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City University of New York PH.D. 1983

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ULTRASTRUCTURE OF AND MONOAMINE LOCATION IN CULTURED HUMAN FETAL SYMPATHETIC NEURONS

by

Gail D. Zeevalk

A dissertation submitted to the Graduate faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, City University of New York

1983

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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ABSTRACT

ULTRASTRUCTURE OF AND MONOAMINE LOCATION IN CULTURED HUMAN FETAL SYMPATHETIC NEURONS

by

Gail D. Zeevalk

Advisor: Professor Katherine M. Lyser

Partially dissociated human fetal sympathetic ganglion cells were cultured in a chemically defined medium for 5 to 40 days. The effects of serum-free growth conditions on several neuronal features were examined by phase contrast and electron microscopy. Serum grown cultures and *in vivo* ganglia were studied for comparison.

Neurons cultured in defined medium or serum-supplemented medium attached readily to the culture dish and extended extensive neurites. In marked contrast to the dense outgrowth in serum-supplemented medium, very few non-neuronal cells were present in defined medium. The fine

structure of the neurons in defined medium was similar to that of neurons *in vivo* or cultured in serum-supplemented medium. While a developmental range of cells from very immature appearing cells to more mature young neurons was found at all time points examined, there appeared to be a larger proportion of the more mature sympathetic neurons in older cultures. Very immature cells had condensed chromatin and scanty cytoplasm while more mature neurons showed increasingly more dispersed karyoplasm and a developmentally advanced organelle population. The ultrastructure of the neurites was typical of sympathetic axons. Varicosities and occasional synapses were found in cultures maintained for at least 20 days. It is concluded that the defined medium used in this study allows the expression of typical neuronal cytology, neurite extension, and synapse formation.

The physiological differentiation of these neurons with respect to neurotransmitter production and storage was examined in serum-supplemented and defined medium cultures by chromate-dichromate cytochemistry. Chromate-dichromate specifically reacts with the monoamines norepinephrine, dopamine and 5'-hydroxy-tryptamine and results in an electron dense precipitate. Electron dense reaction product resembling the cores of dense core vesicles and with a size range compatible with them (50-94 nm) was seen in all chromate-dichromate treated cultures. Chromate positive

vesicles were found in cell somas as well as processes. These vesicles occurred either singly or in small clusters. Clusters of large dense core vesicles were seen more frequently in the 25 day cultures than in the 6 day ones. Small dense core vesicles were never found. When potassium permanganate was employed as the fixative, occasional positive vesicles were found but their frequency was greatly reduced from that seen in the glutaraldehyde-osmium or chromate-dichromate fixed cultures. This is believed to be due to depletion of monoamines with potassium permanganate fixation. The findings demonstrate the ability of these neurons to retain an adrenergic phenotype *in vitro* and the storage of the adrenergic neurotransmitter in large dense core vesicles.

To my very dear friends Bob and Marion

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INTRODUCTION

I. Objectives

Studies of normal and neoplastic autonomic neurons have contributed much to our current knowledge of neural development. However, many questions concerning the mechanisms of differentiation still remain unanswered. Cell, tissue and organ culture provide a valuable tool for developmental studies. The immediate cellular environment is under strict control and variables can be manipulated more readily than *in vivo*. Control of the culture environment has recently been advanced by the development of several chemically defined media. This is of particular importance since serum contains an undefined population of molecules which may interfere with experimental results in an unsuspected manner and make interpretation of results more difficult.

Neurons of both the central and peripheral nervous systems have been studied in culture. Relatively few studies, however, have been concerned with developing human neurons. Study of normal human neurons is important in assessing the applicability of the vast amount of research obtained from other species to human neuronal development. In addition, it provides a basis of comparison for studies using human neuroblastoma cell lines as model systems for development and differentiation of the nervous system. Cell

lines of neuronal origin also have been used for research aimed at understanding the mechanisms involved in the conversion of normal cells to a transformed state. In evaluating the results of such studies it is crucial to know how normal cells of the same developmental origin respond in a similar culture environment. A serum-free culture system was recently established for rat neuroblastoma (Bottenstein and Sato, 1979). The same chemically defined medium has been used successfully for the growth of cells of a continuous human neuroblastoma line (Burmeister et al., 1979; Burmeister, 1982; Carroll, 1982). The first objective of this thesis research was to test the ability of defined medium to support the growth of human fetal sympathetic ganglion cells, the normal counterpart of peripheral neuroblastoma. A second major objective was to assess the extent of morphological and cytological differentiation by means of electron microscopy when cultured in this medium versus "traditional" serum-supplemented medium.

The frequent finding of large dense core vesicles in these cells by electron microscopy led to the third objective of this research, which was to examine the physiological differentiation of these cells with respect to monoamine (neurotransmitter) production and storage. This work has resolved an uncertainty in the literature in regard to the storage site for monoamines in human fetal sympathetic neurons. Dense core vesicles are thought to be the storage

sites for these neurotransmitters. However, specific formaldehyde induced fluorescence (FIF) procedures (Hervonen et al., 1977; Hervonen et al., 1978) have shown that monoamines are present in developing human sympathetic neurons both in vivo and in vitro but fixation techniques used to demonstrate monoamines by electron microscopy have failed to show their presence.

Ultrastructural demonstration of monoamines was performed using an improved method of sodium chromate-potassium dichromate cytochemistry (Tranzer and Richards, 1976). This modification of the chromaffin histological test results in an electron-dense precipitate when monoamines are exposed to sodium chromate-potassium dichromate during fixation. Both model and fixed tissue experiments have shown this to be an extremely sensitive method for monoamine determination (Wood, 1966; Tranzer and Richards, 1976; Hokfelt, 1971). A consideration of the cytochemistry and specificity of the procedure can be found in the Discussion, part VI: Dense Core Vesicles. A. Cytochemistry.

This study is among the first describing the culture of any type of normal neurons in defined medium and is the first report establishing a system for culturing human developing neurons in a chemically defined medium. In addition, the results confirm and extend the relatively small amount of previous work on developing human sympathetic neurons in

serum-containing cultures. The detailed ultrastructural examination of these cells in chemically defined medium provides a basis of comparison for evaluating differentiation and expression of neuronal characteristics by neuronal tumor cells *in vitro*, especially human neuroblastoma. The storage of monoamines within large dense core vesicles has been demonstrated and supports increasing evidence that large dense core vesicles function as storage sites for neurotransmitter.

II. Sympathetic Ganglia - Developmental Background

A. Cell Origin and Neural Crest Migration

Sympathetic ganglia have been shown through a variety of experimental approaches to be derived from cells of the neural crest. Deletion experiments (Detwiler, 1937), radioactive labelling experiments (Weston, 1963), and crest cell transplantation experiments (LeDouarin and Teillet, 1974) all confirm this embryonic origin.

Crest cells arise from dorsal ectoderm and at about the time of closure of the neural tube are found along its dorsal margin. Shortly after neural tube closure, crest cells begin to migrate away from this site. The migratory pathways of crest cells have been determined by the use of two types of experimental approaches. Labelling crest cells

with 3H-thymidine before migration (Weston, 1963) and transplants of the cells between chick and quail embryos (LeDouarin and Teillet, 1974) permit following their passage. These experiments have shown that migration occurs primarily in two directions: a dorsolateral migration into epithelial areas to form melanocytes and a ventral migration in a variety of defined pathways depending on the axial level to differentiate into one of several diverse cell types, including sympathetic ganglion cells. Neural crest derivatives are summarized in Table I.

TABLE I

Derivatives of Neural Crest Cells	ref.
melanocytes	Weston, J.A., 1963
neurons and accessory cells of the sympathetic ganglia	Weston, J.A., 1963; Weston, J.A., and Butler, S.L., 1966
neurons and accessory cells of the parasympathetic ganglia	Le Douarin, N.M. and Teillet, M.A., 1974
neurons and accessory cells of the sensory ganglia	Weston, J.A. and Butler, S.L., 1966
chromaffin cells of the adrenal medulla	Le Douarin, N.M. and Teillet, M.A., 1971
dermal, skeletal and connective tissue of the head and neck	Le Lievre, C.S. and Le Douarin, N.M., 1975; Noden, D.M., 1978

Migration occurs along preferential routes (Weston, 1963; LeDouarin and Teillet, 1974) and the pathway taken depends on the axial level of the crest cells. Crest cells at cranial levels [corresponding to levels S1-7 (1st through 7th pairs of somites) in the chick] will migrate in a ventral and caudal direction to eventually form parasympathetic cholinergic ganglia in the gut. Trunk level crest cells (corresponding to levels S8-27) migrate laterally and ventrally to paravertebral and prevertebral sites and eventually aggregate to form the sympathetic adrenergic chain and prevertebral ganglia.

B. Cell Determination

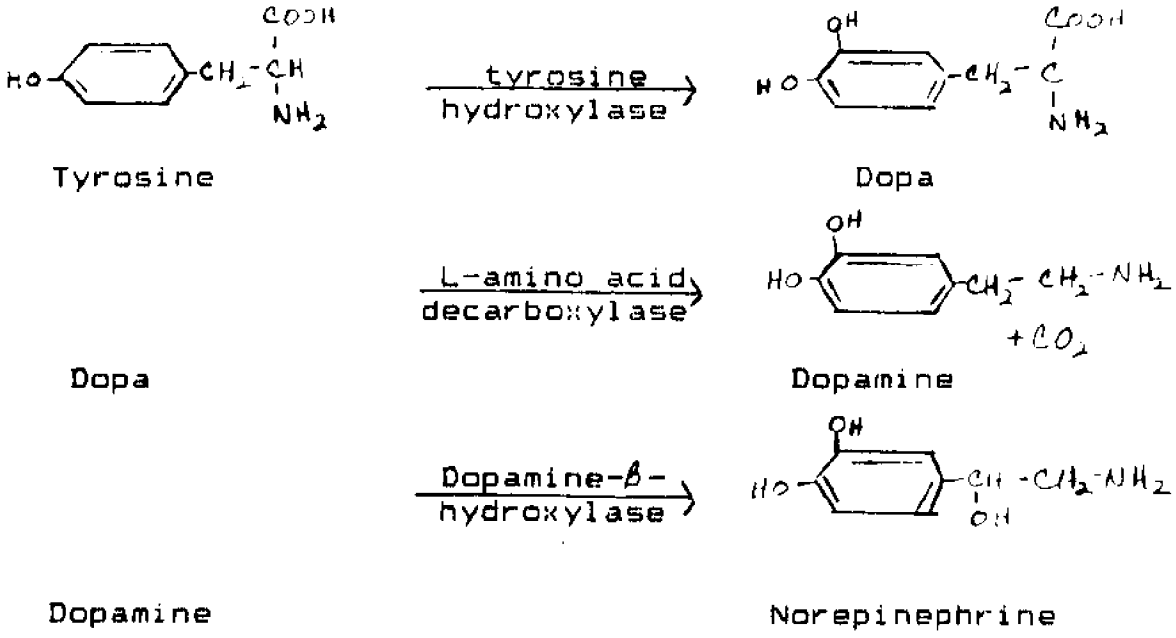
When looking at the diverse final fates of the crest cells an immediate question that arises is whether the final fate of these cells is determined before migration begins. An approach to answering this question has been to transplant crest cells from one axial level to another before their migration begins and to see if these cells respond to cues from the host or differentiate in accordance with their original location. Such transplants have been done between chick and Japanese quail embryos (Le Douarin and Teillet, 1974). Quail donor cells can be distinguished from chick host cells by differential staining of the interphase nuclei using the Fuelgen-Rossenbach technique (LeDouarin, 1973). When cranial level crest cells

(presumptive parasympathetic) are grafted to trunk levels (presumptive sympathetic) these cells will migrate to form the paravertebral sympathetic chains and differentiate as adrenergic neurons. Conversely, trunk level crest cells when grafted to cranial levels will migrate according to their new axial level and colonize the gut. These transplantation experiments show that the final differential fate of neural crest cells is not determined before migration begins. This suggests that environmental factors play a significant role in crest cell differentiation. Interactions between the crest cells and their environment occur along the migratory route and at their final site. Both have been shown to influence crest cell differentiation. Organotypic cultures of crest cells plus different combinations of other embryonic tissues (neural tube, somites, notochord) (Cohen, 1972; Norr, 1973) will allow the develment of crest cells into adrenergic neurons. This occurs even though crest cells have not reached their final destination. Other investigators have provided evidence that interactions with tissues encountered during migration need not occur for maturation into autonomic neurons (Smith et al., 1977; LeDouarin, 1980). When quail donor crest cells from either presumptive sympathetic or parasympathetic levels are transplanted before their migration to chick aneural hindgut, the donor cells migrate to regions indicative of enteric ganglia and acquire significant levels of choline acetyltransferase (CAT) activity. Preventing the

initial phase of migration of the crest cells, therefore, has no significant effect on their differentiation. However, the differential fate of such transplanted crest cells can be influenced by coculturing them with the gut in the presence of notochord and neural tube. When this is done, adrenergic neurons as well as cholinergic plexi are present (Teillet et al., 1978). Thus differentiation may be influenced by environmental factors encountered along the migratory route as well as at the final site. As will be discussed later, it appears that it is not direct interaction with other embryonic tissues but rather a diffusible factor that is perhaps supplied by a particular cell type (see page 13 for discussion of conditioned medium effects on neuronal cell differentiation) that is responsible for this influence.

C. Neurotransmitter development

1. Natural history Much is known about the natural history in vivo of the appearance of the neurotransmitter norepinephrine in sympathetic ganglion cells. The sequence of reaction steps involved in the synthesis of norepinephrine is presented below.



Tyrosine Dopa

Dopa Dopamine

Dopamine Norepinephrine

Tyrosine hydroxylase (TH) is the first enzyme involved in the synthesis of norepinephrine. TH is the rate limiting enzyme in the pathway and is subject to end product inhibition by norepinephrine. L-amino acid decarboxylase (DDC) converts Dopa to Dopamine. Dopamine is hydroxylated to norepinephrine by dopamine-β-hydroxylase (DBH). This enzyme has been located to the vesicle storage granules (DePotter et al, 1980).

In rat sympathetic ganglia there appears to be a synchronous appearance of TH, DDC, and DBH at embryonic day 11.5 (Cochard et al., 1978; Teitelman et al., 1979). This is about the time that the crest cells have begun to aggregate into a ganglion structure and the time at which monoamines can be detected by histofluorescence. In chick, however, Ignarro and Shideman (1968) monitored the appearance of the enzymes and concluded that their appearance was sequential and followed the order of the reaction sequence.

The difference in timing of the order of appearance of enzymes in chick and rat may be accounted for by the fact that enzyme activity in the chick was measured by 3H-tyrosine conversion to L-Dopa, dopamine and norepinephrine in whole embryos. The cellular location of these enzymes, therefore, is not clear and enzyme activity may exist in other embryonic tissues at the times they are measured. Migrating presumptive cholinergic crest cells in the rat, however, can express neurotransmitter enzyme activity. Mesencephalic crest cells destined to give rise to cholinergic ciliary ganglia demonstrate CAT activity (Le Douarin, 1980).

2. Plasticity in vivo and in vitro As mentioned previously, crest cells remain multipotential before and during their migration. This plasticity continues even after ganglia have formed. Neurotransmitter phenotype, even though being expressed at this stage, can be altered by perturbation of the ganglia's environment. If Remak's ganglion (a small mesenteric cholinergic ganglion) is removed from 4-5 day quail embryos and back-transplanted into the trunk neural tube region of a 2.5 day chick, the ganglion cells migrate to paravertebral sympathetic ganglion sites and to the adrenal medulla and contain catecholamines as demonstrated by formaldehyde-induced fluorescence (Le Douarin, 1980).

The pluripotency of crest cells has been confirmed *in vitro*. Explanting neural crest plus neural tube and then removing the neural tube within 24-48 hours allows time for some of the crest cells to migrate away from the neural tube (Cohen and Koningsberg, 1975). This provides an almost pure culture of crest cells. Single isolated clones derived from such cultures give rise to either pigmented, unpigmented or mixed colonies (Sieber-Blum and Cohen, 1980; Sieber-Blum *et al.*, 1981). The unpigmented cells contain catecholamines and are considered adrenergic neurons. The number of catecholamine positive cells can be enhanced by the addition of cellular fibronectin (Sieber-Blum *et al.*, 1981) or by the presence of irradiated extracellular matrix (Sieber-Blum and Cohen, 1980).

The adrenergic phenotype of aggregated embryonic neonatal ganglion cells can be retained in culture but is still capable of being manipulated. Rat superior cervical ganglion cells from 1-2 day neonates can be grown in culture in the presence or absence of nonneuronal cells (Mains and Patterson, 1973). When cultures of ganglion cells are grown alone, they synthesize and accumulate norepinephrine from tyrosine (Mains and Patterson, 1973), store and release norepinephrine (O'Lague *et al.*, 1974; Rees and Bunge, 1974), conduct action potentials and are sensitive to acetylcholine (O'Lague *et al.*, 1975; Patterson, 1978), all characteristics of adrenergic neurons. When cultured

in the presence of nonneuronal cells they produce 1000 times more acetylcholine. The amount of acetylcholine synthesis is found to correlate with the length of time in culture. The older cultures with the larger number of non-neuronal cells show higher levels of acetylcholine synthethesis (Patterson and Chun, 1974). When mitotic inhibitors are added to reduce proliferation of nonneuronal cells this blocks the increase in acetylcholine synthesis but has no effect on catecholamine synthesis. The physical presence of nonneuronal cells is not required to induce this change from adrenergic to cholinergic phenotype. Medium conditioned with nonneuronal cells will induce an increase in choline acetyltransferase activity and a corresponding increase in acetylcholine synthesis as well as a decrease in norepinephrine synthesis (Patterson, 1978).

The presence of nonneuronal cells or medium conditioned with nonneuronal cells appears to influence the formation of synaptic contacts made between the principal neurons (O'Lague et al., 1974; Patterson, 1978). An in vivo counterpart is not known. A correlation is seen between the number of nonneuronal cells, an increase in acetylcholine synthesis and the number of synaptic contacts. Electrophysiologic experiments (O'Lague et al., 1974) indicate that transmission is nicotinic- cholinergic while electron microscopic evidence suggests that a high percent of the presynaptic endings are capable of taking up 5'-

hydroxy dopamine which is a characteristic of adrenergic terminals *in vivo*. This phenomenon of "dual transmitter synthesis" is further evidenced in studies involving microcultures which contain a single neuron and a small number of nonneuronal cells (Furshpan *et al.*, 1976). When single neurons are cultured with a limited number of cardiac myocytes, three types of electrophysiologic responses are recorded. Some electrically excitable cultures contain exclusively cholinergic synapses, others exclusively adrenergic synapses, while a third type of culture appears to contain three different synapses: nicotinic-cholinergic autapses (a neuron synapsing on itself), muscarinic-cholinergic synapses with myocytes, and adrenergic synapses with myocytes. A single neuron then is capable of expressing adrenergic and cholinergic characteristics simultaneously. Recent immunocytochemical studies of cultured rat superior cervical ganglion cells (Iacovitti *et al.*, 1981) indicate that sympathetic ganglion cells are capable of adrenergic and cholinergic transmitter synthesis for as long as 7 weeks in culture.

3. *Age dependence of plasticity* The mutability of the autonomic phenotype declines with increasing age of the cell. If sympathetic ganglion cells are explanted from rats 3 to 22 days after birth, their ability to accrue cholinergic properties diminishes. Three weeks after birth they remain irreversibly adrenergic (Ross *et al.*, 1977).

Adrenergic enzyme levels (TH and DBH) in 21 day embryo and adult rat ganglia explants remain approximately the same over several weeks in culture while CAT activity greatly increases in embryonic cultures but remains undetectable in adult cultures (Johnson et al., 1980).

D. Mitosis and Differentiation

The developmental pattern of differentiation following a final mitosis that is seen in the central nervous system is not characteristic of the peripheral nervous system. Withdrawal from the cell cycle is not a requirement for neurite outgrowth, transmitter enzyme synthesis or monoamine production in developing sympathetic ganglion cells. Chick ganglion cells simultaneously label with 3H thymidine and fluoresce with formaldehyde vapors (Rothman et al., 1978). In addition, 3H thymidine labelled cells show immunoreactivity to TH and DBH antibodies (Rothman et al., 1978). As the embryo matures, more cells are withdrawn from the cycling but labelling of some cells may continue through hatching.

