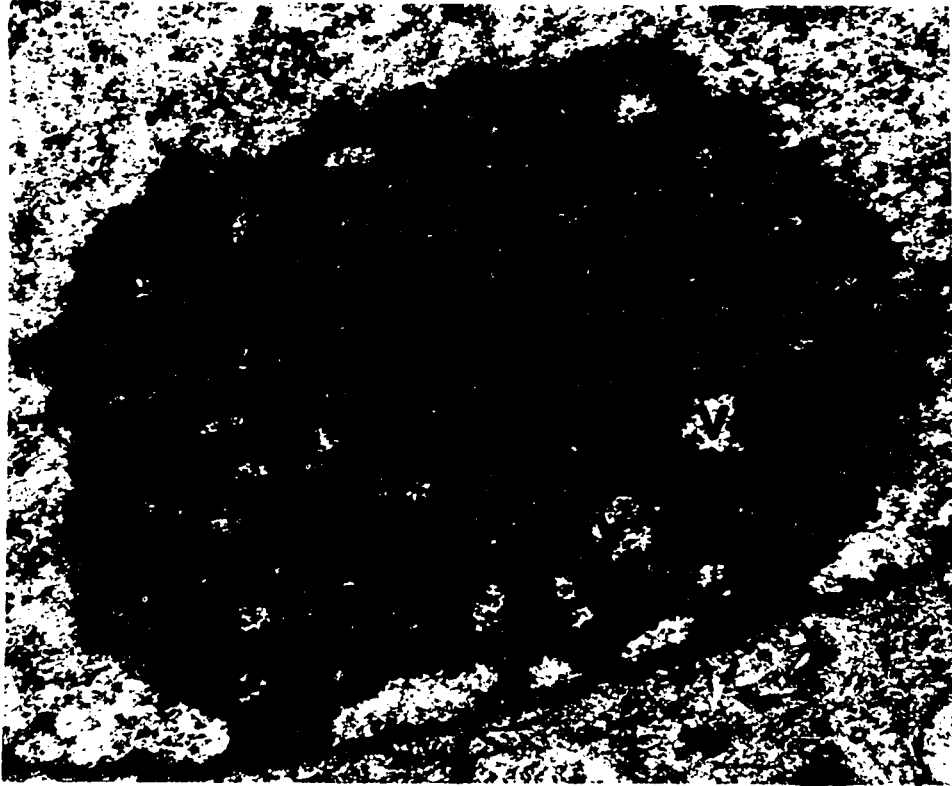
Figure 57

Figure 58. Compact type nucleolus with an adjacent extranucleolar

body in a normal neuron. There are 'halo-like'

vacuoles of regular size. The extranucleolar body

is joined to the nucleolus by 'strips' of chromatin

(arrow), assumed to be nucleolar-associated chromatin.

The size of the granules of the extranucleolar body

is like that of the single dense granules which are

centrally located in the nucleolar vacuoles (X36,450).
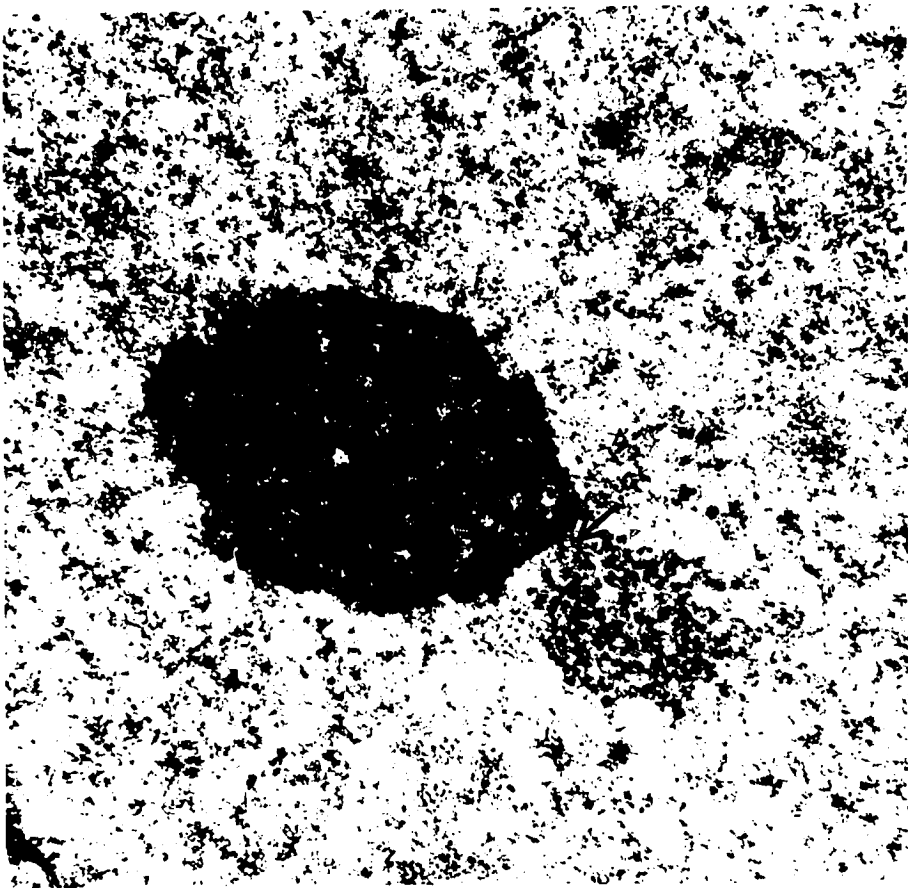
Figure 58
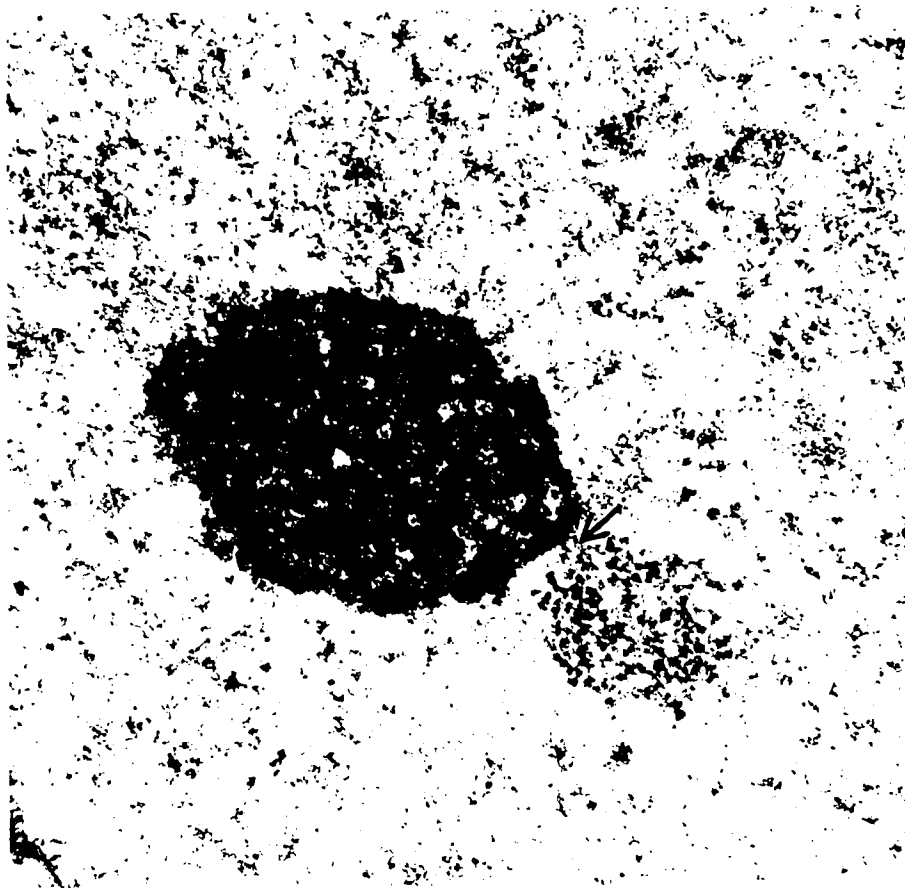
Figure 58

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VITA

NAME: Shiu Hong KUNG

BORN: Haiphong, French Indochina, 1939.

EDUCATION:

    Primary   Canton, China, 1947-1952.

    Secondary   Hongkong, 1952-1958.

    University   Chinese University of Hongkong, 1958-1962.

                   McGill University, 1962-1964.

                   University of Western Ontario, 1964-present.

    Courses and   B.Sc., Biology, Hongkong, 1962.
      Degrees
                   M.Sc., Anatomy, McGill, 1964.

PUBLICATIONS: Synthesis and migration of pancreatic enzymes as

                   shown by radioautography using tritiated amino

                   acid. M.Sc. thesis, McGill University, 1964.

                Synthetic activities associated with keratohyalin

                   granules. Anat. Rec., 154: 469, 1966.

               Quantitative cytology of chromatolytic primary

                   sensory neurons. Anat. Rec., 157: 369, 1967.

              La Incorporacion de tirosina-H^3 al Epitelio Escamaso

                   Estratificado de la Rata.