E. Presynaptic and Target Organ Effects

Once presumptive sympathetic crest cells have reached their final destination, regulation of their continued

development depends on input from the central nervous system as well as interactions with their target organs. When preganglionic cholinergic axons from the spinal cord are transected in neonatal mice or rats (Black et al., 1972; Black et al., 1976), the ganglion cells fail to show the normal increase in TH activity. Similar results can be achieved by treating mouse neonates with ganglionic blocking agents (chlorisondamine or pempidine) which compete for acetylcholine receptor sites on sympathetic ganglion cells (Black and Green, 1973). This suggests that the presynaptic neurons regulate development of TH activity via acetylcholine. The effects of acetylcholine blockade, however, may be the result of blocking depolarization and subsequently Ca++ influx. Cultured ganglion cells grown under conditions which promote cholinergic differentiation retain an adrenergic phenotype if high K+ (which induces depolarization) is included in the medium (Walicke et al., 1977). Ca++ antagonists plus K+ fail to prevent the cholinergic induction. The normal development of target organ innervation by sympathetic ganglion cells is also affected by decentralization (Mytilineou and Black, 1976). Innervation density, 3H-norepinephrine uptake, TH activity and catecholamine fluorescence all fail to develop normally within the adrenergic nerve terminals in the iris.

The target organ itself can have a profound effect on ganglion cell development. Iridectomy or sialectomy in 3

day old rats prevents the normal increase in TH activity (Dibner et al., 1977). The reduction in enzyme activity is a result of a decrease in the population of adrenergic neurons by 30%, but it is not known whether the population decrease is the result of a decrease in adrenergic neuron production or of an increase in destruction.

In vivo experiments also show a loss of these effects with age. Severing the preganglion connections to sympathetic ganglia in adult rats does not affect TH or DBH activity (Molinoff et al., 1972).

F. NGF Dependence

Nerve growth factor (NGF) was discovered through observation of the reactions of sensory (Beuker, 1948) and sympathetic (Levi-Montalcini and Hamburger, 1951) ganglia to transplants of mouse sarcomas into the body walls of chick embryos. Both ganglia respond by producing enormous numbers of nerve fibers that not only invade the tumor but embryonic viscera as well. Evidence for a diffusible growth factor was provided by grafting tumors to the chorio-allantoic membrane in 4-6 day chick embryos (Levi-Montalcini and Hamburger, 1953). An excessive outgrowth of neurites from sympathetic and sensory ganglia was observed even though the host circulation was the only contact between the sarcoma and the ganglion cells. Since then NGF

has been isolated from a variety of sources: snake venom (Cohen and Levi- Montalcini, 1956) and mouse submaxillary salivary gland (Cohen, 1960).

NGF has been characterized as a hormone, with a molecular weight of 130,000 (sedimentation coefficient of 7S) and consists of 2α, 2β, and 2γ subunits. The β subunit (2.5S) is the biologically active one.

The effects of NGF in embryonic and neonatal sympathetic ganglia have been shown to be 1) an increase in mitotic activity, 2) hypertrophy of differentiated nerve cells, 3) a requirement for survival and maintenance, 4) promotion of certain neuronal differentiated characteristics. In addition, it may also play a role as a chemotropic agent and guide the establishment of appropriate neuron-target connections.

Hyperplasia and hypertrophy Daily injections of NGF in chick embryos at 7 to 10 days of incubation and in newborn mice causes an increase in mitotic activity (Levi-Montalcini and Cohen, 1956; Levi-Montalcini and Cohen, 1960; Levi-Montalcini and Angeletti, 1968). Total nerve cell population is 2 to 3 times greater in the NGF treated than in control mice. The overall size increase of the NGF ganglia is 6 times that of the controls. Ultrastructural examination of NGF treated ganglia (Angeletti, et al.,

1971) shows an increase in neurotubules and neurofilaments in the cytoplasm of the enlarged neurons. The effects on mitotic activity in sympathetic ganglia decrease with increasing age of the animal (Levi-Montalcini and Booker, 1960a). NGF treatment 9 days after birth or later fails to elicit increased mitotic activity but does cause an increase in the volume of preexisting ganglion cells.

Survival and maintenance Administration of antiserum to NGF in newborn mice causes an almost complete destruction of the sympathetic nerve cells (Levi-Montalcini and Booker, 1960b). Examination of the ganglion cells between 2 and 9 days after birth shows only a few mitotic figures and an increase in the number of dead and degenerating cells. Dissociated chick sympathetic and sensory ganglia (7-10 day embryos) require the presence of NGF for their survival (Levi-Montalcini and Angeletti, 1963). In vitro the cytotoxic effects of NGF antiserum on ganglion cells can be seen within hours of its administration (Sabatini, et al., 1965).

Effects on differentiation NGF's effects on neurite outgrowth has been well documented (for review see Levi-Montalcini and Angeletti, 1968). NGF causes extensive neurite outgrowth from explanted sympathetic ganglia. Its appearance has caused it to be termed the "halo effect". The neurite promoting effects of NGF are also demonstrated

in chromaffin cells (Aloe and Levi-Montalcini, 1979), in a pheochromocytoma cell line (PC12) (Greene, 1978), and in human neuroblastoma (Burmeister et al., 1979; Burmeister, 1982; Carroll, 1982).

TH and DBH activity in rat neonates is increased by the presence of NGF (Thoenen et al., 1971; Stoeckel et al. 1974). PC12 cells, while showing a morphological response to NGF, do not show this biochemical induction (Edgar and Thoenen, 1978). Hence the mechanisms involved in the morphological and biochemical maturation induced by NGF need not be dependent on one another and are separable. Also separable is the morphological response induced by NGF from its effects on mitosis. Some morphologically differentiated PC12 cells are still capable of 3H-thymidine incorporation (Gunning et al., 1981).

Chemotropic effects Coculture of the sympathetic ganglia with a variety of target tissues (Chamley et al., 1973; Ebendal and Jacobson, 1977) suggests that NGF may play a chemotropic role since fiber outgrowth from the side of the explant facing the target tissue is greater and there is an enhanced fiber outgrowth toward certain target tissues. Perhaps the most direct evidence that implicates NGF in guiding the "directionality" of neurite outgrowth comes from a recent experiment by Gunderson and Barrett (1980). In this experiment the growth cones of chick

sensory ganglion cells were made to repeatedly and consistently turn towards the direction of an NGF gradient supplied by a micropipette.

G. Summary

From the abundance of experiments discussed above, several general conclusions can be made regarding the development of sympathetic ganglion cells:

1. Neural crest cells are not determined as to their final differential fate before or during their migration or for a variable time after they have reached their final site.
2. The adrenergic neurotransmitter phenotype (in rats) is not expressed until the cells reach their final site. Conversely, presumptive cholinergic crest cells may express cholinergic enzyme activity while migrating.
3. Environmental cues encountered along the migratory route and at the final site can greatly influence neurotransmitter phenotype.
4. These environmental cues may be due to a diffusible factor or factors.
5. The plasticity of neurotransmitter phenotype diminishes with increasing age of the cell.
6. Preganglionic input and target cell interactions probably serve to augment and stabilize neuronal differentiation.
7. Several neuronal differentiated features can be

expressed in the developing peripheral neuron before it undergoes a final mitosis.

8. The expression of neurotransmitter phenotype *in vitro* is manipulatable.

9. Cultured ganglion cells may show "dual transmitter synthesis".

10. NGF affects the survival, maintenance, mitotic activity, growth and differentiation of developing sympathetic ganglion cells.

III. Ultrastructural Differentiation of Sympathetic Neurons

The pattern of development of sympathetic neruons is similar in many respects for several mammals studied (rat, Eranko, 1972; mouse, Kim and Munkasca, 1974; rabbit, Tennyson and Mytilineau, 1975; Papka, 1972; man, Hervonen and Kanerva, 1972; Kondo and Fujiwara, 1979). Electron microscopic examination shows cells at different stages of maturation within a developing ganglion. There appear to be some differences among species in regard to the degree of maturation of the sympathetic neurons at various fetal and neonatal stages of the animals. However, the overall sequence of events in the differentiation of the neuron as seen ultrastructurally is remarkably similar.

The youngest appearing neuronal precursor cell is relatively undifferentiated. The cell is small in size

with only a slight amount of cytoplasm containing few organelles. Free ribosomes, mitochondria and a few multivesicular bodies are the predominant organelles. A few large dense core vesicles (100-150 nm) may be present (Tennyson and Mytilineou, 1975). The nucleus contains varying amounts of condensed chromatin. In some species such as human, this is very pronounced, but is less so in others. This cell type has been referred to as a stem cell (Tennyson and Mytilineou, 1975), an early sympathicoblast (Eranko, 1972) or a primitive sympathetic cell (Hervonen and Kanerva, 1972).

More mature neuronal precursors have increased amounts of cytoplasm and a less dense appearing nucleus. Organelles are more numerous including a moderately developed and extensive Golgi complex, rough endoplasmic reticulum, an increase in mitochondria and large dense core vesicles. Processes are extended at this time. Cells with these characteristics have been called principal cell precursors (Tennyson and Mytilineou, 1975), late sympathicoblasts (Eranko, 1972) or neuroblasts (Hervonen and Kanerva, 1972). They appear in recently formed rabbit ganglia and continue to be found throughout gestation in the rabbit and mouse. They appear as well in neonatal rat. Maturation of the neuroblast involves further increases in cytoplasmic volume with a corresponding increasse in organelle development. The number of large dense core vesicles in the cytoplasem of the developing neuroblast appears to at first increase

and then decreases as the cell matures (Machado, 1971; Kim and Munkasca, 1974).

The most mature stage of the developing neuron has been referred to as the young sympathetic neuron (Eranko, 1972; Hervonen et al., 1978) or the principal cell (Tennyson and Mytilineou, 1975). In rats, this cell is at least twice the diameter of the youngest cell type. Golgi complexes of two or more per cell are frequent and rer is arranged in short stacks known as Nissl bodies. Large dense core vesicles in the cell somas are less frequent; in the rat, small dense core vesicles also have been found in the cell somas and processes in this stage neuron. (No mention of small dense core vesicles in the somas of rabbit and mouse neurons has been made.) Young neurons are multipolar, extending several short dendritic processes and a single axon. The mature adult neuron is similar to the young sympathetic neruon in all respects except that cell size is even greater and the cytoplasm even more densely packed with organelles.

Human fetal sympathetic ganglia from second trimester fetuses contain cells that range in development through all the stages described above. These include very immature appearing cells and cells at different stages of neuronal maturation. The major ultrastructural difference noted between the human and rat sympathetic neurons is the absence of small dense core vesicles in the former at the young sympathetic neuron stage. This may reflect differences in

the degree of maturation of the neurons at the time of fixation. It is also possible that small dense core vesicles are not an ultrastructural feature of the somas of human sympathetic neurons since several reports on the ultrastructure of the sympathetic ganglia from adults make no mention of the presence of small dense core vesicles in the perikaryon (Kondo, 1979; Pick, 1967; Hervonen et al, 1980). It is known, however, that the processes of these adult neurons in vivo contain small dense core vesicles (Kyosola et al., 1976; Schulman, 1975; Ishii, 1972). For consistency, the developmental classification of human sympathetic cells by Hervonen and Kanerva (1972) was used as a guideline for this study. The classification and description of the different developmental stages has been summarized in Table II and is accompanied by a pictoral summary of the predominant features of each developmental stage (Text Fig. 1).

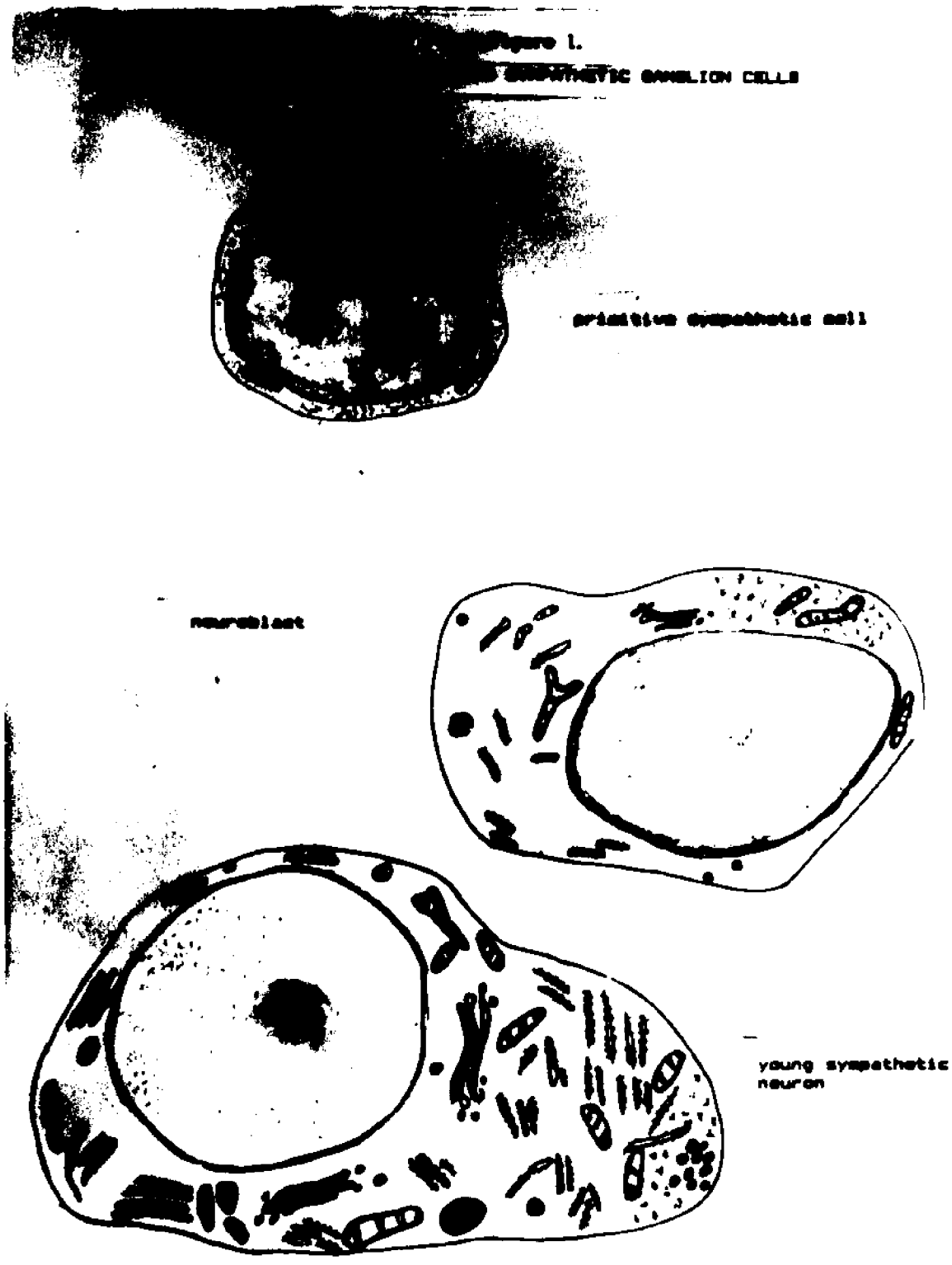

primitive sympathetic cell
neuroblast
young sympathetic neuron

TABLE II

CELL STAGES IN DEVELOPING SYMPATHETIC GANGLION CELLS*

Type	Description
primitive sympathetic cell	rounded or ovoid nucleus with condensed chromatin, sparse cytoplasm with few organelles, abundant free ribosomes
neuroblast type I	nuclear chromatin more dispersed, thinner perimembranous rim, cytoplasm contains occasional rer, many ribosomes present in polysomal aggregates, dense core vesicles
neuroblast type II	homogeneous nucleoplasm, little or no perimembranous rim, increase in cytoplasmic volume, cytoplasm contains developing Golgi complexes, moderate amounts of rer, dense core vesicles and an increase in mitochondria
young sympathetic neuron	large nuclei with evenly dispersed chromatin and no perimembranous rim, extensive cytoplasm with well developed Golgi, Nissl bodies, dense core vesicles

*stages taken from Hervonen, A. and Kanerva, L., 1972, and Hervonen, H., et al., 1978

IV. Monoamines in Sympathetic Neurons

Electron microscopic examination of most mature adrenergic nerve endings shows vesicles containing electron dense material. These vesicles fall into 2 size categories: small vesicles of approximately 50 nm and large vesicles of approximately 100 nm. The appearance of dense core vesicles is associated with the presence of monoamines. Pretreatment of animals with monoamine-depleting drugs (reserpine or α-methyl metatyrosine) results in empty appearing vesicles (Tranzer et al., 1969). Electron microscopic autoradiography in conjunction with pharmacological experiments has also provided evidence for the location of adrenergic neurotransmitters within dense core vesicles (Wolfe et al., 1962; Bloom and Barrnett, 1966). In addition, clusters of granular vesicles as seen in the electron microscope appear to correspond to fluroescent granules demonstrated by treatment with formaldehyde vapors (Eranko, 1976). Catecholamines condense with for- formaldehyde to produce a specific fluorescence (Falck et al., 1962).

Human fetal sympathetic ganglion cells both in vivo (Hervonen and Kanerva, 1972) and when cultured in serum containing medium (Hervonen et al., 1978) contain large dense core vesicles. Formaldehyde treatment of the ganglion cells demonstrates a specific fluorescence (Hervonen

et al., 1977; Hervonen et al., 1978), an indication of the presence of monoamines. However, the subcellular location of monoamines in this embryonic tissue was unclear since dense core vesicles were not found following potassium permanganate fixation (Hervonen et al., 1977; Hervonen et al., 1978). Potassium permanganate is a fixative that is known to react with the biogenic amines norepinephrine (NE), epinephrine, dopamine and 5'-hydroxy tryptamine (Richardson, 1966; Hokfelt and Jonsson, 1968) and has been used frequently to demonstrate their presence within dense core vesicles.

The second part of this thesis was done to try to clarify this issue. Monoamine storage was examined using a different and more specific cytochemical approach, chromate-dichromate fixation. Since previous work on human fetal sympathetic ganglion cells was performed on cells cultured in serum containing medium, the presence and location of monoamines was first tested for in comparably grown cultures. Ultrastructural studies of serum-free cultures of the ganglion cells during the first part of this thesis project demonstrated the presence of dense core vesicles. It was of interest, therefore, to determine if the presence and storage of monoamines was similar when grown under the two medium conditons tested. In addition, the partial selectivity of defined medium in reducing the nonneuronal cell population permitted examination of

possible sites of monoamine stores in somas and processes over short and long term culture.

V. Neoplastic Tissue for Developmental Studies

Cell lines have been employed widely in biological research for many years. The convenience of a large source of material which is easily maintained in culture as well as a homogeneous population of cells often permits investigation in certain areas when the use of normal cells is not feasible. Neuroblastomas are believed to be tumors of development. They are of neural crest origin and are usually diagnosed at birth or in infancy. They comprise approximately 15% of all childhood cancers (Wilkerson, 1967). The most common site for this tumor is the adrenal medulla. There is a high incidence of spontaneous regression of this tumor to a more benign form, ganglioneuroma. Cells within ganglioneuromas appear as mature neuronal cells and no longer continue to divide. Because of the high incidence of spontaneous regression, cell lines derived from this tumor are thought to be appropriate as "model" systems for the study of neuronal development. Such studies are usually aimed at pushing differentiation in these cells through culture manipulation. Caution, however, must be used when interpreting results. It is necessary to assess whether these cells are responding in a manner similar to normal cells or if results are a consequence of their "trans-

formed" state. Because these cells are "transformed," they also may provide information that enables a better understanding of the mechanisms involved in this conversion.

The most frequently studied neuroblastoma cell line is C1300. This line was derived from a spontaneous tumor in a Jackson mouse (Dunham et al., 1953). A wide variety of sublines is available today and possess different neuronal characteristics. Neurite outgrowth and neurotransmitter enzyme activity can be elicited in C1300 by the addition of agents to the medium. Bromodeoxyuridine, cyclic-3', 5'-adenosine monophosphate (cAMP), and serum deprivation are some of the factors used (Prasad and Hsie, 1971; Waymire et al., 1972; Schubert and Jacob, 1970; Seeds et al., 1970; Schubert, 1971). Several human neuroblastoma cell lines also have been studied, but to a lesser extent (Biedler et al., 1973; Ross et al., 1980). One advantage of human lines is that their karyotype is near diploid and has remained stable over many years in culture. Two such lines have been extensively studied in my laboratory. SK-N-SH SY5Y and SK-N-BE(2) M17 have been well characterized with respect to their ultrastructure and neurotransmitter enzyme activity (Carroll, 1982) and process-forming ability (Burmeister, 1982) in both serum-supplemented and defined media. My initial interest in examining the ultrastructure of human fetal sympathetic ganglion cells in the different media was to provide a

frame of reference for ultrastructural studies using human neuroblastoma. Only by studying the ultrastructure of normal developing ganglion cells in a similar environment can the extent of ultrastructural differentiation in the neuroblastoma be assessed in a meaningful manner.

VI. Culture Systems

A. Nervous System Culture

The first successful tissue culture was also the first successful attempt at maintaining neurons *in vitro*. Neuroblasts from frog neural tube were cultivated in clotted lymph (Harrison, 1907) and the observation of neurite elaboration in these cultures solved a long standing controversy in neurobiology: that the developing nerve cell was the contributor of the long axonal cylinder.