                   Dermatologia Ibera Latina-Americano, in press.

According to classical cytogenetic theory, the formation of a nucleolus is determined by a genetically active organizer region which is a specific chromosomal locus (Hertz, 1931; McClintock, 1934; Stewart and Bramford, 1942; Brown, 1949; Lin, 1955; Longwell and Svihla, 1960; Mirsky and Ssawa, 1961; Sirlin, Tandler and Jacob, 1963). Clear-cut evidence of genetic control of nucleolar formation was afforded by the discovery of the Oxford mutant of <u>Xenopus Laevis</u> (Elsdale, Fischberg and Smith, 1958).* Wild-type diploid cells of this organism have the potentiality of containing two nucleoli, while individuals heterozygous for the Oxford mutant contain only one nucleolus per cell. Cells that are homozygous for the mutant have no organized nucleolus and this is a lethal genetic error. Karyotype studies demonstrated secondary constrictions on a certain pair of chromosomes in the wild-type organism and the presence of the mutant factor was correlated with the absence of this secondary constriction (Kahn, 1962). As noted above, cells in wild-type individuals may contain only one nucleolus. It is not known whether the single nucleolus is formed by one nucleolar organizer or by both nucleolar organizers. Physical association of one nucleolus with organizer regions of several chromosomes was observed in human cells by Ohno <u>et al</u>. (1961) and Ferguson-Smith and Handmaker (1963), yet the authors suggested that there can be one active and one inactive organizer region on a pair of chromosomes. In the rat, chromosomes 3 and 13 have been shown to contain satellites, which are usually associated with nucleolar organizer regions (Hungerford and Nowell, 1963). In the present experiment, the number of nucleoli or combination of nucleoli and extranucleolar bodies was four or less, an observation which may have a bearing on the number of satellites on chromosomes of the rat.

* It has been claimed by Schultz, Caspersson and Aquilonius (Proc. N.A.S. 26: 515-523, 1940) that clear-cut evidence of genetic control of nucleolar formation has been presented.

Peterson (1966) found an inverse relationship between the size of ventral horn neurons in the rat and the rate of uptake of phenylalanine-H^3 as measured over 100 μ^2 of cytoplasm. Since Henneman, Somjan and Carpenter (1965) have shown that there is an inverse relation between the size of motor neurons and their excitability, Peterson (1966) suggested that his results could best be explained by the need of a higher rate of protein synthesis in more excitable and more active neurons.

Differences in the level of activity of certain enzymes in large and small sensory neurons have been recorded. For example, there is an inverse correlation between the size of these cells in the rat and rabbit and levels of acid phosphatase and acetylchoinesterase activity. (Colmant, 1959; Tewari and Bourne, 1962; Galabov, Penev and Manolov, 1964; Kokko, 1965). The significance of these observations is still obscure, although possible explanations were suggested by Kokko (1965).

In the present study, it was found that the level of radioactivity in the cytoplasm of small neurons exceeded that of large neurons. With the uptake of radioactive precursors in the cytoplasm of normal and reacting large neurons as the baseline, the increase of cytoplasmic radioactivity in normal small neurons and reacting small neurons was 70% and 41% higher, respectively, following injection of cytidine-H^3, and 21% and 24% following arginine-H^3. High acid phosphatase activity was demonstrated in endocrine secretory cells, and in neurosecretory cells (Eranko, 1951, Novikoff, 1961; Osinchak, 1964), suggesting that the enzyme is probably related to active protein synthesis. In relation to our results, it is of interest that the smaller neurons of dorsal root ganglia have a higher level of acid phosphatase activity than the larger neurons (Colmant, 1959; Tewari and Bourne, 1962; Galalov, Penev

and Manolov, 1964; Kokko, 1965).

In neurons undergoing axon reaction, the difference in synthetic activity of small and large neurons is not as great as it is in their normal counterparts. This may be caused by a greater variation in the synthetic activities of individual neurons in a population of reacting cells. There is considerable variation, microscopically, in the intensity of chromatolytic phenomena among individual neurons undergoing axon reaction in a given cell population. Another possibility is that neurons of different sizes may respond differently with respect to changes in synthetic activities during axon reaction. This view is not supported by the data obtained in the protein experiments, in which the uptake of arginine-H^3 in small normal and experimental neurons was 21% and 24% higher, respectively, than it was in large neurons. However, Colmant (1959) found that division of the rat's sciatic nerve resulted in an increase of acid phosphatase activity in the large cells, while the reverse was true for the small cells.

D. Synthesis and Metabolism of Proteins in the Neuronal Cytoplasm

After injection of a labelled amino acid, the newly-synthesized proteins are labelled and may be detected in histological sections by the autoradiograph technic. The first site to show radioactivity should be the site at which the proteins are synthesized by the cellular synthetic "machinery". The radioactivity which appears later at other sites may indicate the location of subsequent metabolic activities, either fast turnover of proteins to be exported or slow turnover of proteins that are metabolized in situ. In practice, however, it is often difficult to decide whether the level of radioactivity in a given region is related to protein synthesis or metabolism.

With electron microscopy and autoradiography combined, Droz (1965) found that 15 min after injection of tritiated leucine, radioactivity in the cytoplasm of the rat's semilunar ganglion was confined to the endoplasmic reticulum or Nissl substance, where the ribosomes are sites of protein synthesis. By 30 min after injection of the same precursor, radioactivity was detected in both endoplasmic reticulum and the vesicles and flattened sacs of the Golgi zone. After 24 hr, the proximal regions of the axons contained silver grains. Droz concluded that proteins were synthesized in the endoplasmic reticulum or Nissl substance, migrated to the Golgi zone and then entered the axon. Thus, the Golgi zone, axon hillock and axons are sites in which proteins synthesized in the Nissl substance are metabolized.

Studies of non-nervous tissues, again using electron microscopy and autoradiography in combination, indicated that at early time intervals, such as 10 or 15 min after injection of labelled amino acids, radioactivity appears over the endoplasmic reticulum only (Revel and Hay, 1963; Caro and Palade, 1964; Ross and Benditt, 1965; Nadler et al., 1965).

In the present experiments, the level of radioactivity in the cytoplasm of reacting cells proved to be lower than in normal cells, 5-10 min after injection of arginine-H^3. Results in the opposite direction were reported when the animals survived for longer periods after the administration of radioactive precursors (Brattgård, Hydén and Sjöstrand, 1958; Fischer, Lodin and Kolousĕk, 1958; Scott, Gutmann and Horsky, 1966). For example, 6 days after nerve section and 3 hr after administration of leucine-H^3, Scott, Gutmann and Horsky (1966) found a higher level of radioactivity in the cytoplasm of reacting cells compared with normal cells. As pointed out above, the radioactivity detected 3 hr after

injection of a precursor is believed to represent protein in a metabolic phase. Our experiments on the fate of newly-synthesized proteins showed that, at 2 and 4 hr time intervals, the level of radioactivity was lower in the cytoplasm of reacting neurons than it was in normal cells. The discrepancy may result from the difference in 'pool size' of the amino acids leucine and arginine in the cytoplasm of neurons, i.e. faster turnover proteins may contain more leucine than arginine and slower turnover proteins the reverse. Further, after crushing the peripheral processes of the spinal ganglion cells of the rabbit and adding radioactive amino acids in vitro for autoradiographic studies, Miani, Rizzoli and Buccinante (1961) found an increased incorporation of amino acids into the cytoplasm of experimental neurons at various stages of reaction. It is difficult to compare the present findings with the foregoing because of differences in laboratory animals and technics.

When the axon of a neuron is injured, the nerve cell endeavours to repair the injury by regenerating the axon. However, the cellular response to injury may not include hyperfunction of the neuronal synthetic mechanism at all stages of neuronal response, as suggested by earlier investigators (Hydén, 1943; Brattgård, Hydén and Sjöstrand, 1958; Fischer, Lodin and Kolousěk, 1958; Takano, 1964; Mackey, Spiro and Weiner, 1964), although such a possibility cannot be ruled out. Whatever the nature of the repair mechanism may be, the existence of a special regenerative component requires evidence that the synthetic "machinery" of the neuron has manufactured the materials necessary for repair in addition to what the neuron needs for simple maintenance of its altered integrity. In other words, proteins with the characteristics of structural proteins have to be synthesized. Our data indicate that there is a higher level of radioactivity in the cytoplasm of reacting cells

16 and 96 hr after injection of a protein precursor, compared with the normal cells, while the reverse is true 5-10 min after injection of the precursor. Therefore, proteins synthesized in the cytoplasm of neurons undergoing axon reaction have a slower turnover rate than those of normal neurons. This conclusion is in line with the ultrastructural evidence that the dispersion of the rough surface endoplasmic reticulum or Nissl aggregates in reacting cells is analogous to the ultrastructure of differentiating and neoplastic cells, which are presumably synthesizing structural or cell-bound proteins, rather than secretory proteins (c.f. Mackey, Spiro and Wiener, 1964). However, reduced axoplasmic flow following nerve section may be a minor factor contributing to the retention of newly-synthesized proteins in the cell body.