Culture of nerve cells has always been a difficult task. This is particularly true of mammalian nerve cells since these cells lack the embryonic yolk found in amphibian cells. The medium used in early studies was usually a complex mixture of balanced salts, serum, chick embryo extract and glucose. Small pieces of nervous tissue were cultured either on plasma clots or on collagen coated dishes. These tissue pieces could be maintained lying flat or inverted in a hanging drop of medium (Murray, 1965).

While these studies permitted observation of the tissue over relatively long periods of time, they did not lend themselves too readily to culture manipulation.

Probably the most important advance in the ability to culture sympathetic ganglia came with the discovery of NGF (see II, section F, of Introduction). NGF is now known to be a requirement for the survival and maintenance of fetal sympathetic ganglion cells both *in vivo* and *in vitro*. Since then much has been done with dissociated sympathetic ganglion cells (mainly from rat superior cervical ganglia), particularly in regard to neurotransmitter expression *in vitro*.

These studies also employ the use of serum in the culture medium. Although great strides have been made in maintaining nerve tissue *in vitro* for longer periods of time while allowing for a continuation of the differentiation process, the complex nature of the medium makes experimental investigation and interpretation difficult. Recent trends have strived to eliminate as many unknown variables as possible from the culture medium. The ideal culture environment would be a chemically defined medium which would maintain the nerve cell for long periods of time.

B. Defined Medium

The culture of several cell lines and primary explants from a variety of tissues in serum-free medium is now possible (Bottenstein and Sato, 1979; Mather and Sato, 1979; Orly et al., 1980; Taub and Sato, 1979; for review see Barnes and Sato, 1980). The elimination of serum from the medium permits control of the cellular environment to a greater extent than was previously possible. Thus, in vitro studies such as the examination of the effects of trophic agents on development and differentiation can be done without the complications contributed by unknown serum factors. Serum is generally replaced by hormones, binding proteins, trace elements and nutrients thought to be needed for survival and growth.

An additional advantage of using serum-free medium for primary cell cultures is that in some cases it allows for selection of a specific cell type and eliminates the problem of overgrowth by unwanted cells. A greater than 95% pure culture of neurons can be obtained from dissociated dorsal root ganglion cells when grown in a chemically defined medium (Bottenstein et al., 1980).

When normal differentiated cells are grown in serum-free medium, their ability to either retain or repress certain differentiated characteristics as compared to their

growth in serum-supplemented medium varies depending on the cell type tested. Chick sensory ganglion cells continue to show morphological differentiation (Bottenstein et al., 1980) while rat granulosa cells do not express a differentiated state unless grown in serum-free medium (Orly et al., 1980). Since human fetal sympathetic ganglion cells have not been cultured in a serum-free medium before and since few papers have described their ultrastructure in serum-containing medium, these cells were cultured in both media to assess such differentiated features as neurite extension, fiber bundle formation and extent of ultrastructural maturation. The morphological and cytological maturation of human sympathetic ganglion cells in vivo was extensively examined for comparison.

MATERIALS

Progesterone, putrescine, 5'-hydroxy dopamine, transferrin (human, iron-free) and soybean trypsin inhibitor were obtained from Sigma Chemical Company. Dulbecco's modified Eagle's medium, Ham's nutrient mixture F12 and penicillin-streptomycin were obtained from Grand Island Biological Company. Sodium chromate, potassium dichromate, potassium permanganate, uranyl acetate and sodium citrate were obtained from Fisher Scientific Company. Ethylene-(dinitrilo) tetraacetic acid and lead acetate were obtained from J. T. Baker Chemical Company. Glutaraldehyde, osmium, and the components of the Epon 812 embedding mixture were purchased from Electon Microscopic Sciences. The sodium selenium used was obtained from either Bacto or Alfa Chemical Company. Trypsin was purchased from Difco Laboratories, fetal bovine serum from either Microbiological Associates or Flow Laboratories, and bismuth subnitrate from E. Merck.

The films used for phase and electron micrographs were Kodak Tri-X Pan and Kodak electron microscopic film #4489 respectively. The culture dishes used were Falcon 35mm tissue culture dishes #3001.

Nerve growth factor (NGF), 2.5S, was the kind gift of Dr. Mark Bothwell.

METHODS

I. Preparation of Cultures

The thoracic and lumbar sympathetic paravertebral chains from 9 human second trimester fetuses (CR length 10.5 to 17 cm) were obtained from prostaglandin induced interruptions of pregnancy according to protocol approved by the Committee for Human Rights in Research at New York Hospital-Cornell Medical Center. The outer connective tissue was removed by dissection and several ganglia from 4 of the fetuses were processed immediately for electron microscopy. The ganglia to be cultured were incubated in 0.05% crude trypsin in 0.02% ethylene(dinitrilo) tetraacetic acid, tetra sodium salt (EDTA) for 15 minutes at 37oC. Trypsinization was halted by rinsing the ganglia for 15 minutes at 37oC in a medium composed of equal parts Ham's nutrient mixture F12 and Dulbecco's modified Eagle's medium (F12:DME) containing 0.25 mg/ml soybean trypsin inhibitor. Ganglia were then placed in F12:DME until plated in experimental medium. Individual ganglia (1-2 per dish) were mechanically dissociated either by teasing between 2 watchmaker forceps or by trituration with a pasteur pipette onto serum coated 35 mm Falcon culture dishes containing 1 ml of experimental medium. Culture dishes were coated prior to use by the addition of 1 ml of fetal bovine serum (FBS) for 1 minute, removal of the serum and rinsing of the dish with

F12:DME. Cells were fed with complete medium replacement 24 hours after plating and then every 2-3 days until the completion of the study. Incubation was carried out in a 5% CO2, 95% air humidified atmosphere at 37oC.

II. Media

A. Defined Medium The N2 medium of Bottenstein and Sato (1979) consisted of a 1:1 mixture of F12:DME containing 5 μg/ml insulin, 20 nM progesterone, 100 μM putrescine, 100 μg/ml transferrin and 30 nM selenium plus 10 ng/ml 2.5S nerve growth factor (NGF).

B. Serum-supplemented Medium Equal parts F12 and DME were supplemented with 10% fetal bovine serum (FBS) plus 10 ng/ml NGF.

In addition, several cultures were grown in defined medium or serum-supplemented medium lacking NGF.

Culture media were mixed in the laboratory from dry powders using glass distilled water.

Penicillin (100 units/ml) and streptomycin (100 μg/ml) were added to all cultures.

III. Microscopy

A. Phase Microscopy

Living cultures, 73 from 5 fetuses, were examined daily and some were photographed on days 3, 5, 12 and 20 using a Nikon inverted phase microscope, model number MSS, with a Nikon Microflex photographic unit and a Leitz 35mm camera back.

B. Electron Microscopy

Cultures were fixed for electron microscopy by one of the methods described below. Ganglia that were not cultured (10 ganglia from 4 fetuses) were fixed only with glutaraldehyde-osmium. Ultrastructural studies of glutaraldehyde-osmium fixed cultures included 20 cultures maintained in defined medium and serum-supplemented medium for 5, 10, and 20 days and in defined medium for 40 days. A total of 23 cultures from 2 fetuses were used for chromate-dichromate cytochemistry. Five cultures in serum-supplemented medium and 10 in defined medium were fixed on day 6; 8 cultures in defined medium were fixed on day 25. Longer term serum-supplemented cultures were not used for chromate-dichromate cytochemistry due to overgrowth by nonneuronal cells. In addition, 4 cultures were fixed with $KMnO_4$ on days 6 and 25. At least 2 cultures for each fixation treatment for

cytochemical studies were incubated in 1 x 10-5 M 5' hydroxy dopamine (5' OHDA) before fixation. Fixation and embedding were carried out directly in the culture dishes.

1. Glutaraldehyde-osmium Cultures were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, for one hour, and post-fixed in 1% OsO4 for 30 minutes at room temperature in the same buffer. Washing of the cells between fixations and before dehydration was done in 0.1 M phosphate buffer, pH 7.2. The dehydration schedule included 10 minute washes in 30% and 50% ethanol, staining with 2% uranyl acetate in 70% ethanol for 20 minutes, and 2 ten minute washes in 95% ethanol and in absolute alcohol. (Propylene oxide was omitted from the dehydration protocol because it dissolves the plastic culture dish.) Cultures were infiltrated with a 3:1 mixture of absolute alcohol and Epon 812 mixture for several hours, followed by a 1:1 mixture of absolute alcohol and Epon 812 for another several hours and finally straight Epon 812 overnight. On the following day, the epon was changed and polymerization was carried out in a 60oC oven for at least 18 hours.

2. Chromate-dichromate The medium was removed and 2 ml of cold fixative containing 1% glutaraldehyde, 0.4% paraformaldehyde in 0.1 M sodium chromate-potassium dichromate, pH 7.2, were added directly to the culture dish and allowed to remain for 10 minutes at 4oC. After

removal of the fixative, 0.1 M sodium chromate-potassium dichromate, pH 6, was added and the cultures stored overnight at 4oC. They were washed with 0.1 M phosphate buffer, post-fixed in 2% OsO4 in 0.1 M sodium chromate-potassium dichromate, pH 7.2, for 1 hour at 4oC, dehydrated as with the glutaraldehyde-osmium (except that staining with uranyl acetate was omitted) and embedded in Epon 812. In order to verify the specificity of the reaction, several cultures were fixed according to the chromate-dichromate method except that post-fixation in osmium was omitted.

3. Potassium permanganate The medium was replaced with 2 ml of cold 3% KMnO4 in 0.1 M phosphate buffer, pH 7.2, and allowed to remain for 1 hour at 4oC. KMnO4 was removed by washing in 0.1 M phosphate buffer. Dehydration and embedding were performed as with the chromate-dichromate fixed tissue.

Thick sections (2-5 μm) and thin sections showing silver or light gold interference colors (500-1500 Å) were cut using glass or diamond knives on a Sorvall Porter-Blum MT-2 ultramicrotome. Thick sections were stained with 1% Toluidine blue in 1% borax and viewed with a light microscope. Thin sections were either unstained or stained with one or more of the following: 2% uranyl acetate for 30 minutes, lead citrate for 5 minutes (Reynolds, 1963), bismuth subnitrate for 3 minutes (Riva, 1974). Sections

were viewed in an RCA-EMU 3H electron microscope operating at 100 kV.

C. Fluorescence Microscopy

A modification of the Falck et al., (1962) formaldehyde-induced fluorescence (FIF) procedure was used. The medium was removed and the culture dishes washed briefly with 2 ml of F12:DME. Culture dishes with the cells were dried overnight in a vacuum dessicator containing phosphorous pentoxide and then placed in a sealable jar over 5-6 grams of paraformaldehyde. The jar was sealed and put in an 80oC oven for 1 hour. One culture received the same treatment as above except that it was not exposed to paraformaldehyde. The paraformaldehyde used for gassing had been previously brought to 70% relative humidity by storing in a dessicator over H_2SO_4 (100 ml H_2O to 27.8 ml of 96% H_2SO_4) for 1 week or longer. Culture dishes were removed from the paraformaldehyde vapors and viewed immediately with a Leitz fluorescence microscope equipped with epifluorescence. The wavelength of excitation was 405 nm. A Leitz barrier filter KP 510 was used between the stage and ocular lens. Photographs were taken with Tri-X pan film using exposure times of 15 to 60 seconds.

IV. Developmental Staging

Neurons derived from 3 fetuses and cultured in defined medium or serum-supplemented medium for 5, 10 and 20 days were classified into one of the categories described by Hervonen and Kanerva (1972): primitive sympathetic neurons, neuroblasts and young sympathetic neurons. All neurons were examined in 8x10" electron micrographs of approximately the same magnification (X15,000 - 20,000). Each neuron was scored for each of the following parameters and classified on the basis of its total score.

Parameters	Description	Score
nuclear differentiation	dense karyoplasm with thick perinuclear rim	+1
	intermediate	+2
	homogeneous karyoplasm with little or no peri-nuclear rim	+3
cytoplasmic differentiation	cytoplasm contains mainly free ribosomes and mitochondria	+1
	intermediate	+2
	well developed Golgi, Nissl bodies, dense core vesicles	+3
cytoplasmic size	small rim around nucleus	+1
	intermediate	+2
	ratio of cytoplasm to nucleus>1	+3

Neurons with scores of +3 or +4 were classified as primitive sympathetic cells, +5 to +7 as neuroblasts and +8 to +9 as young sympathetic neurons. In addition to the cultured cells, neurons from 4 ganglia taken from different embryos that were not cultured but fixed immediately were analyzed and staged as above. In all, a total of 252 neurons were studied, 57 of which were from 5 day cultures, 62 from 10 day cultures, 44 from 20 day cultures and 89 from *in vivo* ganglia.

V. Nonneuronal Cell Counts

Nonneuronal cells were counted from photographs taken on a phase microscope using a 10x objective. Counts were done on cultures maintained in defined medium for 5 and 12 days. Only areas where cells were present were used for counts although many areas of the defined medium culture dishes were devoid of any cells. At least 25 photographs from each time point were examined and counted. Results were reported as the number of nonneuronal cells per 0.17 mm^2 area of the culture dish. Counts were not done on serum-supplemented cultures because the large number of nonneuronal cells made accurate counting impossible.

RESULTS

I. Phase Microscopy

A. Defined Medium

Partially dissociated human fetal sympathetic ganglion cells settled and attached to the tissue culture dish within the first 24 hours. Neurite extension was observed shortly thereafter. Neurites emerged from the cells without any evidence of investment by supporting cells and in many instances before any migration of nonneuronal cells from the small explants had begun. In the first 72 hours there was little branching and anastomosis of fiber bundles (Fig. 1) but by 5 days this was extensive (Fig. 2). In many instances, nerve fibers appeared to emerge from the small clumps of neurons in bundles (Fig. 3). The distal portion of the growing axon as observed by phase showed the typical morphology of a growth cone (Fig. 4): fine filopodia extending from an enlarged area at the base of the growing neurite. Terminal portions of the neurites could be found in contact with the substrate as in Fig. 4, in contact with nonneuronal cells as in Fig. 1, or in contact with other neurons as in Fig. 2. Many individual neurons seen within the first 48 hours after plating either failed to survive or aggregated into small groups since their occurrence was much less evident in older cultures. Indi-

vidual neurons could be found in older cultures (Fig. 5) but most neurons were in varying size clumps. The neurites at first appeared firmly attached to the substrate but observations 72 hours and later revealed that slight movements of the dish would cause the neurites to "sway" within their central portion suggesting that only the somas and terminal portions of the neurites were attached. This was later confirmed in thick and thin sections of the cultures and was also evidenced in phase micrographs of older serum-supplemented cultures in which fiber bundles appeared above the confluent monolayer of nonneuronal cells. The cell bodies of the neurons remained rounded and very refractile. They did not flatten out over the surface of the culture dish throughout the experiment. Their rounded morphology, refractiveness and neurite extension made them very easy to recognize (see Figs. 1-19).

Observations 48 to 72 hours after plating revealed the presence of nonneuronal cells spread out on the surface of the culture dish. In areas where small groups of neuronal cells had attached, migration of nonneuronal cells from the clumps was first evident at about 48 hours. Two morphologically different nonneuronal cells were present. A large, flat, pleomorphic cell with abundant cytoplasm, centrally located nucleus and prominent nucleolus resembled that of fibroblasts (Figs. 3 and 4). Defined medium cultures older than 10 days had fibroblast-like cells with a slightly

different morphology. These cells were large, flat, but usually circular and their cytoplasm appeared stretched over the surface of the culture dish (Figs. 6 and 7). Flat cells, intermediate in size and shape, also were found and suggested that the large, flat circular cells seen in the older cultures were of the same type as those seen earlier and that some change in morphology of these cells was occurring in defined medium. The fibroblast-like cells did not associate with the outgrowing neurites and in later cultures were often isolated by a short distance from other nonneuronal cells. Fiber bundles were seen to course above the plane of fibroblasts. It could not be determined for certain if nerve endings terminated on this type of nonneuronal cell, although in some instances it appeared to do so (See Fig. 7).

The second type of nonneuronal cell was smaller in size and its cytoplasm appeared much denser. Frequently, these cells would form a "chain" of several cells stretched end to end (Fig. 8). In a few instances, this smaller nonneuronal cell appeared to associate with the neurite along its length (Fig. 2), and at its ending (Fig. 1). Most neurites in the cultures from all the time points examined and in both media, however, appeared devoid of any investment by nonneuronal cells.

After 2 weeks in defined medium, the neurons appeared

healthy and fiber formation was elaborate with extensive branching and anastomosis of fiber bundles (Figs. 9 and 10). Some fibers were as long as 2 mm (Fig. 11). Non-neuronal cells were still present on the surface of the dishes. The presence of nonneuronal cells in defined medium cultures at 12 days and later appeared less than that seen in the earlier cultures and markedly reduced from the number of nonneuronal cells in the same age serum-supplemented cultures (compare Figs. 9 and 14). While both types of nonneuronal cells persisted, the most frequently encountered nonneuronal cells in these later cultures were the large, flat, circular fibroblast-like cells (Fig. 6). Although the number of nonneuronal cells in defined medium cultures appeared to decrease between 1 and 2 weeks, one isolated area containing fibroblast-like cells was followed closely over a 9-day period of time and cell numbers within that area increased from 9 cells on day 3, to 24 cells on day 12. Counts of nonneuronal cells from phase micrographs also suggested an actual reduction in the number of nonneuronal cells present. On day 5, background cells averaged 18 per 0.17 mm^2 while on day 12 they averaged 7 for the same area of dish. Only areas of the dish where cells were present were used for counts. Many areas of the dish were devoid of any cells and therefore this value does not represent the average number of nonneuronal cells per unit area of the dish but rather gives an estimate of the density of nonneuronal cells present whenever they were

encountered. In addition, any glial cells which formed close associations with the neurons would not be counted.

B. Serum-supplemented Medium

Sympathetic ganglion cells plated in serum-supplemented medium also attached readily to the culture dish. Neurite extension was noted within 24-48 hours after plating. The morphology and extensive neurite elaboration were similar to that found in the neuronal cells plated in defined medium (Figs. 12-17). Nonneuronal cells were present (Figs. 12 and 13) and at 5 days were about equal in number to those seen in defined medium cultures. The population of nonneuronal cells increased rapidly in serum-supplemented medium and by 12 days formed a confluent layer (Figs. 14-17). The abundance of nonneuronal cells often obscured viewing of neurons and neuronal profiles. The nonneuronal cells observed by phase microscopy appeared spindle-shaped or triangular in the early cultures but it was impossible to discern their shape once crowding began.

C. Thick Sections

The rounded morphology of the ganglion cells cultured in either medium was evident in thick sections (2-5 μm). This was seen in sections cut either parallel (Fig. 18) or perpendicular (Fig. 19) to the substrate and was also true

of neurons found either singly or in clumps. Very often, there was a prominent nucleolus located eccentrically. Glial cells, much smaller in size than the neurons, could occasionally be found adjacent to them. Most neurites were "naked", that is, they were not ensheathed by glial cells. Neurites emerging from the small groups of neuronal cells were situated well above the substrate throughout their course (Fig. 21). This was consistent with their "clothes-line" appearance as seen in the living cultures. Small groups of neuronal cells were often only attached to the substrate by a few neuronal cells while the remainder did not make contact with the substrate. These neuronal cell somas were in direct contact with the substrate (Fig. 21) and did not need a layer of nonneuronal cells for attachment.

D. Medium Minus NGF

When NGF was omitted from either chemically defined or serum-supplemented medium, the human ganglion cells attached to the substrate but either failed to extend neurites or showed only sparse neurite outgrowth (Figs. 22 and 23). (At this time, sister cultures plated in medium with NGF showed extensive neurite formation - see Figs. 2 and 12). The neurons maintained a rounded morphology. Nonneuronal cells proliferated in serum-supplemented medium but they were sparse in defined medium. Cultures without

NGF were only maintained for 5 days.

II. Electron Microscopy

A. Defined Medium

1. Fine structure of neuronal cells Neuronal cells representing various stages of differentiation (Fig. 24) were seen at 5, 10, 20 and 40 days in culture. The most immature appearing cells had dense karyoplasm with a prominent ring of condensed chromatin inside the nuclear envelope (Figs. 24-26). Cytoplasm was scant and formed a thin rim around the nucleus. Free ribosomes were abundant in the cytoplasm and there were a few mitochondria. Golgi complexes were sparse or not evident. When they were found, they had only a few cisternae and were short in length. Rough endoplasmic reticulum (rer) was only rarely found and consisted of a few scattered cisternae. An occasional large dense core vesicle (LDCV), measuring 70 to 130 nm, was also seen in the cytoplasm. These cells correspond to the primitive sympathetic cells described by Hervonen and Kanerva (1972) (see Table II).