E. Synthesis and Metabolism of RNA and Proteins in Neuronal Nuclei.

It is well established that RNA is synthesized in nuclei (nucleoli and nucleoplasm) and then migrates into the cytoplasm (c.f. Goldstein and Plaut, 1955; Woods and Taylor, 1959; Prescott, 1959; Perry, 1960; Taylor, 1960; Leblond and Amano, 1962; Shimada and Nakamura, 1966) where different types of RNA are metabolized (Penman, 1967). As expected, 20 min after injection of cytidine-H^3 radioactivity was localized predominantly over the nucleolus and nucleoplasm in both normal and experimental neurons. The level of radioactivity in the nucleolus and nucleoplasm of reacting cells was less than that of normal cells, indicating that there is a depression* of RNA synthetic activity in situ in axon reaction. This interpretation does not

* As noted in the previous section, the nature of proteins synthesized in the cytoplasm of reacting cells changed from normal into a slower turnover type resembling structural proteins. The slower turnover proteins can involve types of proteins not synthesized before, or an increased synthesis of existing slow turnover proteins when the demand is high. At the same time, the synthesis of fast turnover proteins such as those for axoplasmic flow must decrease. An overall amount of RNA necessary for cytoplasmic synthesis of these two categories of proteins, namely fast and slow turnover proteins, happens to be less in reacting cells than the normal neuron needs. Such a phenomenon of reduced nuclear RNA synthesis is referred to as 'depression' in this thesis.

agree with the view proposed by Hydén (1943) and shared by others, that axon reaction is consistently a state of hyperfunction of synthetic mechanisms involving RNA and proteins. Previous demonstrations, with the autoradiographic technic, of RNA synthetic mechanisms in neurons undergoing axon reaction were done on motor neurons (Brattgård, Hydén and Sjöstrand, 1958; Fischer, Lodin and Kolousĕk, 1958). Sensory neurons and motor neurons may respond differently, in several respects, to trauma to a cell process.

The presence of proteins in cell nuclei was recognized by Miescher (1897), who found a protein-containing nuclear fraction which he called "nuclein" in cells of pus. Histone, a basic protein, was first extracted with dilute HCl from blood cells of the goose (Kossel, 1884). Stedman and Stedman (1944) found that, besides acid-extractable proteins, nuclei contain another protein fraction which is soluble in dilute NaOH. They called this alkaline-extractable protein "chromosomin". In view of their location, basic and acidic nuclear proteins are thought to have something to do with the regulation of genetic transcription. Indeed, histone has been shown to depress DNA-template RNA synthesis (Bonner and Haung, 1962; Allfrey, Littau and Mirsky, 1963), and the synthesis and metabolism of non-histone nuclear proteins change during tissue differentiation or in response to hormones (Ürspring, 1964; Sporn and Dingman, 1966).

It has been suggested that axon reaction involves a change in gene action in the nerve cell nucleus (Brattgård, Edström and Hydén, 1958). If this is so, quantitative analysis of the synthesis and metabolism of nuclear proteins may be useful in providing a better understanding of basic aspects of axon reaction. The present autoradio-

graphic analysis showed that: (1) in nucleoli and nucleoplasm of reacting

cells, the level of radioactivity due to newly-synthesized protein is

not as high as it is in normal neurons and, (2) nuclear proteins (in

nucleoli and nucleoplasm) synthesized in neurons undergoing axon reaction

have a slower turnover, compared with normal cells. Therefore, during

axon reaction, either new types of nuclear proteins with slower turnover,

or an increase in synthesis of existing slower turnover nuclear proteins,

has occurred.

The changes in quantity and turnover of nucleoplasmic proteins

during axon reaction is in line with the thought that nuclear proteins

have something to do with genetic transcription (Bonner and Haung, 1962;

Allfrey, Littau and Mirsky, 1963; Ürsprung, 1964; Sporn and Dingman,

1964). In vivo as well as in vitro studies present no evidence that

histone is synthesized in nucleoli in quantities large enough to be

demonstrated by present biochemical technics (Ro, Muramatsu and Busch,

1964). However, it could be shown that acidic nuclear proteins are

synthesized actively (Birnstiel and Hyde, 1963; Birnstiel, Chipchase

and Flamm, 1964). Biochemical evidence indicates that histone and

acidic proteins control the 'readout' ability of nucleolar DNA (Liau,

Hnilica and Hurlbert, 1965), a finding in line with the present auto-

radiographic studies on the significance of changes in quantity and

turnover of nucleolar proteins and nucleolar RNA synthetic activity

during axon reaction. The present demonstration of a lower level of

nuclear protein and RNA synthesis, and a change in the turnover rate

of nuclear protein as a result of axon reaction, leads to the

conclusion that the synthesis and metabolism of nuclear proteins in

the neuron is related to its ability to synthesize RNA. On the other

hand, the level of nuclear protein is maintained by some species of RNA metabolized _in situ_.

Let us compare this conclusion with biochemical observations. The anti-tumor agents such as uracil mustard, Myleran, 6-mercaptopurine, Leukeran, 5-fluorouracil and NH_2 have been shown to affect nuclear protein synthesis. The synthesis of the acidic proteins, in particular, is affected early by these anti-tumor agents. Later, there is a suppression of biosynthesis of cytoplasmic proteins (Busch, Amer, Nyhan, 1959; Busch _et al._, 1961). Barton, Cerny and Tracy (1965) reported that increased nuclear volume induced by thioacetamide administration is accompanied by an increase in the acidic nuclear proteins. Sporn and Dingman (1967) showed that total acidic proteins of chromatin showed significant variations from normal when animals were fed with two carcinogens (N-hydroxy-2-acetylaminofluorene and 3'-methyl-4-dimethyl-aminoazobenzene). They also found an increase of RNA and total acidic protein content of liver chromatin in response to pituitary hormone stimulation. Malec _et al._ (1961) found an increase in labelling with glycine and orotic acid in nuclear proteins and RNA of leucocytes engaged in phagocytosis.

Thus various factors may cause an elevation or depression of nuclear protein synthesis, and cytoplasmic synthetic activities correspondingly. This suggests that in neurons undergoing axon reaction, lower nuclear protein synthesis with corresponding lower nuclear RNA and cytoplasmic protein synthetic activity may be a phenomenon of some generality and not an isolated example.

F. Ultrastructure of the Neuronal Nucleoli.

The particulate and fibrillar components of nucleoli are believed to be of universal occurrence, although some nucleoli may

possess regions predominantly fibrillar and others predominantly particulate. In dorsal root ganglion cells of the rat, the ultrastructure of nucleoli differs from cell to cell in spatial relationship of the particulate and fibrillar components. The cortical type nucleoli have a fibrillar medulla. The cortex of the cortical type nucleoli, and the compact and loose types of nucleoli, are composed of a mosaic of fibrillar and particulate components. The former resembled nucleoli in plant tissues, the fibrillar component of which is generally surrounded by the particulate component, both occupying distinct areas (Lafontaine and Chouinard, 1963; Chouinard, 1966; Hyde, 1967). However, such a spatial arrangement is not often seen in animal cells (David, 1964; Jézéquel and Berhard, 1964; Stevens, 1964; Karasaki, 1965; Brinkley, 1965; de Man and Noorduyn, 1967). That the ultrastructural organization of nucleoli is to some extent species-specific was demonstrated by Miller (1962), who found that peripheral oocyte nucleoli of Rana clamitans and Triturus viridescens have particulate or fibrillar cortical components, respectively. In brief, the ultrastructure of nucleoli of fully differentiated tissues resembled each other, while the nucleoli of tissues at various stages of differentiation differed to some extent (Karasaki, 1965; Hyde, 1966). It was of interest to see that a highly differentiated tissue such as the spinal neurons had different types of nucleoli.

As will be recalled from the light microscopy studies, the number of nucleoli or combination of nucleoli and 'extranucleolar bodies' per neuron was four or less. This result can best be related to the number of satellite chromosomes of the rat (Hungerford and Nowell, 1963). With the electron microscope, we found two types of 'extranucleolar bodies'. They are fibrillar and particulate types, the ultrastructural characteristics of which were

similar in some aspects, but not identical to, the fibrillar and particulate
components of the nucleoli. An extranucleolar organelle of fibrillar
nature, which the author called a 'karyosome', was described in root
tip cells of Plantago ovato (Hyde, 1966). It was suggested that
'karyosomes' which contain more DNA than the fibrillar region of the
nucleolus are a form of under-developed nucleolus. They may be products
of genes other than, but functionally related to, those from nucleolar
organizer regions. After further studies in the same material in serial
sections, Hyde (1967) concluded that 'karyosomes' are attached to the
fibrillar region of the nucleolus, often sharing a common opening
through the particulate periphery, with an entering extension of
heterochromatin. We did not study dorsal root neurons in serial sections.
However, our electron micrographs showed granular and fibrillar extra-
nucleolar bodies structurally associated with the compact type nucleoli.
The fibrillar extranucleolar body looks like a 'bud' of the nucleolus
proper. The granular extranucleolar body was joined by 'strips' of
material to the nucleolus. There are no cytochemical studies to
indicate the nature of such 'strips' of material. But the periphery
of a nucleolus is usually associated with nucleolar-associated chromatin.
Furthermore, the size of the granules of the granular extranucleolar
body is like that of the single dense granules, which are centrally
located in the nucleolar vacuoles to give a 'halo-like' appearance.
Aggregates of granules resembling the granular extranucleolar bodies
have been described previously in dorsal root ganglion cells of the
rat, after treatment with osmium tetroxide (Hisaoka and Breemen, 1964).
Before the cytochemical nature of the fibrillar and granular extra-
nucleolar bodies are clarified, any attempt to explain their functional

significance can only be speculative. However, their close topographical relationship with, and the ultrastructural similarities to, the nucleolus suggest that the extranucleolar bodies and the nucleolus are functionally interrelated.