More mature neuronal cells comparable to Hervonen and Kanerva's neuroblast types I and II had nuclei with more evenly dispersed chromatin (Figs. 27-28). The condensed perimembranous rim was greatly reduced or no longer evi-

dent. The cytoplasm was generally increased in size and showed a more advanced degree of differentiation. Ribosomes existed mainly as polysomal aggregates or were found associated with endoplasmic reticulum. More mature Golgi complexes were frequently found. The laminar components of the Golgi were greater in number and more extensive in length than in the primitive cells. There tended to be an increase in the number of mitochondria. The dense core vesicle population was randomly dispersed throughout the cytoplasm or in small clusters (Figs. 29-34). The cytoplasm also contained scattered microtubules as well as 10 nm filaments.

The most mature neurons seen in the cultures had more extensive cytoplasm, which contained numerous mitochondria, LDCVs and frequent stacks of rer arranged as Nissl bodies. Golgi complexes had many cisternae and vesicles. Cells frequently contained 2 or more such complexes (Fig. 35). The nucleus was large and had a typically vesicular appearance (Fig. 35), with little or no condensed chromatin. These cells were classified as young sympathetic neurons according to the terminology of Hervonen and Kanerva (1972).

In addition to the cytological features noted above, cell somas of many of the ganglion cells had large vesicles containing swirls of membranes (Fig. 35). These were found scattered throughout the cytoplasm and were more prevalent

in the 20 and 40 day cultures. The vesicles are referred to as laminar bodies and their significance will be addressed in the discussion.

2. Neuritic profiles At all the time periods examined, the cultured neurons exhibited extensive neurite formation. Processes were often found between the tightly packed neuronal cells and extending to adjacent groups of cells. Transverse and longitudinal sections through these bundles showed the neurites to contain microtubules, 10 nm neurofilaments, occasional LDCVs, smooth endoplasmic reticulum and mitochondria (Figs. 36-38). The average diameter of the neurites was 0.4 μm but ranged from 0.2 to 1 μm. Neurite bundles (Fig. 36) extending between adjacent groups of neurons were not wrapped by cytoplasmic extensions from glial cells and were almost always found as naked bundles. Nerve cells cultured for 20 and 40 days had processes which frequently showed varicosities along their length. Small clear vesicles (30-50 nm) and occasional LDCVs were contained within the varicosities. Elongated clear vesicles resembling profiles of smooth endoplasmic reticulum were also seen (Figs. 39-42). Synapses were only found in cultures maintained for 20 days or longer. The synapses terminated on nerve cell perikarya and contained small clear vesicles and a few large dense core vesicles. Mitochondria were present in some of the profiles. Membrane thickenings were evident on the pre- and post-

synaptic sides of the apposed membranes (Figs. 43-46).

3. Developmental stages All the different stages of neuronal maturation were found at 5, 10, 20 and 40 days. Neuronal cell differentiation ranged from an immature embryonic appearing cell to a mature young neuron. While all stages were found at all time points, there appeared to be a shift toward the more mature sympathetic neuron over time in culture. Classifying cells into one of several developmental stages as described by Hervonen and Kanerva (1972) confirmed this. Cells were designated as either primitive sympathetic cells, neuroblasts or young sympathetic neurons according to the criteria described in Methods. At 5 days in culture, 2% of the cells counted were labelled as young sympathetic neurons while at 20 days, 50% of the cells displayed characteristics that allowed them this designation (Table III). There were, however, even at 40 days in culture, cells which could be classified as primitive sympathetic neurons, but the proportion of these cells decreased over time in culture. These observations suggest that some of the sympathetic neurons progressed to more mature developmental stages during the period in culture. No differences were found between serum-supplemented and defined media cultures when examining the number of cells in different stages over time in culture. The results in Table III are the combined populations of neurons in both media.

TABLE III

NEURONAL MATURATION*

% of cells

stage	days in culture 5	10	20	in vivo
primitive sympathetic cell	49	39	5	62
neuroblast	49	58	45	36
young sympathetic neuron	2	3	50	2
n	57	62	44	89

*Classification of neurons was based on scoring for 3 independent observations. These were nuclear differentiation, cytoplasmic differentiation and ratio of cytoplasm to nucleus. The different stages of neuronal maturation were taken from the classification of Hervonen and Kanerva, 1972

4. Nonneuronal cells A few nonneuronal cells were seen in thick and thin sections of cultures from all time periods. One type of nonneuronal cell, most likely glial, was smaller in size and had nuclear and cytoplasmic material much denser than any neuronal cell (Figs. 47-49). This cell type was occasionally found within a small group of neuronal cells (Fig. 47) as well as isolated from them (Figs. 48 and 49). When not in association with neuronal somas, this cell type could either form attachments to the substrate (Fig. 48) or to the surface of other nonneuronal cells (Fig. 49). The nucleus frequently had an irregular contour with cytoplasmic indentations which gave it a kidney-shaped appearance. In some sections, although rarely, the nucleus appeared ovoid or lobular. In all cases, the nucleus contained very dense chromatin which formed a broad band around the nuclear membrane (Fig. 48). Because of the patchy appearance of the nucleus, it was not possible to visualize a nucleolus. The cytoplasm, also dense, contained many small polyribosomal aggregates, a few scattered cisternae of rer, a Golgi complex and mitochondria. Quite often the cytoplasm also contained large electron dense vacuoles. In some instances, cytoplasmic projections from these cells could be seen to partially envelop neighboring neurites (Figs. 47 and 49) and some evidence of investment of neuronal somas also was found.

A second type of nonneuronal cell, resembling that of

fibroblasts, was usually found lying flattened and adjacent to the substrate. This cell was most frequently found in monolayer as in Fig. 50, but occasionally cytoplasmic processes from nearby cells would extend to and traverse the upper surface of the cell. The nucleus of this cell was usually ovoid and frequently extensive in length. Indentations of the nucleus were occasionally observed. Nuclear material was homogenous in appearance with only a thin rim of condensed perimembranous chromatin. Usually one prominent nucleolus was noted. A striking feature of the cytoplasm was the long elaborate lengths of endoplasmic reticulum, both smooth and rough (Figs. 50 and 51). Golgi complexes (often 2 per cell), mitochondria, free ribosomes, and electron dense vacuoles were present also. Cytoplasmic blebs on the surface of the cell opposite the substrate were seen in some sections.

B. Serum-supplemented Medium

1. Fine structure of neuronal cells and processes

A similar range of neuronal cell types was found in cultures grown in the presence of serum (Fig. 52). Ultrastructural characteristics of cell somas and processes at these various stages of neuronal maturation were comparable to those seen in the serum-free cultures. The primitive sympathetic cell had condensed chromatin and scanty cytoplasm while the neuroblast showed increasingly more dis-

persed karyoplasm and a developmentally advanced organelle population (Figs. 52-54). Short stacks of rer (Nissl bodies) were a frequent finding in the extensive cytoplasm of young sympathetic neurons (Figs. 55 and 56). As in the defined medium cultures, varicosities and synaptic profiles were evident in the 20 day cultures but were not found in earlier cultures. Occasional synaptic profiles with clear synaptic vesicles, a few large dense core vesicles and membrane thickenings were seen to terminate on neuronal perikarya. Varicosities containing small agranular vesicles and a few large dense core vesicles but lacking any membrane specializations were more common (Figs. 57 and 58).

2. Nonneuronal cells Nonneuronal cells were frequently encountered in serum-supplemented cultures at all time periods. The confluent monolayer of nonneuronal cells that covered the entire surface of the culture dish, as observed by phase microscopy, was in actuality multilayered when viewed in sections cut perpendicular to the substrate (Fig. 60). It was not surprising to find 10 or more cells layered on top of one another in later cultures. Cells in this multilayered arrangement had the same general cytology as the fibroblast-like cells seen in defined medium cultures. The most striking features were an ovoid, elongated and centrally located nucleus lacking any appreciable condensed chromatin except for a thin rim just below the nuclear envelope; extensive and elongated rer; an abundance

of free ribosomes and extended lengths of flattened cytoplasm. Although the cytology of these cells was the same in both media, only serum-supplemented cultures contained multilayers of this cell type.

Cells classified as glial were occasionally found within small groups of neuronal cells (Fig. 61) or at some distance from any neuronal cell. These cells had the same fine structure as those seen in defined medium. Although no extensive quantitation was done, it appeared that these cells were encountered no more frequently in serum-supplemented than in defined medium and that glial investment of neuronal processes and perikarya was the same under both conditions.

C. Sympathetic Ganglion Cells In Vivo

The ultrastructure of neurons fixed immediately did not differ from the cultured neurons (Figs. 62-66). The primitive sympathetic cell was the most common type of neuronal cell encountered. Cells classified as young sympathetic neurons were only rarely seen. A greater degree of glial investment of the neuronal perikarya and processes was noted although it was considerably less than that seen in the adult. Varicosities and synaptic profiles were occasionally found. Synaptic profiles contained numerous small clear vesicles (30 to 50 nm) and membrane densities

but were usually devoid of dense core vesicles (Figs. 67-69). The profiles terminated on either neuronal perikarya or adjacent neurites and were thought to be the endings of the preganglionic fibers.

Small granule containing cells (SIF cells) were not found in the in vivo material examined although they were specifically sought. Small dense core vesicles were never encountered in the in vivo ganglia or in vitro ganglia cultured for up to 40 days.

III. Cytochemistry

A. Chromate-Dichromate

A positive chromaffin reaction was observed in the ganglion cells of all cultures fixed by the chromate-dichromate method (6 and 25 day defined medium cultures and 6 day serum-supplemented cultures) (Figs. 70-81).

1. Osmicated material Ultrastructural examination of unstained but osmicated material revealed dense precipitate within vesicles of a size range similar to the dense core vesicles found in the glutaraldehyde-osmium fixed cells (70-180 nm). Staining was omitted from these sections to insure that the densities seen were not due to staining artifacts. The amount of precipitate within

vesicles was variable. Some vesicles were filled such that it was difficult to discern their membrane, whereas other vesicles contained only a small dense core of material (Figs. 70-71). Vesicles containing a dense core were located in cell somas (Figs. 70 and 73) and processes (Figs. 72 and 74). Consistent with the first part of this study, clusters of large dense core vesicles were seen more frequently in the 25 day cultures than in the 6 day ones (Figs. 70 and 73). All other fine structural features of these cells were similar to those found in the glutaraldehyde-osmium fixed cultures.

Sections of this material were stained by a variety of techniques, including uranyl acetate plus lead citrate, uranyl acetate plus bismuth subnitrate, and uranyl acetate and lead citrate plus bismuth subnitrate. Optimal conditions were difficult to achieve and often resulted in coarse grained images (Fig. 75). The best results were obtained by staining for 20 minutes with 2% uranyl acetate followed by immersion in bismuth subnitrate for 3 minutes.

2. Unosmicated material

To insure that any electron dense material seen in the chromate-dichromate treated cultures was not due to the oxidizing effects of osmium, several cultures were reacted with chromate-dichromate but were not post-fixed in osmium. These cultures were either viewed unstained or stained with

uranyl acetate and bismuth subnitrate. Unosmicated, unstained material was difficult to view due to lack of contrast. Six day (defined medium and serum-supplemented medium) cultures and 25 day (defined medium) cultures, however, showed areas where reaction had occurred (Figs. 76-81). Older cultures were easier to scan for electron dense reaction product since they frequently had clusters of dense material. Clusters of electron dense material were not limited to 25 day cultures and were occasionally seen in younger cultures. These clusters resembled the cores of dense core vesicles and had a size range compatible with them (50-94 nm). No other cellular constituents with the same degree of electron density were seen.

Although the contrast in all the unosmicated material was poor, it was sufficient to visualize the outline of cells, processes, and even the nucleus within cells (Fig. 76). It could be discerned that the electron dense product was in cell somas (Figs. 76, 78, and 80) as well as processes (Fig. 79). Cellular organelles (except perhaps an occasional mitochondrion) and membranes could not be visualized. Staining of the unosmicated sections with one or more of the stains mentioned above increased contrast so that placement of the reaction product within the perikaryon or neuritic process could be confirmed. Post-staining without osmication did not improve visualization of vesicular membranes (Fig. 81).

The size range of the electron dense material (50-94 nm), its presence in cell somas and processes, its occurrence either singly or in clusters, and its persistence in unosmicated, unstained material is consistent with the size, location and distribution of large dense core vesicles seen in osmicated material. Cultures preincubated with 5' OHDA before chromate-dichromate fixation contained electron dense reaction product with the same size range, morphology, and distribution as untreated cultures.

B. Potassium Permanganate

Cultures treated with $KMnO_4$ showed only occasional single vesicles such as those seen in chromate-dichromate fixed cultures (Figs. 82-86), measuring 70 to 150 nm and with a dense core. Those vesicles which were found were usually seen in the cell soma. Clusters of dense core vesicles, as seen in chromate-dichromate treated and conventionally fixed cells, were not found in the permanganate fixed cultures. Other cellular organelles such as Golgi complexes, mitochondria, and endoplasmic reticulum were very apparent (Fig. 83). Varicosities with numerous small clear vesicles were seen (Figs. 85-86). Small dense core vesicles were never present in the varicosities, while large dense core vesicles were found only rarely.

Very large, electron dense, usually membrane-bound material (400-800 nm) was occasionally seen in the cell bodies of 6 day cultures and more frequently encountered in the 25 day cultures. These vesicles are most likely lysosomal in nature. Plasma membranes were often disrupted, suggesting that tissue preservation was generally poorer than that fixed with the other methods.

IV. Fluorescence Microscopy

After exposure to paraformaldehyde vapors, the ganglion cells exhibited a green to yellow fluorescence, characteristic of catecholamines (Figs. 87 and 89). The fluorescence appeared diffuse throughout the cytoplasm and of moderate intensity. Some ganglion cells were more strongly fluorescent than others. Processes were fluorescent also but their intensity was less than that seen in the cell somas. Background nonneuronal cells (Fig. 89) and ganglion cells not exposed to paraformaldehyde (Fig. 88) showed faint autofluorescence with occasional large brightly fluorescent orange-red granules.

V. KEY

(av) agranular vesicles

(c) centriole

(dcv) dense core vesicle

(er) endoplasmic reticulum

(f) filaments

(fb) fiber bundle

(g) Golgi complex

(G) Glial cell

(lb) laminar bodies

(m) mitochondria

(mt) microtubules

(mv) microvilli

(mvb) multivesicular bodies

(n) neuron

(nf) neurofilaments

(NB) neuroblast

(Nu) nucleus

(p) process

(PSC) primitive sympathetic cell

(rer) rough endoplasmic reticulum

(ser) smooth endoplasmic reticulum

(YSN) young sympathetic neuron

PLATE I

Figure 1. Ganglion cells grown in defined medium for 3 days. The neurons (n) are rounded and very refractile. Processes (p) with some evidence of fasicle formation are already present. A few non-neuronal cells can be seen in the background. One process (►) appears to be making contact with one of the nonneuronal cells. X 250

Figure 2. Ganglion cells grown in defined medium for 5 days. Branching of the neurites is common at this time. Many small groups of neuronal cells send processes to adjacent neurons. Most neurites appear "naked," but some processes are surrounded by nonneuronal cells, most likely glial (►). Another type of nonneuronal cell, larger and flatter in appearance, is indicated by (*). X 250

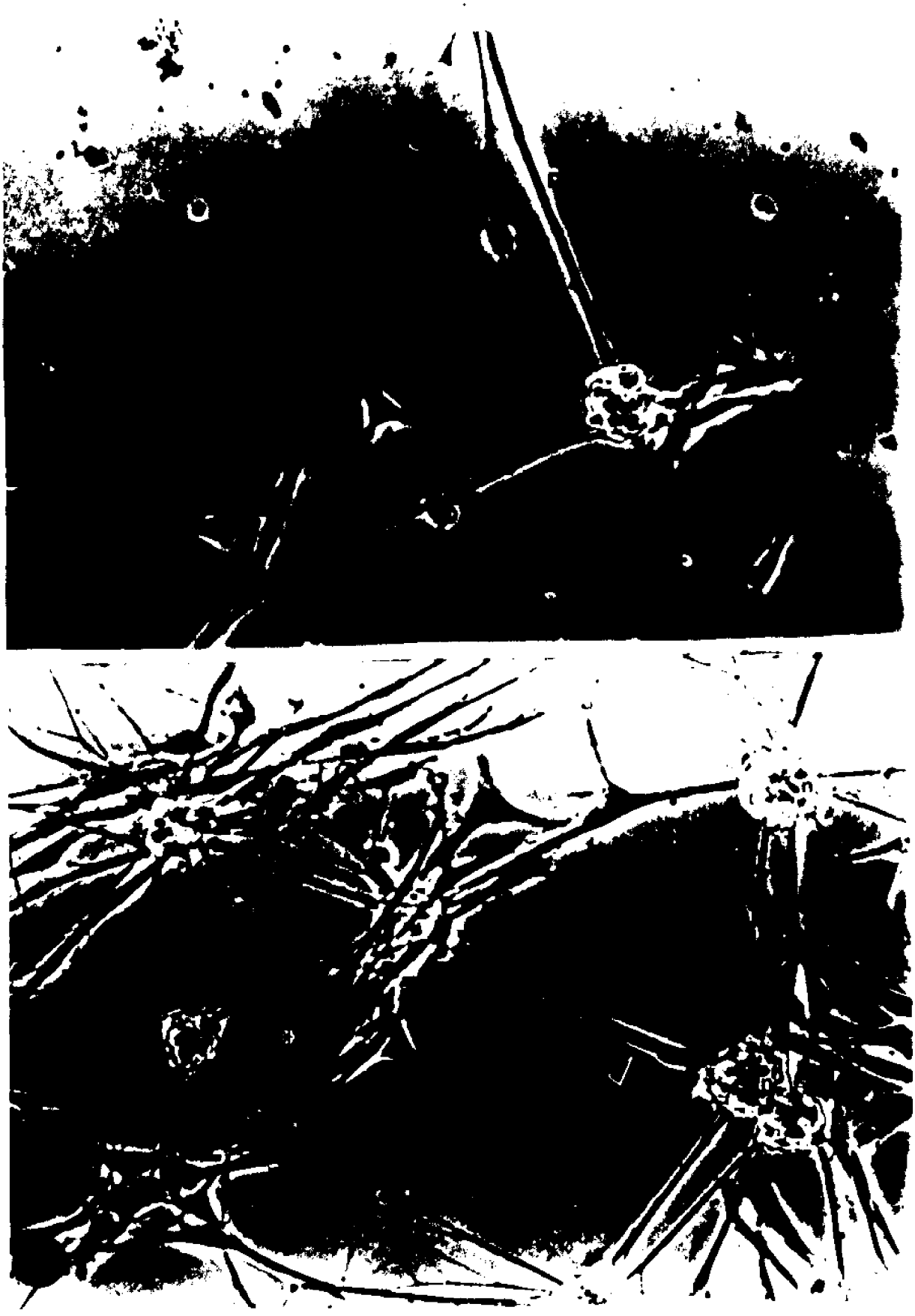


PLATE II

Figure 3. Neurons (n) at 5 days in defined medium extend processes (p) which emerge from the clumps in small bundles (►). X 250

Figure 4. The growing tips of the neurites (►) show the typical growth cone morphology. At the base of the neurite is an enlarged area from which extend fine "finger-like" projections, the filopodia. (*) indicates background nonneuronal cells. Defined medium, 5 days *in vitro*. X 250