As a result of a three-dimensional reconstruction of nucleoli based on serial sections, Hyde (1967) found that nucleolar vacuoles are continuous with the nuclear chromatin. Presumably because of the loose architecture, the loose type of nucleoli have peripheral vacuoles continuous with the nucleoplasm.

Since a nucleolus is made up of particulate and fibrillar components and vacuoles, one naturally asks when a nucleolus increases in size during axon reaction (c.f. Brattgård, Edström and Hydén, 1958) or is reduced in size after treatment of differentiating amphibian cells with actinomycin D (Eakin, 1964), which of the nucleolar components is responsible for changes in size? The question cannot at present be satisfactorily answered. In studying chromatolytic motor neurons with basic dyes, Barr and Bertram (1951) and Crouch and Barr (1954) found that the enlarged nucleoli showed spherical, pale-staining areas more conspicuously than nucleoli of control cells. Lafontaine and Chouinard (1963) found that the pale-staining areas within the nucleoli of Vicia faba are equivalent in electron density to the nucleoplasm. The particulate and fibrillar components identified by electron microscopy cannot be distinguished in material stained with basic dyes. In the present study, the enlarged nucleoli of reacting cells showed a decrease in RNA and protein synthetic activity and had a normal ultrastructure. These apparently contradictory observations indicate that the synthetic role of the nucleolus, viewed as a functional unit, is a very complicated matter indeed. Current research suggests that the synthetic functions of nucleoli may be determined by the balance of

a number of factors, including nucleolar organizer regions and other DNA-containing materials.

Lafontaine and Chouinard (1963) suggested that the growth of the nucleolus during telphase of the mitotic cycle results mainly from incorporation of material that accumulates in the interchromosomal space from early to late telophase. However, in the studies of the ultra-structure of embryogenesis of _Triurus pyrrhogaster_ (Karasaki, 1965) and the mitotic cycle of _Allium cepa_ (Chouinard, 1966), it was found that the nucleoli enlarged during successive developmental stages by the acquisition of a component made up of ribosome-like 150 Å granules, which form a layer around the growing nucleoli. Subsequent growth is brought about, at least in part, by the addition to the nucleolar mass of a component characterized mainly by the presence of 150 Å granules resembling cytoplasmic ribosomes. Electron microscopy and autoradio-graphy, used in combination, indicated that after the administration of a RNA precursor, radioactivity first appeared in the nucleolar-associated chromatin and in the fibrillar region of nucleoli, and later in the particulate region (Granboulan and Granboulan, 1965; Karasaki, 1965; La Cour and Crawley, 1965). It was thought, therefore, that the partic-ulate component may be a chromosomal product, the biogenesis of which is related to nucleolar-associated chromatin and/or the fibrillar component of the nucleolus.

An unusual opportunity was provided by the so-called anucleolate mutant in _Xenopus laevis_ to investigate the fine structure and function of the component parts of the nucleolus (Elsdale, Fischberg and Smith, 1958). The homozygous mutant does not develop true nucleoli at gastrulation and the heterozygote acquires only one nucleolus per

nucleus, instead of the two of the wild-type animal. Fischberg and

Wallace (1960) showed that progeny of heterozygotes segregate in proportions

expected by a single Mendelian factor, indicating that the development

of nucleoli is under genetic control. When the nucleoli of normal and

mutant Xenopus embryos were examined with the electron microscope, the

Xenopus mutant was found to contain nucleolus-like spherical bodies,

structurally and chemically similar to the fibrillar region of the

nucleoli (Wallace, 1962; Jones, 1965; Hay and Gurdon, 1967). Use of the

DNA-RNA hybridization technic showed that anucleolate nuclei lost

ribosomal RNA cistrons through mutation (Wallace and Birnstiel, 1966).