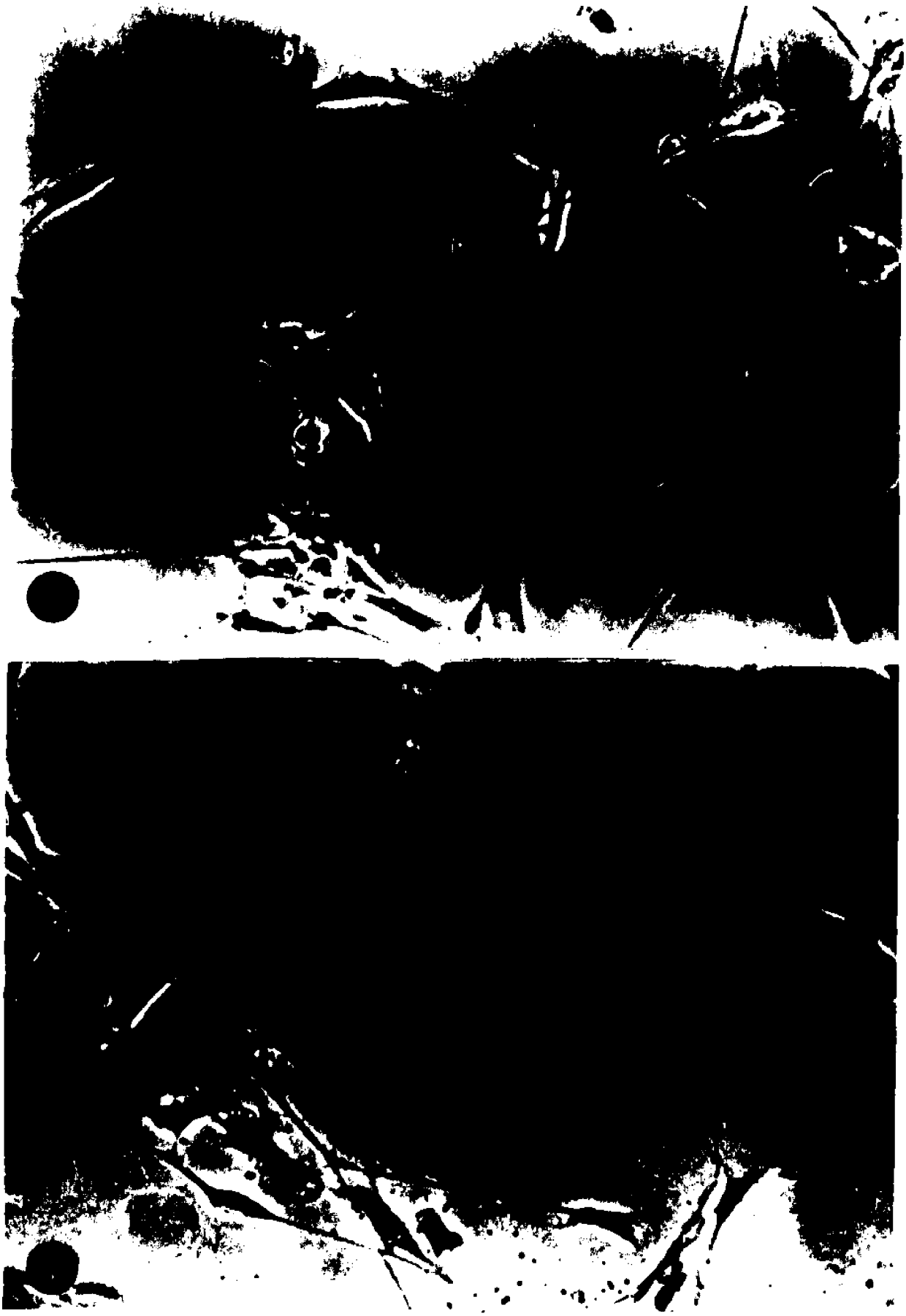


PLATE III

Figure 5. Defined medium culture of ganglion cells 12 days *in vitro*. Individual neurons (n) sending out fine processes (p) are still present in older cultures but most neurons are found in association with other neurons in varying size clumps. X 250

Figure 6. Nonneuronal cells (*) from a 20 day defined medium culture. In cultures older than 10 days, the substrate-adhering cells are usually round and have cytoplasm that covers extensive areas. This cell, although slightly different in morphology from those in earlier cultures, is thought to be the same type as that seen in Figure 4. X 250

PLATE IV

Figure 7. Nonneuronal cells adhering to the substrate in older cultures are usually large, flat and circular in shape. The cytoplasm has little contrast and stretches over the surface of the culture dish. Nonneuronal cells, even in 20 day defined medium cultures, as in this micrograph, are few in number. Fine processes appear to be making contact with 2 of the cells. X 250

Figure 8. Another type of nonneuronal cell has a very different morphology than that in Figure 7. This cell type is much smaller, denser, and frequently forms a "chain" of cells as at (►). It is occasionally found in association with neuritic processes, as in Figure 2, and is thought to be glial. Defined medium culture, 5 days *in vitro*. X 250

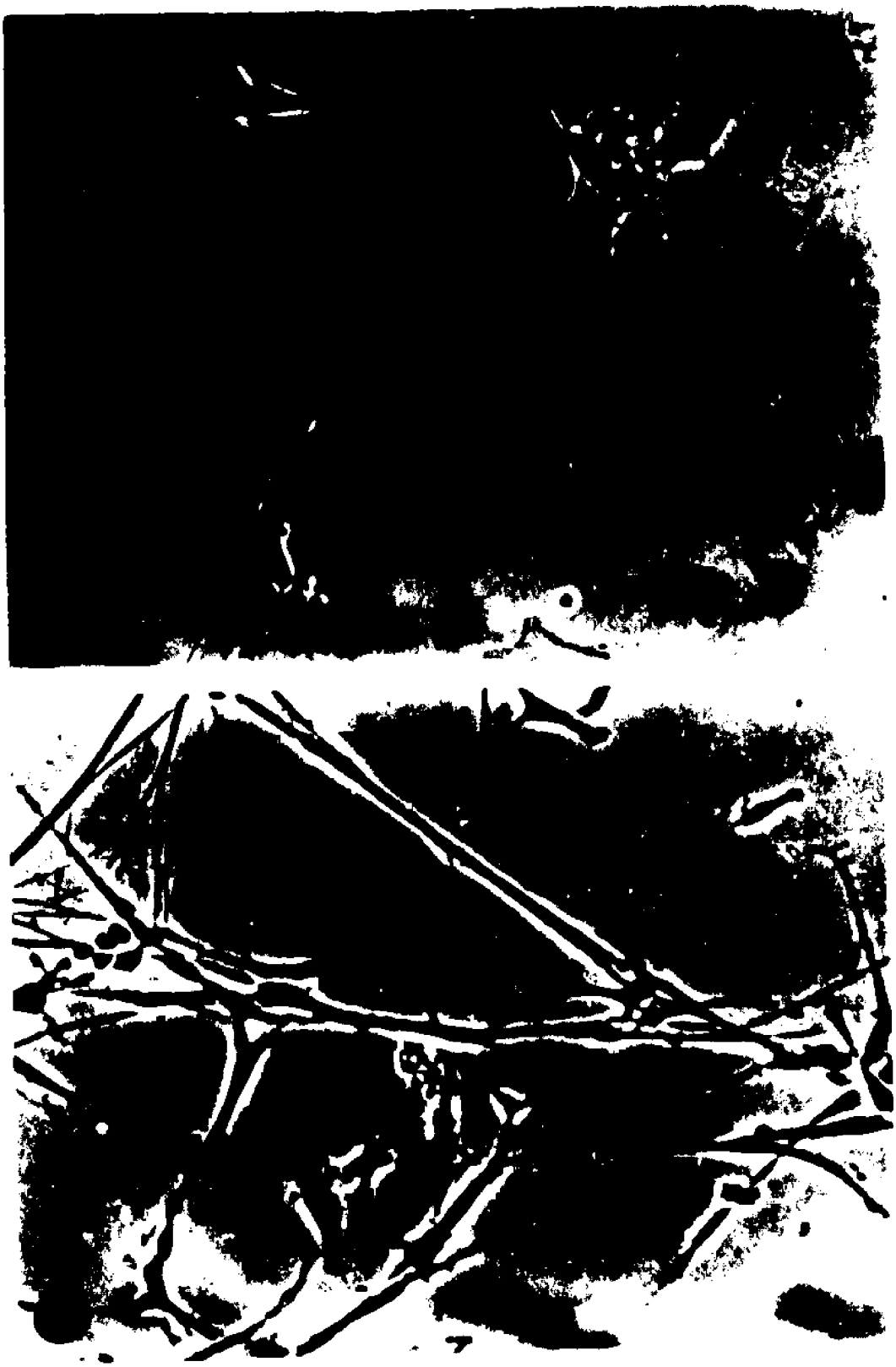


PLATE V

Figure 9. Neurons at 12 days in defined medium show elaborate and extensive processes that associate into fiber bundles (fb). The nonneuronal cells seen in the background appear fewer in number than those in 5 day defined medium cultures (Figure 2) and dramatically fewer than those in 12 day serum-supplemented cultures (Figure 14). X 250

Figure 10. Neurons maintained in defined medium for 12 days. X 250

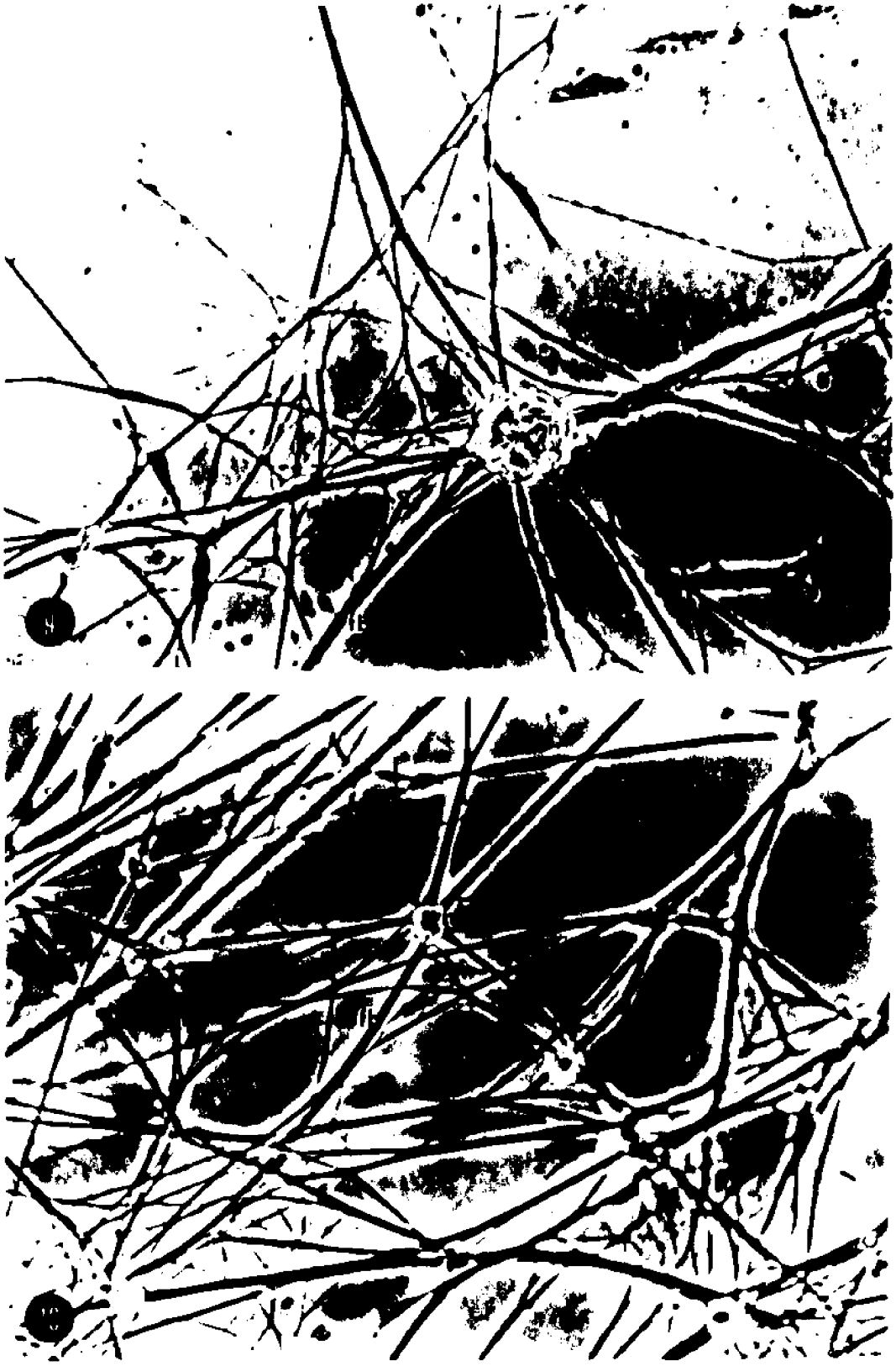


PLATE VI

Figure 11. Neurites are emerging from a group of neuronal cells at lower right and by 12 days in culture extend several mm in length. There are only a few nonneuronal cells in the background, in contrast to similar age serum-containing cultures. Defined medium culture, 12 days *in vitro*. X 150

PLATE VII

Figure 12. Culture of ganglion cells in serum-supplemented medium for 5 days. The general morphology of the neurons (n), the extent of process formation (p), and the relative density of the background nonneuronal cells is similar to that of the same age defined medium cultures. X 250

Figure 13. Ganglion cells cultured in serum-supplemented medium for 5 days. X 250

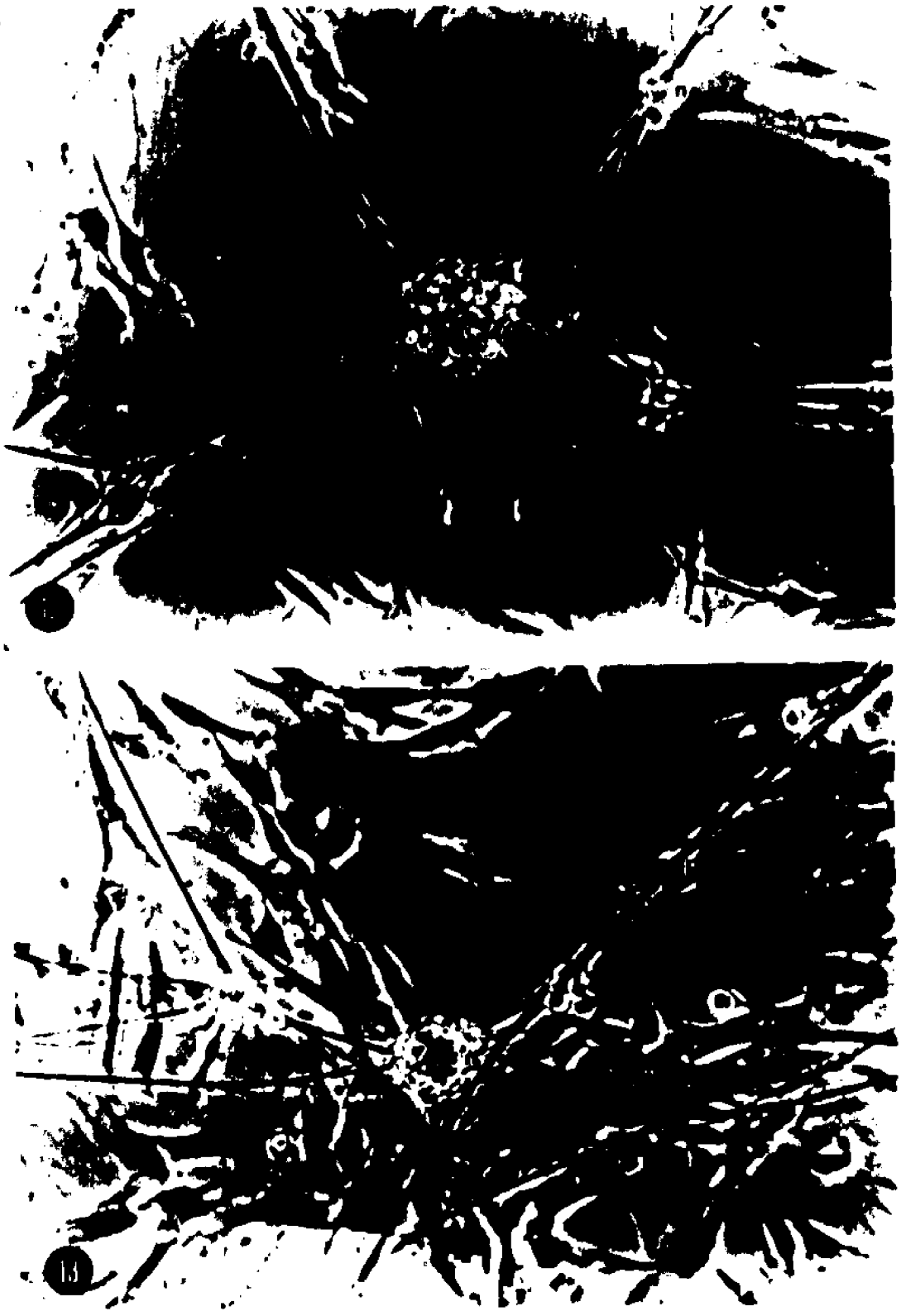


PLATE VIII

Figure 14. Neurons (n) with elaborate processes (p) course above the background nonneuronal cells. The nonneuronal cells are confluent by 12 days in serum-containing medium, much in contrast to their limited numbers in similar age defined medium cultures. Serum-supplemented medium, 12 days *in vitro*. X 250

Figure 15. Individual neurons and background nonneuronal cells from a 12 day serum-supplemented culture. X 250

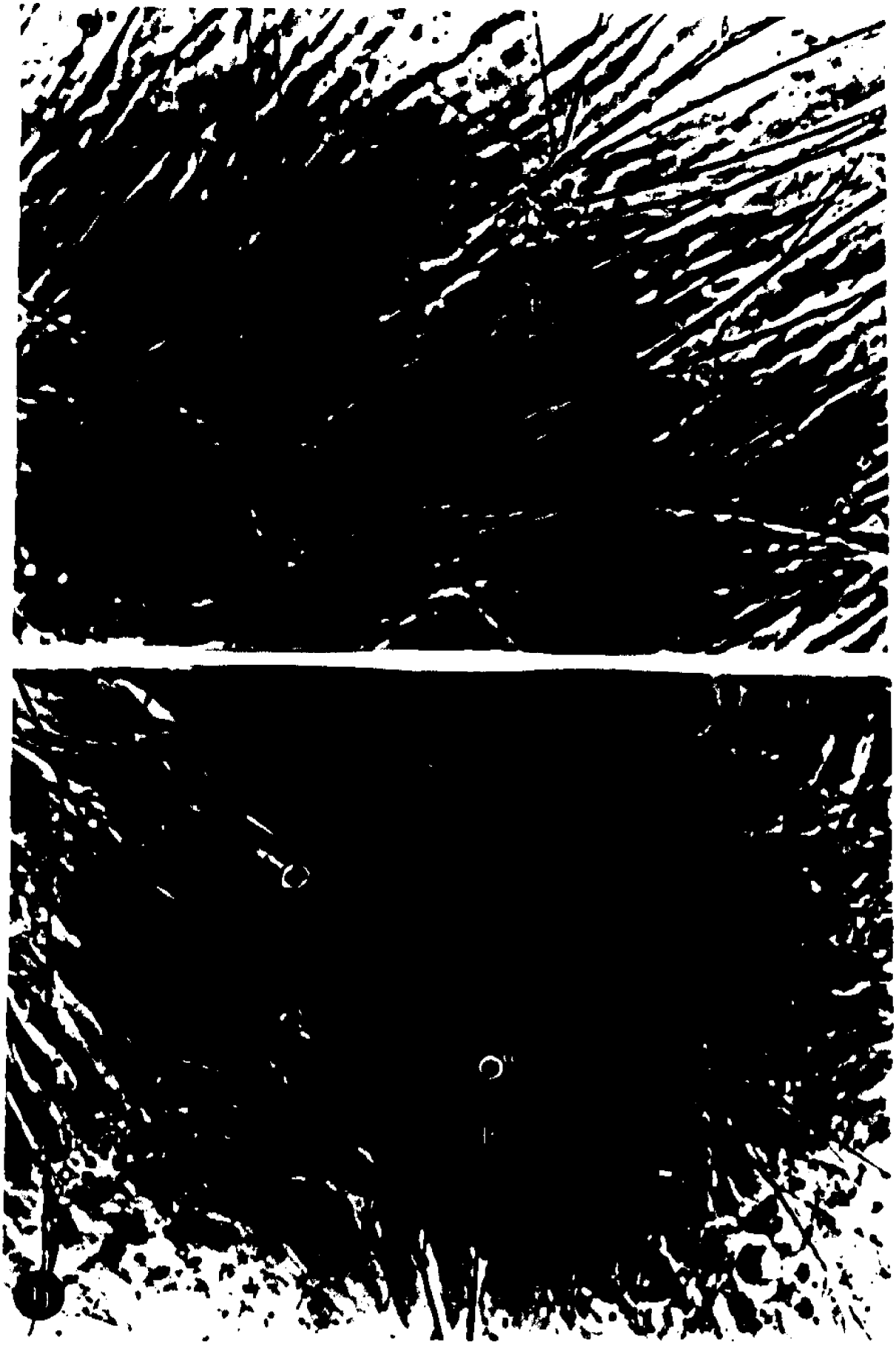
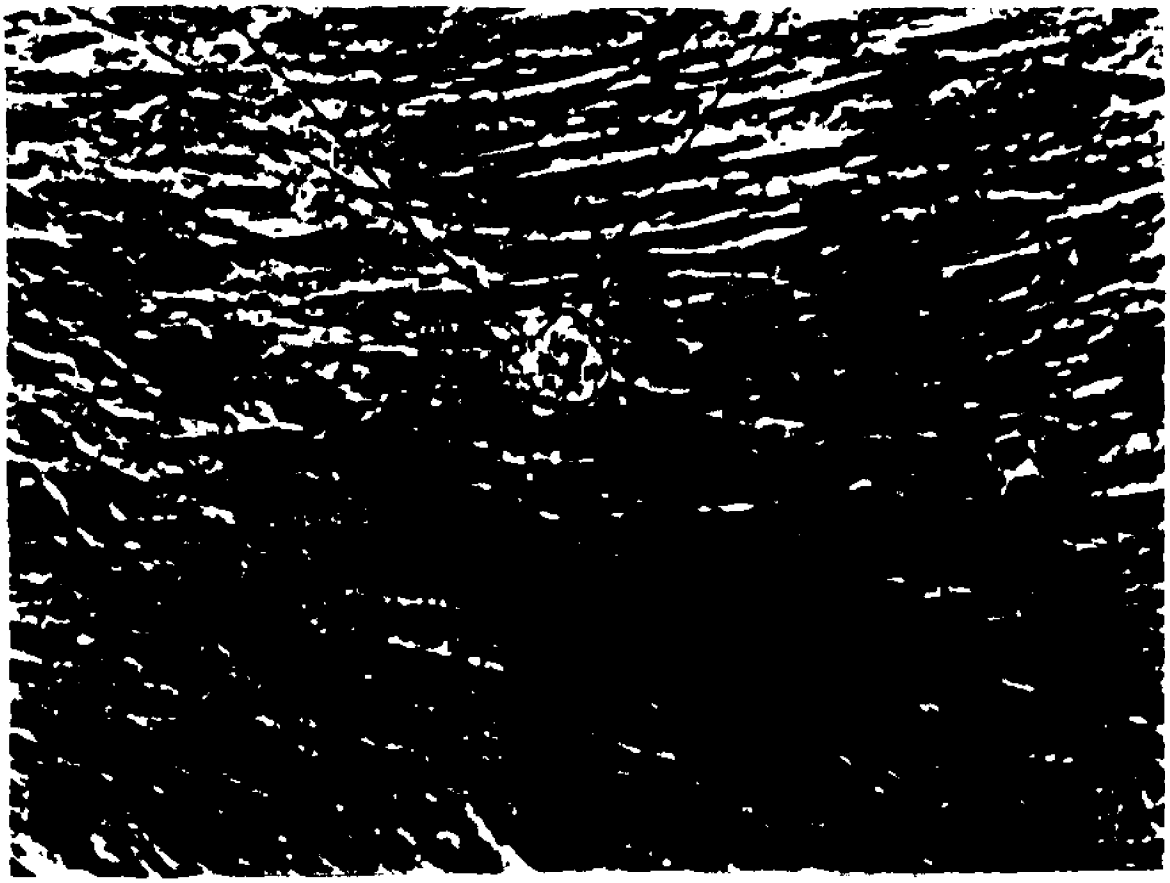


PLATE IX

Figure 16. Small group of neurons (n) with extended processes (p) are situated over a confluent layer of nonneuronal cells. Serum-supplemented medium, 12 days *in vitro*. X 250

Figure 17. Serum-supplemented culture at 12 days. Compare the number of background nonneuronal cells to that in Figures 9 and 10. X 250



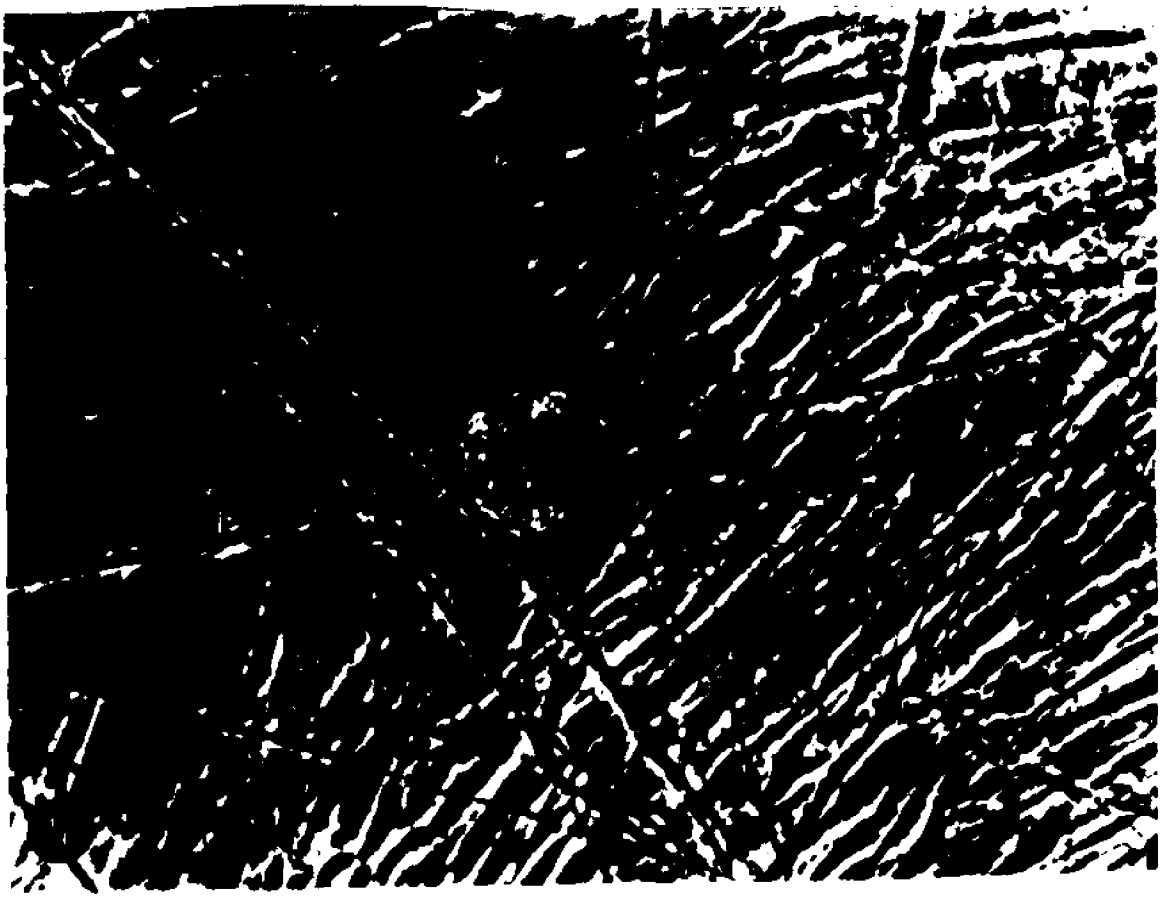


PLATE X figures have been stained in 1% toluidine in 1% borax

PLATE X

Figure 18. Thick section of a neuronal culture cut parallel to the substrate. The neurons remain rounded, have prominent nucleoli and were never seen to flatten during their time in culture. Defined medium culture, 10 days *in vitro*. X 1500

Figure 19. Thick section of cells cut perpendicular to the substrate show the same general morphology as those cut parallel. Defined medium culture, 10 days *in vitro*. X 1600

Figure 20. Perpendicular section of a multilayered arrangement of nonneuronal cells from a 20 day serum-supplemented culture. Substrate is indicated by arrows. X 1500

Figure 21. A large group of neuronal cells have processes that emerge from the clump in one general area at left. The processes are situated well above the surface of the dish and are not found to make contact with the substrate (arrows) except at their growing tip. Defined medium culture, 10 days *in vitro*. X200

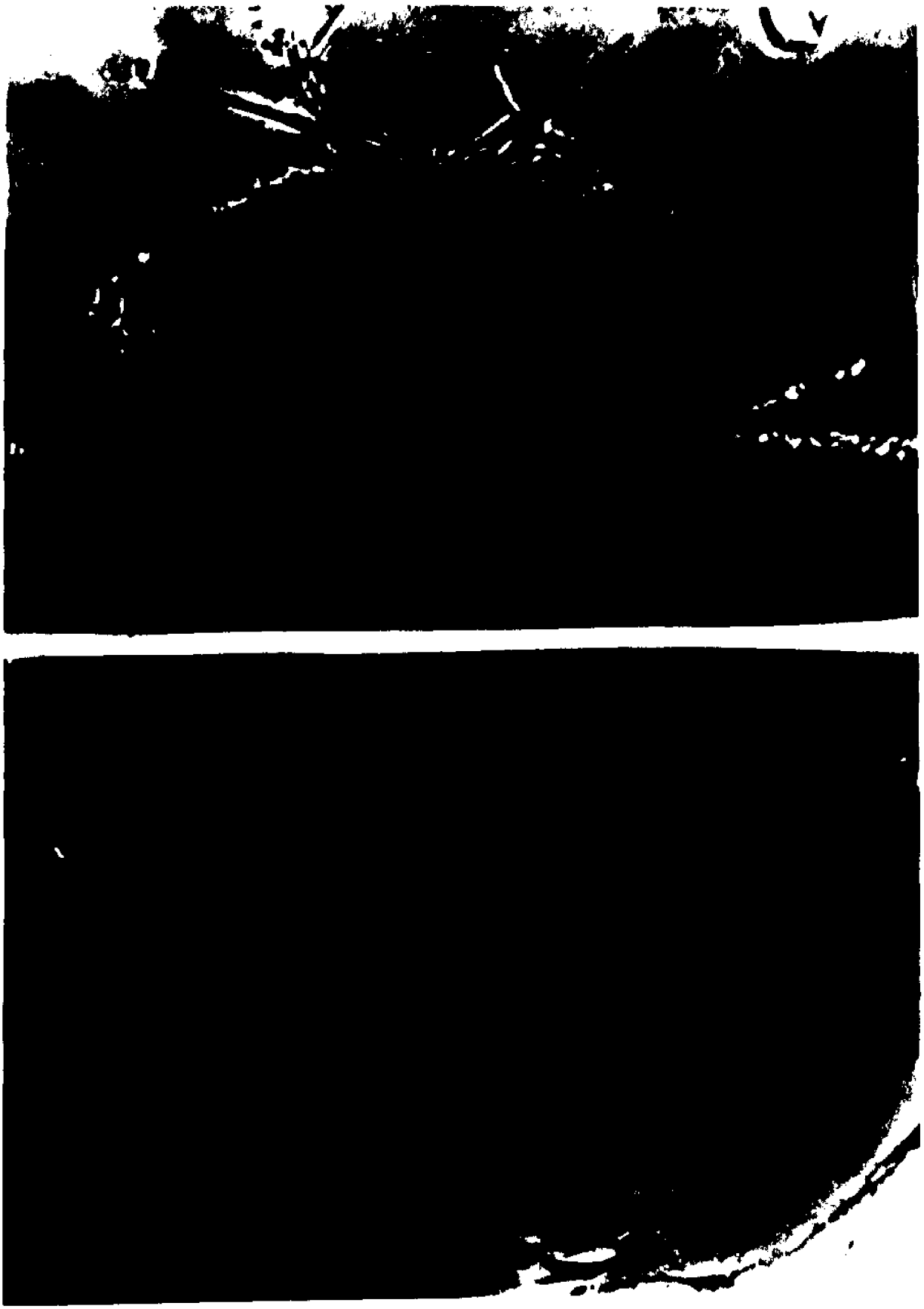
20

21

PLATE XI

Figure 22. Neuronal cells cultured in defined medium lacking NGF show little evidence of neurite formation. The same age defined medium cultures containing NGF have extensive processes (Figure 2). 5 days *in vitro*. X 250

Figure 23. Culture of ganglion cells maintained in serum-supplemented medium without NGF for 5 days. Compare with Figure 12. X 250



PLATES XII through XXXIV have been fixed in glutaraldehyde, post-fixed in osmium and stained with uranyl acetate and lead citrate.

PLATE XII

Figure 24. Electron micrograph of sympathetic neurons maintained in defined medium for 5 days shows a primitive sympathetic cell (PSC) surrounded by neighboring neuroblasts (NB). The primitive sympathetic cell is usually smaller in size, has little developed and scant cytoplasm, and a denser appearing nucleus. Plasma membranes of most of the adjacent neurons are in direct contact with one another. Processes (p), seen in longitudinal and transverse section, course in and around the neurons. X 9000

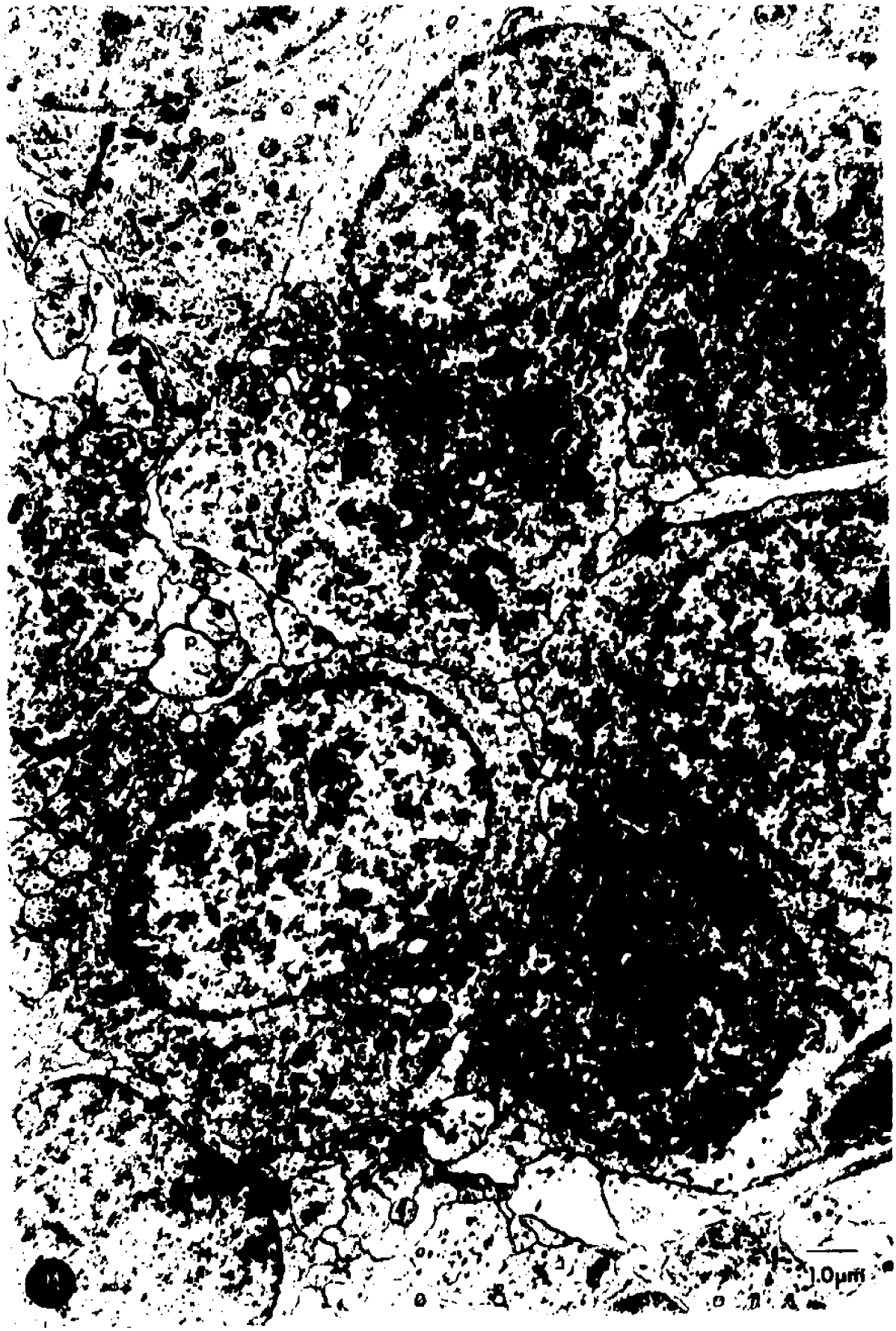

NB
P
1.0μm

PLATE XIII

Figure 25. Cell classified as a primitive sympathetic cell (PSC) has a nucleus with very condensed nuclear material giving it a dense, patchy appearance. The cytoplasm forms a thin rim around the nucleus and usually contains only free ribosomes and mitochondria. Defined medium culture, 5 days *in vitro*. X 17,000

Figure 26. Another primitive sympathetic cell showing its typically dense nucleus and scant cytoplasm is surrounded by several processes (p). Defined medium culture, 5 days *in vitro*. X 17,000

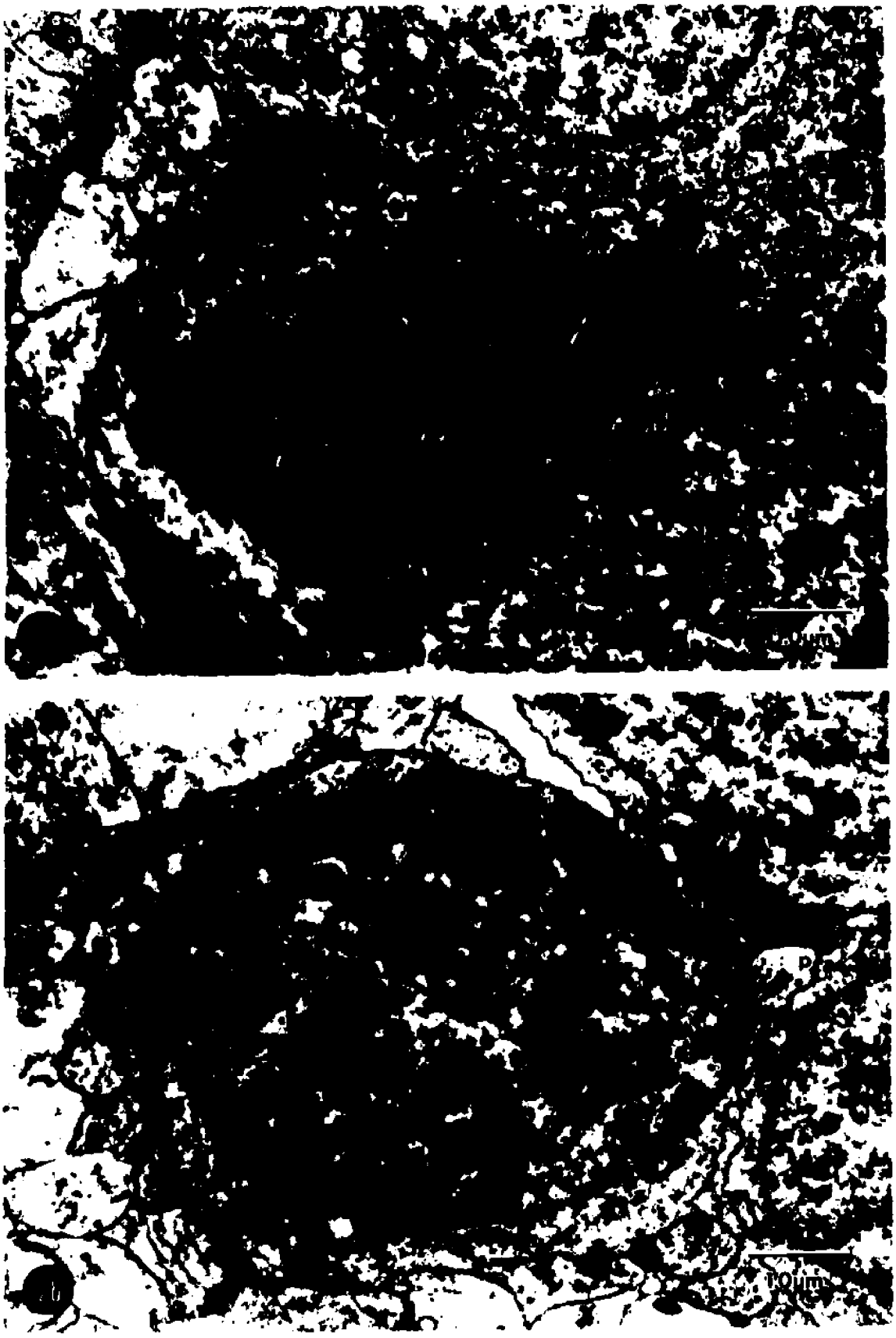

P
P

PLATE XIV

Figure 27. Cell classified as a neuroblast (NB) has condensed heterochromatin but the nucleus appears less dense than that of the primitive sympathetic cell. Rough endoplasmic reticulum (rer) is found in the cytoplasm of this stage cell but consists of only a few scattered cisternae. A Golgi complex (g), mitochondria (m), and a few large dense core vesicles are other usual cytoplasmic features. Defined medium culture, 5 days *in vitro*. X 18,000