It is therefore apparent that fibrillar nucleolus-like spherical bodies

are extra-organizer in origin. Similar pseudo-nucleolar spherical

bodies have been described in a Chironomus hybrid (Beermann, 1960),

maize pollen tetrads (McClintock, 1934), maize translocation microspores

(Swift and Stevens, 1966) and micronuclei lacking nucleolar organizers

(Heitz, 1931; Das, 1962). It was assumed that in normal nucleoli, the

two RNA-containing zones (particulate and fibrillar components) interact

with each other to determine the rate of synthetic activities. The

concept of involvement of extra-organizer genes or the so-called 'latent

organizers' (Gates, 1942; Beermann, 1960; Wallace, 1962, Sirlin, 1962)

was seen as 'the capacity of all chromosomes to produce the fibrillar

RNA associated with the dense amorphous substance' (Tandler, 1966).

The nucleolus is therefore envisaged as a mosaic product which interacts

with the ribosomal cistrons of the organizer locus. Furthermore, any

changes in morphology and synthetic function should be determined by

a balance of nucleolar organizers and other loci.

In cytometric and autoradiographic studies, it was shown that

the number of nucleoli has no bearing on the rate of recovery from axon reaction or on cytoplasmic radioactivity due to RNA and proteins. In other words, there is a delicate balance between the nucleolar organizer and other loci to provide an appropriate synthetic function, even though, morphologically, the number of nucleoli is different. These morphological differences might then be interpretated as a shift in the 'equilibrium' of various physiological factors. In the present electron microscopy studies, we found various types of nucleoli and extranucleolar bodies. The varied spatial arrangement of macromolecular components is interpretated as a morphological reflection of a complex balance of the synthetic 'machinery' of the nucleolus.

VII. SUMMARY

Certain aspects of the basic mechanisms involved in the response of nerve cells, as it occurs in spinal ganglion neurons of Sprague-Dawley rats after cutting the sciatic nerve, were investigated. The following observations are considered to be of significance in understanding the basic nature of axon reaction.

1. At sites of RNA synthesis (nucleoplasm and nucleoli), radioactivity in reacting cells following injection of tritiated cytidine was lower than at similar sites of normal cells. The same relationship was true for the site of RNA metabolism (cytoplasm).

2. After injection of tritiated arginine, the level of radio-activity due to newly-synthesized proteins in cytoplasm and nuclei was lower in the experimental neurons than in normal cells. In addition, the turnover rate of proteins was slower in the cytoplasm and nuclei of reacting neurons, compared with control cells.

The foregoing observations may be of considerable importance in relation to the basic nature of axon reaction, at least as it occurs in primary sensory neurons of the rat. It appears that under the conditions of the experiments, synthesis of RNA and protein is depressed following section of the peripheral nerve process. This is not in accord with the view expressed by Hydén (1943), namely, that axon reaction involves, in general, a state of hyperfunction of nucleoprotein metabolism. In addition, the results are strongly suggestive of a qualitative change in the proteins of reacting neurons, leading to

longer retention of radioactivity in the cell.

The following observations, although perhaps of lesser significance, are original findings which are believed to be of interest with respect to the phenomenon of axon reaction.

1. Single nucleoli of normal neurons had a larger volume than two nucleoli together, in neurons having two nucleoli. However, the surface areas of the two categories of nucleoli (single or double) were approximately the same. In reacting neurons, the volume and surface area of double nucleoli combined increased faster than those of a single nucleolus.

2. The number of nucleoli did not influence the rate of recovery from chromatolysis. Cytoplasmic synthetic activities of RNA and protein, in neurons with one nucleolus and in those with double nucleoli, was the same in so far as could be detected by the autoradiographic method.

3. The number of fibroblasts increased in the experimental ganglia. However, there was no change in the size of fibroblast nuclei or satellite cell nuclei in ganglia containing reacting cells.

4. The size of neurons varied considerably and a diameter of 30 μ was selected arbitrarily as the dividing line between 'large' and 'small' cells. After injection of cytidine-H^3, the cytoplasmic radioactivity of normal and chromatolytic small neurons was 70% and 40%, respectively, higher than that of large neurons. After injection of arginine-H^3, the cytoplasmic radioactivity of normal and chromatolytic small neurons was 21% and 24%, respectively, higher than that of large neurons.

5. The spatial relationship of fibrillar and particulate components of nucleoli was of cortical, loose or compact types.

6. Two types of extranucleolar bodies were recognized. One

was a fibrillar type and the second was a particulate type.

7. The number of nucleoli or combination of nucleoli and extranucleolar bodies per cell was four or less.

The following observations confirmed previous findings.

1. The average number of nerve cells in L5 and L6 ganglia was larger in L5 than L6 and of the order of ten thousand and eight thousand per ganglion respectively.

2. Cell bodies and nucleoli were larger in reacting neurons than in those of normal cells. Nuclei of reacting neurons appeared to be undergoing a minimal amount of swelling.

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