Figure 28. Neuroblast from a 5 day defined meduim culture. X 18,000

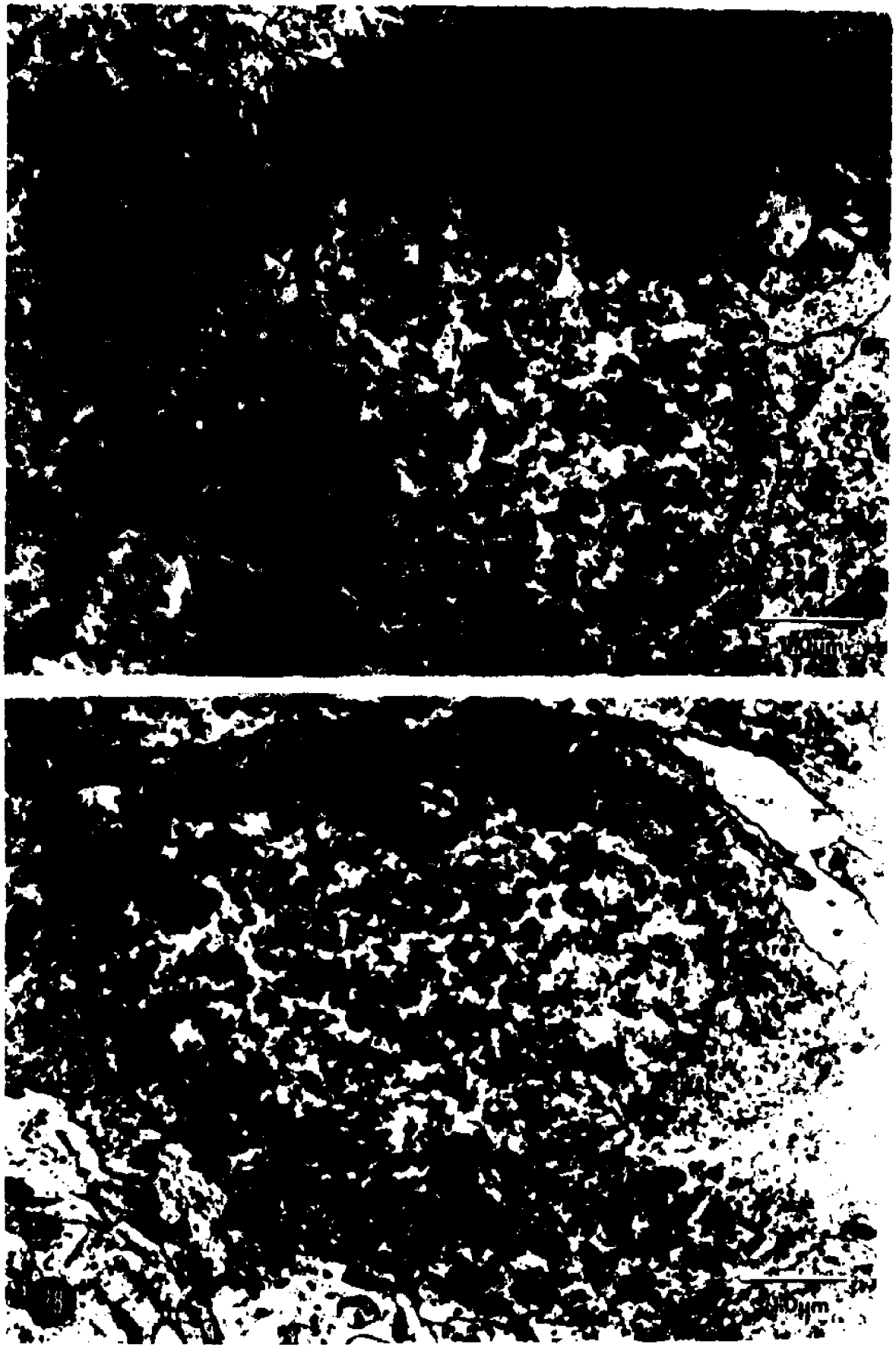


PLATE XV

Figure 29. Neuroblast (NB) from a 10 day defined medium culture has moderately well developed cytoplasm containing a few cisternae of rough endoplasmic reticulum (rer), a Golgi complex (g), mitochondria (m), and dense core vesicles (dcv). The nucleus has a thin rim of condensed chromatin near its periphery. Adjacent to the cell soma are cross sections through numerous processes (p), which make direct contact with the neuroblast's plasma membrane and show no evidence of glial cell association. X 12,000

Figure 30. Enlargement of area (*) from Figure 29 showing 2 large dense core vesicles (dcv). These vesicles are found randomly throughout the cytoplasm and occur either singly or in small clusters. X 26,000


1.0μm

PLATE XVI

Figure 31. Electron micrograph of a developing ganglion cell classified as a neuroblast (NB). The nucleus (Nu) of this cell contains more uniform appearing chromatin but still contains a prominent perimembranous rim of dense material. Defined medium culture, 10 days *in vitro*. X 23,000

Figure 32. Enlargement of area (*) from Figure 31 showing a cluster of large dense core vesicles (dcv), some of which appear completely filled and others which contain only a small dense core. These clusters are occasionally found in cells from 5 day cultures, but appear more frequently in the older ones. X 40,000


0.5µm

PLATE XVII

Figure 33. Cluster of large dense core vesicles in the cytoplasmic extension of a neuronal cell from a defined medium culture, 10 days *in vitro*.
X 23,000

Figure 34. Enlargement of area (*) from Figure 33. The dense core vesicles (dcv) are all of the large variety measuring 70 - 130 nm. They are usually round but occasionally appear ovoid as at (►).
X 32,000

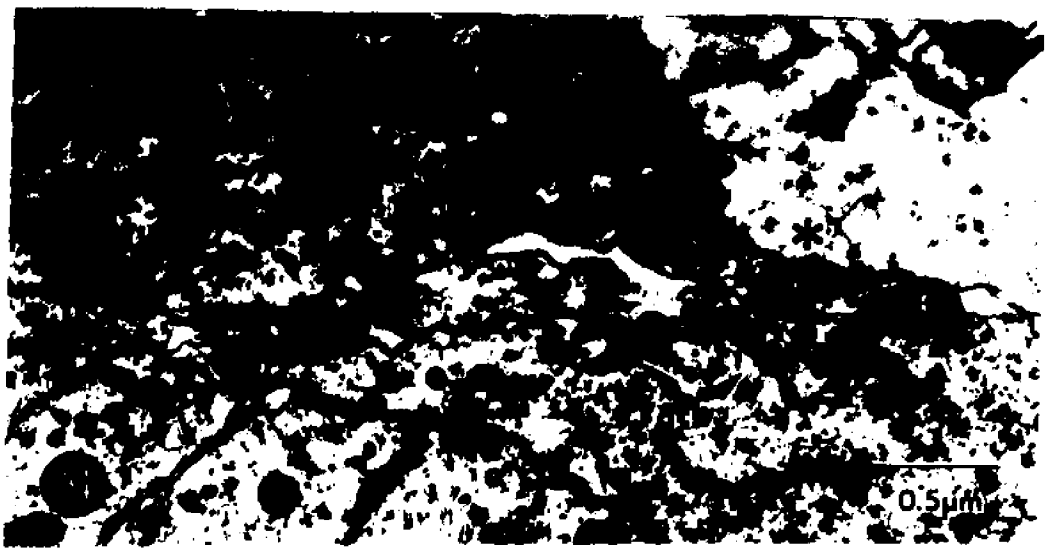

0.5μm

PLATE XVIII

Figure 35. Young sympathetic neuron (YSN) from a 20 day defined medium culture. The nucleus (Nu) contains homogeneous chromatin and there is no condensed material forming a perimembranous rim. The extensive cytoplasm (only part of which is shown here) is packed with organelles and in this section contains 2 Golgi complexes (g), rough endoplasmic reticulum (rer), mitochondria (m), multivesicular bodies (mvb), dense core vesicles (dcv), microtubules (mt), and 10 nm filaments (f). Several laminar bodies (lb) and moderately electron dense vacuoles are present also. X 22,000

35

PLATE XIX

Figure 36. A bundle of neurites seen in cross section shows them to be free of any Schwann cell ensheathment. Neurites emerging from small groups of neuronal cells frequently associate with other processes and form discrete bundles. Defined medium culture, 10 days *in vitro*. X 23,000

Figure 37. Longitudinal section through a process (p) which contains a parallel arrangement of microtubules (mt), and 10 nm filaments (f). A laminar body (lb) is seen in the cytoplasm of an adjacent cell. Defined medium culture, 20 days *in vitro*. X 24,000


1.0µm

PLATE XX

Figure 38. Transverse section through a neurite bundle maintained for 10 days in defined medium. The neurites contain numerous 10 nm neurofilaments (nf), microtubules (mt), smooth endoplasmic reticulum (ser), and an occasional mitochondrion (m). A dense core vesicle (dcv) is seen in one of the neurites. X 85,000

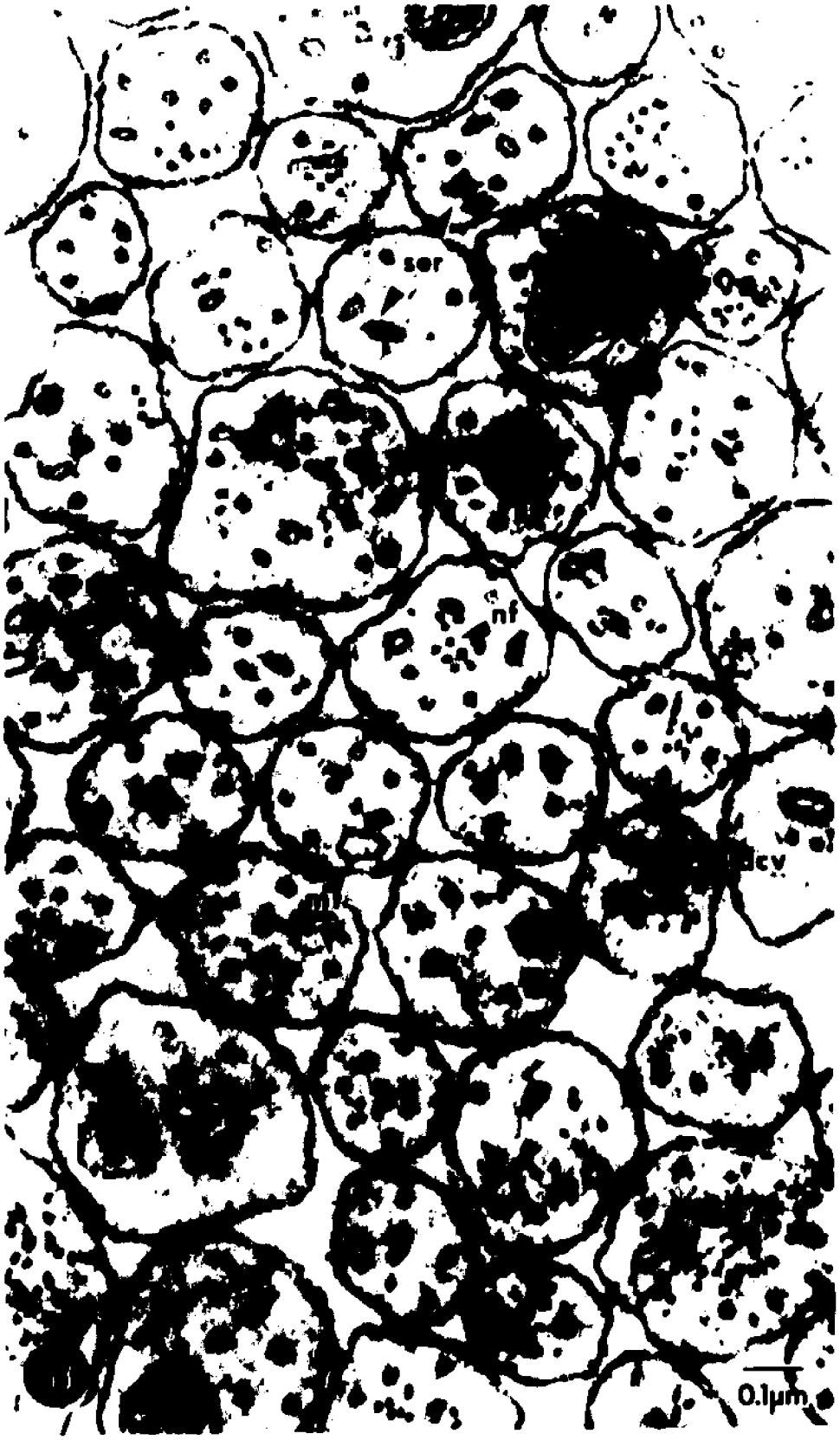

ser
nf
dcv
0.1µm

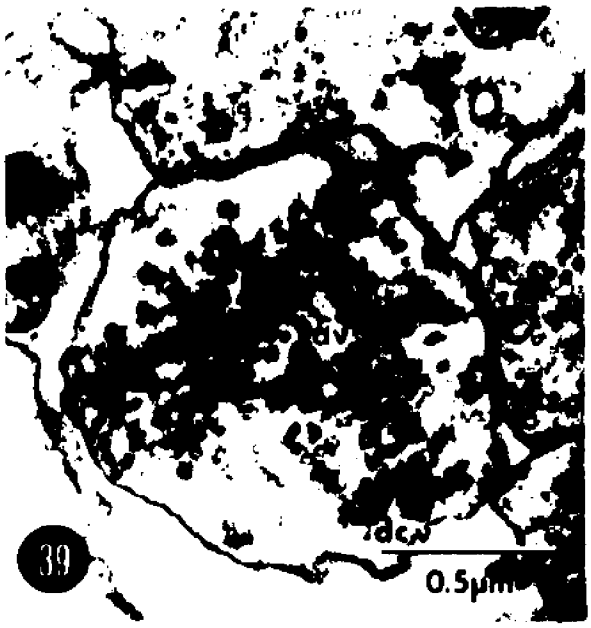
PLATE XXI

Figure 39. Varicosity seen in a 20 day defined medium culture. Agranular vesicles (av) and occasional large dense core vesicles (dcv) are the usual ultrastructural features. Membrane densities are not found and apposed membranes have irregular contours. X 44,000

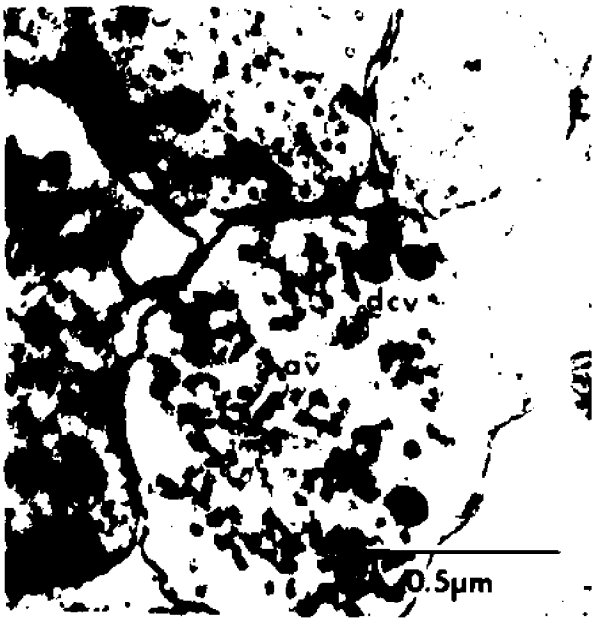
Figure 40. Varicosity similar to that in Figure 39. Defined medium culture, 20 days in vitro. X 48,000

Figure 41. Another varicosity from a 20 day defined medium culture. Some profiles of smooth endoplasmic reticulum are found in the varicosity. X 32,000

Figure 42. Varicosity from a 20 day defined medium culture shows close association of some of the vesicles with the membrane and more regular contours of the apposed membranes, perhaps indicating the development of a synapse. A multivesicular body (mvb) is seen in the lower half of the micrograph. X 32,000



39



41

PLATE XXII

Figure 43. A young sympathetic neuron from a 20 day defined medium culture is contacted at left by a synapse (*). X 13,000

Figure 44. Enlargement of area (*) from Figure 43 shows a synaptic profile filled with small agranular vesicles (av), and a few large dense core vesicles (dcv). Pre- and post-synaptic membrane densities are present at (►). Defined medium culture, 20 days *in vitro*. X 25,000

Figure 45. Synaptic profile with an unusually large number of dense core vesicles (dcv). Membrane densities are indicated by (►). Defined medium culture, 20 days *in vitro*. X 40,000

Figure 46. Another synaptic profile from a 20 day defined medium culture. The synapse shows typical fine structural features except for a small cluster of what appear to be ribosomes at left (►). X 36,000

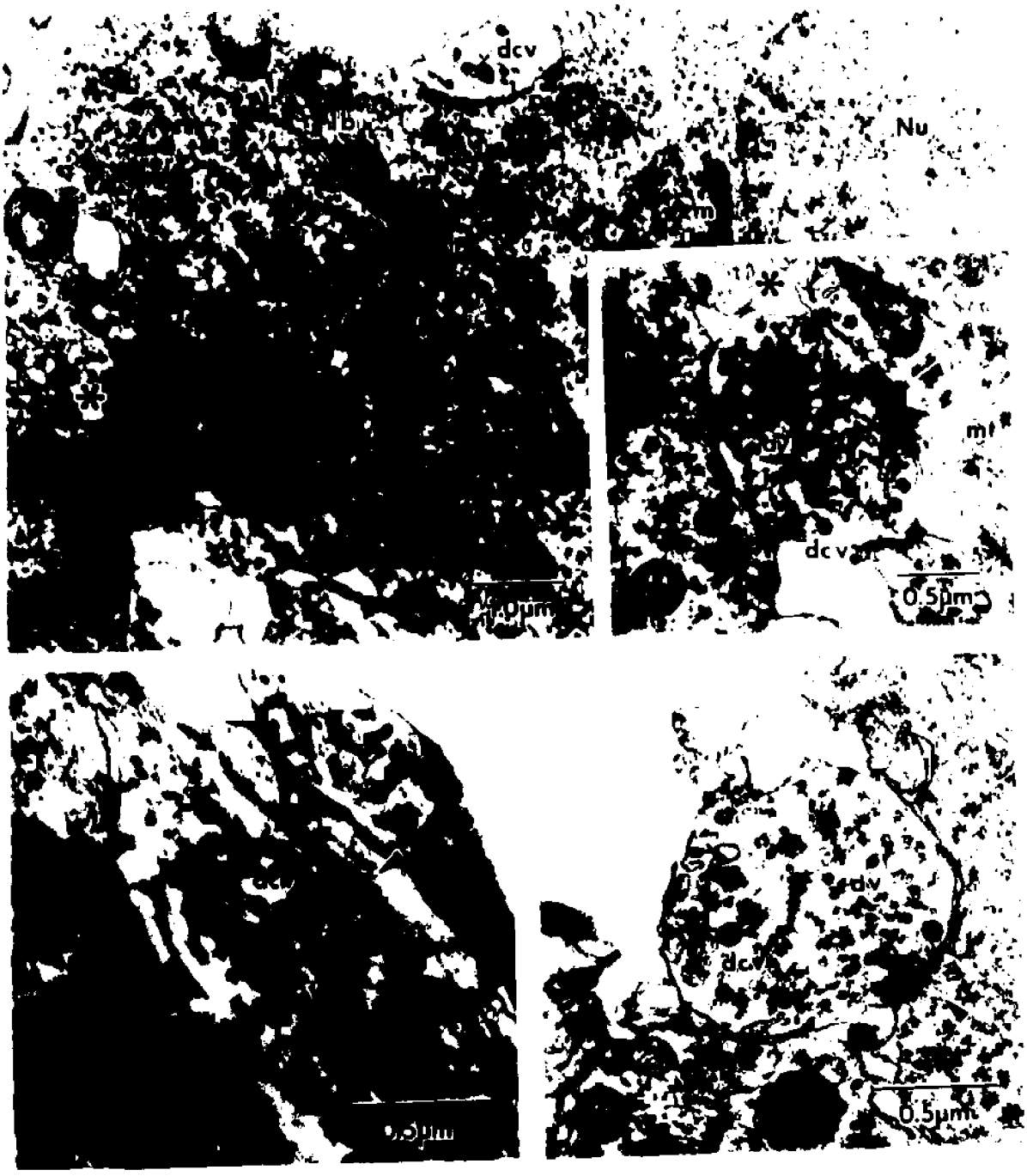

dcv
Nu
0.5μm
dcv
0.5μm
0.5μm
dcv
dcv
0.5μm

PLATE XXIII

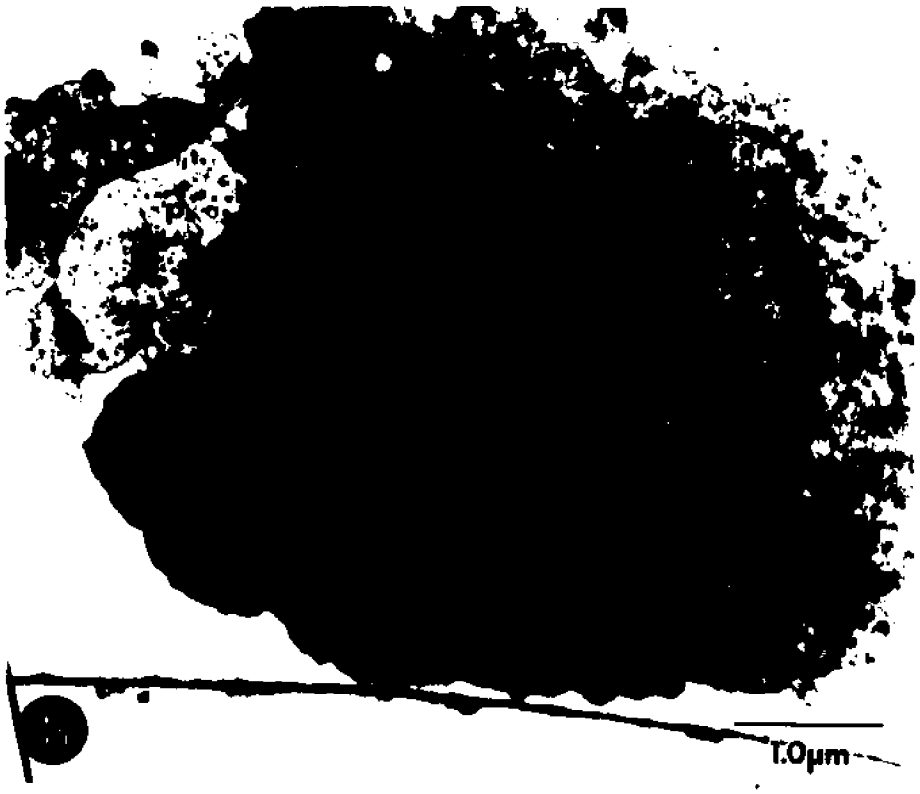
Figure 47. A glial cell (G) at upper left is seen within a small group of neruonal cells (NB). The small size of the cell as compared to the neurons and its dense nucleus and cytoplasm make it easy to distinguish. Areas marked by (*) indicate projections of glial cell cytoplasm. Although some evidence of glial investment of neuronal cells and processes is found, it appears disorganized and poorly developed. Defined medium culture, 10 days in vitro. X 10,000


1μm

PLATE XXIV

Figure 48. A nonneuronal cell, thought to be glial (G), is found attached to the substrate and isolated from neurons. The nucleus and cytoplasm of this cell are much denser than those of the neurons. The nucleus, usuually kidney-shaped, is centrally located. Cytoplasmic contents include mitochondria (m), rough endoplasmic reticulum (rer), Golgi, lysosomes and free ribosomes. Processes (p) lie in close association with the glial cells. Defined medium culture, 10 days in vitro. X 18,000

Figure 49. Electron micrograph showing 2 different nonneuronal cells. The upper cell has a much denser nucleus (Nu 1) and its cytoplasm is seen to surround and envelop adjacent neuronal processes (p). The lower cell that is attached to the substrate is much larger in size with cytoplasm extending from either side of the nucleus, much like that in Figure 50. The nucleus of this cell (Nu 2) has more homogeneous appearing chromatin. Defined medium culture, 10 days in vitro. X 20,000


1.0μm


1.0μm

PLATE XXV

Figure 50. Electron micrograph of a typical nonneuronal cell lying flattened and adjacent to the substrate. The nucleus (Nu) has a prominent nucleolus. Stacks of endoplasmic reticulum (er) are found in the cytoplasm along with Golgi (g), mitochondria, and abundant free ribosomes. Multiple blebs (►) project from the upper surface of the cell. Defined medium 10 days *in vitro*. X 5,000

Figure 51. The upper surface of this nonneuronal cell is traversed by cytoplasmic projections from neighboring cells. Defined medium culture, 10 days *in vitro*. X 16,000


1.0μm
1.0μm

PLATE XXVI

Figure 52. Developing sympathetic ganglion cells show different stages of neuronal maturation. At right is a primitive sympathetic cell (PSC) with dense nuclear material which is surrounded by several neuroblasts (NB). The nuclei of the neuroblasts are larger and contain more homogeneous chromatin. Their cytoplasm and content, although not as extensive as in young sympathetic neurons, are more advanced developmentally than those of the PSC. Serum-supplemented medium, 5 days *in vitro*. X9,000

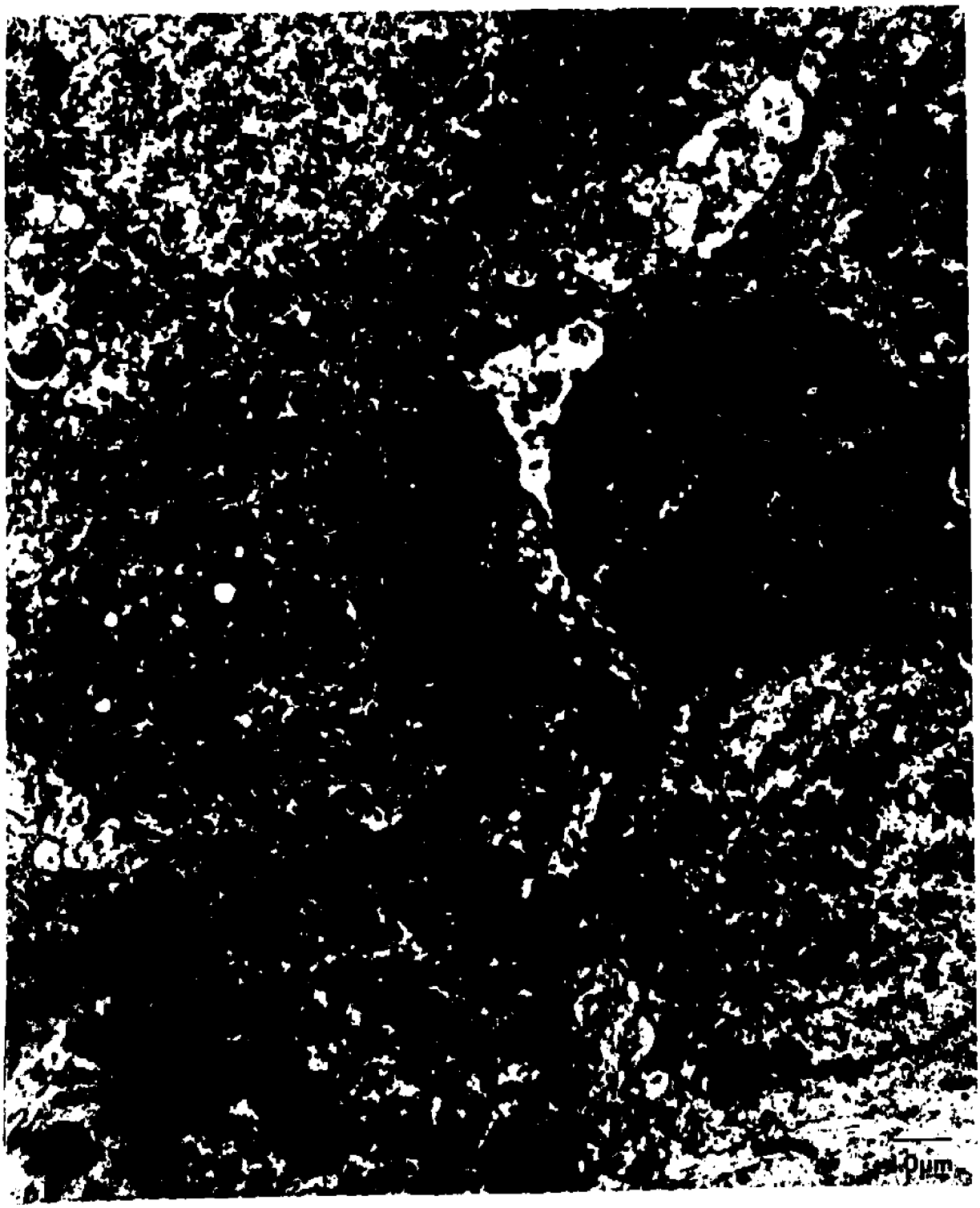

1μm

PLATE XXVII

Figure 53. Enlargement of area (*) from Figure 54, showing a longitudinal section through processes. Microtubules run parallel to the long axis of the process. X 27,000

Figure 54. Neuroblast from a 10 day serum-supplemented culture. The neuron lies adjacent to the substrate. Its cytoplasm and organelle population are more extensive than those seen in the primitive sympathetic cell. A process (p) is seen in close association with the substrate. A small amount of glial cytoplasm is seen at (►) but most neurons are lacking glial investment. X 11,000


(a)
10μm

PLATE XXVIII

Figure 55. Young sympathetic neuron 20 days in serum-supplemented medium. There is little evidence of condensed chromatin or perimembranous rim. The cytoplasm contains numerous short stacks of rer (Nissl bodies), characteristic of mature neurons. At least 3 Golgi complexes (g) are present in the organelle-rich cytoplasm. X 14,000

Figure 56. Enlargement of area of Figure 55 indicated by (*) to show transverse section through nuclear pores (+) and dense core vesicles (dcv) which appear to be budding from Golgi (►). Several laminar bodies (lb) are present. X21,000

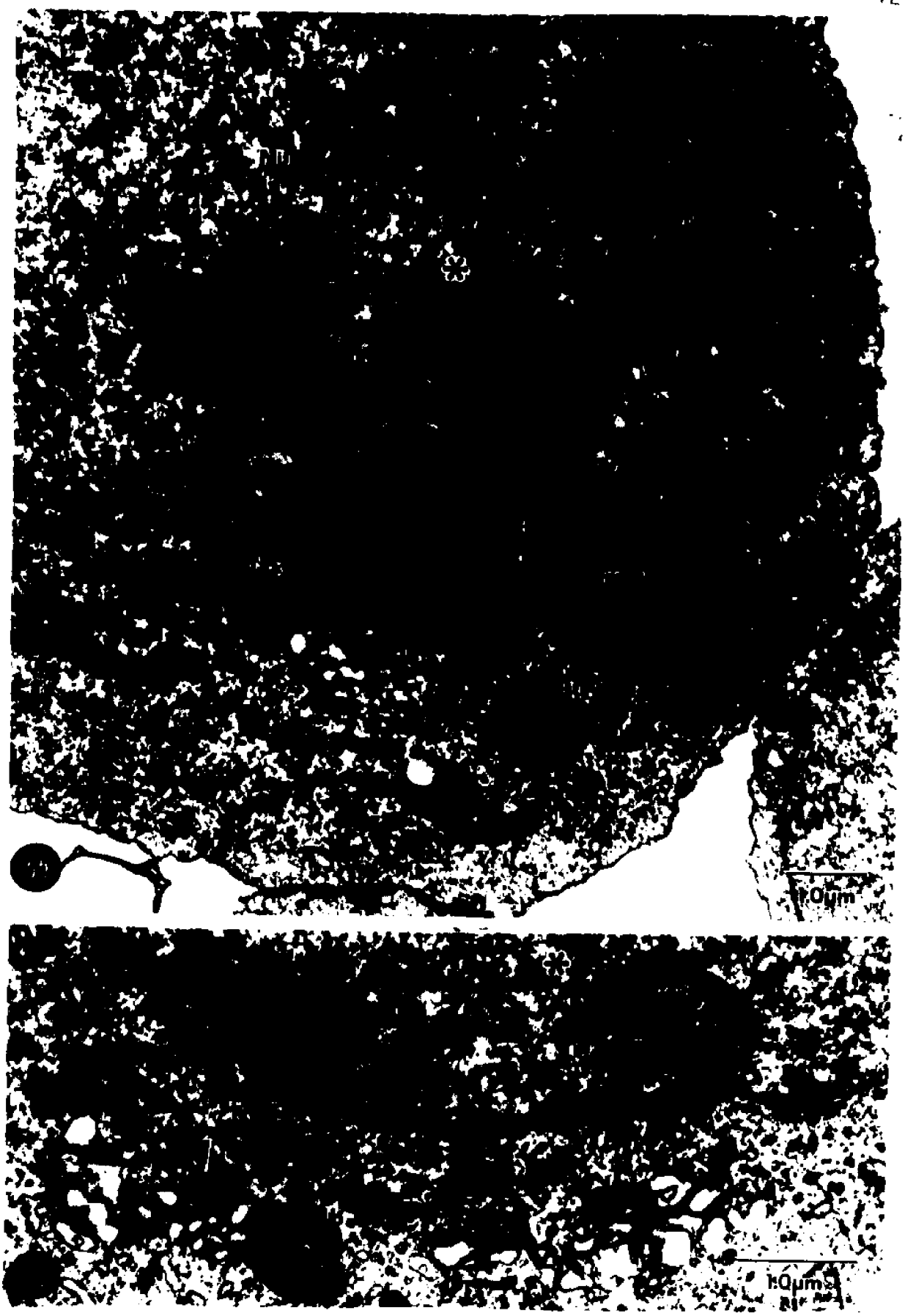

1.0μm
1.0μm

PLATE XXIX

Figure 57. Varicosity from a 20 day serum-supplemented culture containing 30 to 50 nm agranular vesicles and a few large dense core vesicles. X 30,000

Figure 58. More varicosities from a 20 day serum-supplemented culture. X 34,000

Figure 59. Transverse section through a neurite bundle from a 10 day serum-supplemented culture. Processes contain 10 nm neurofilaments, usually centrally located, and numerous microtubules that often surround the periphery. Other organelles that are found are mitochondria, smooth endoplasmic reticulum and dense core vesicles. X 50,000

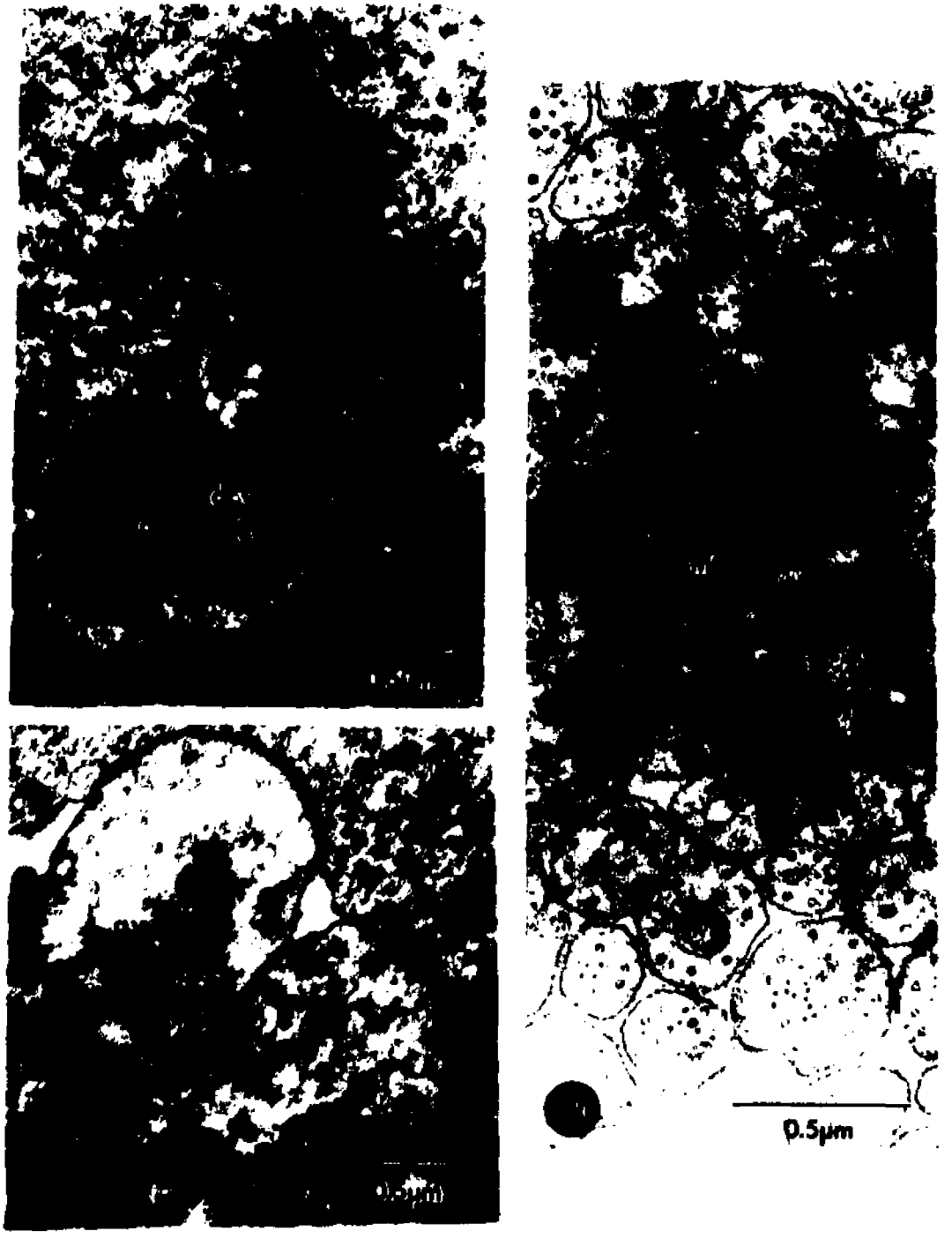

0.5μm

PLATE XXX

Figure 60. Micrograph of nonneuronal cells from section cut perpendicular to the substrate. The nuclei (Nu) of these cells are usually centrally located with extended lengths of cytoplasm found on either side. Elaborate arrays of rough endoplasmic reticulum (rer) are a prominent feature of the cytoplasm. Serum-supplemented culture, 20 days *in vitro*. X 8,000

Figure 61. The neuron at left is situated next to a glial cell (G). Some investment of processes (p) by glial cell cytoplasm occurs but is much less than that seen *in vivo*. Serum-supplemented culture, 10 days *in vitro*. X 16,000

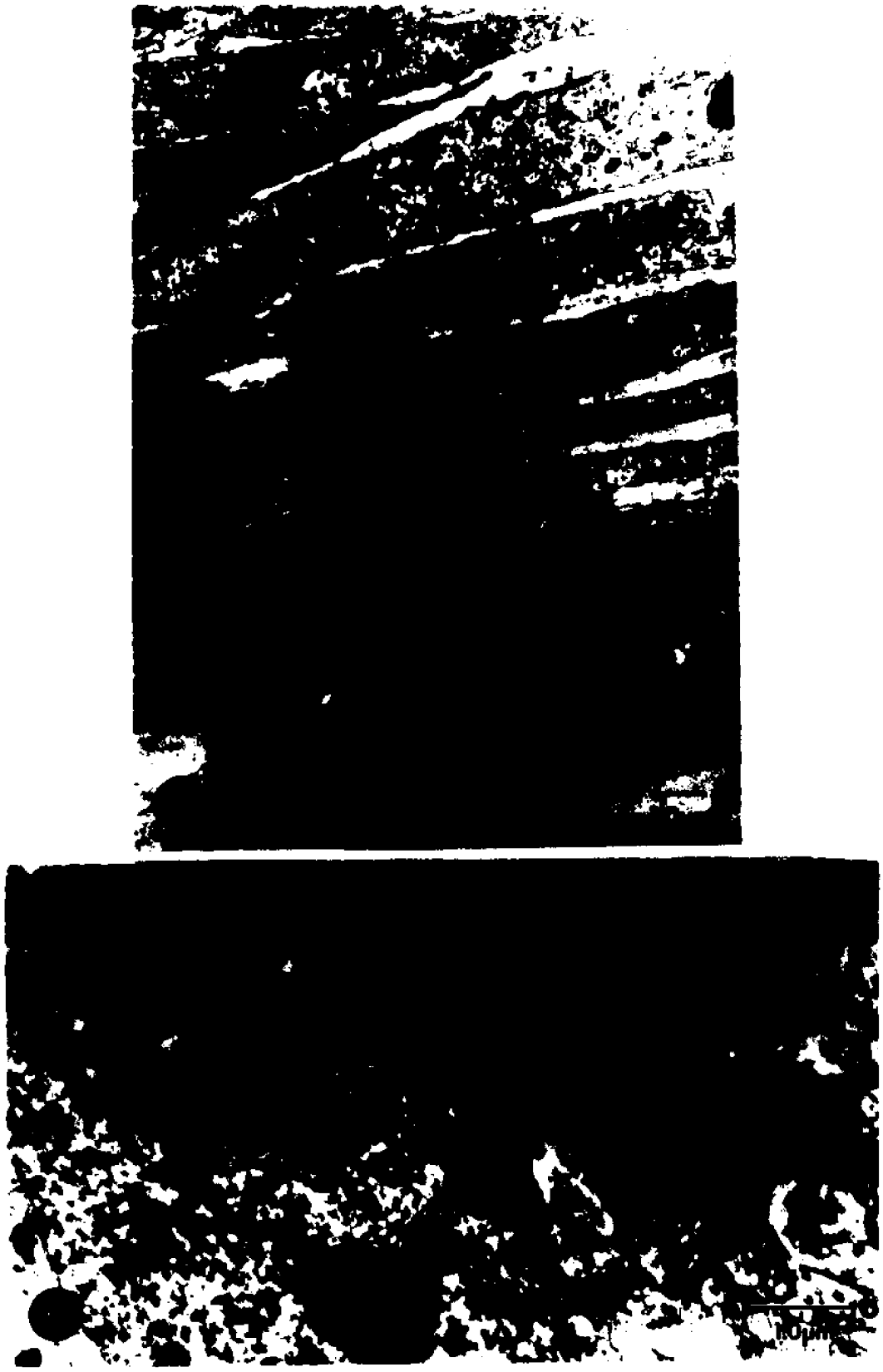


PLATE XXXI

Figure 62. Ganglia fixed *in vivo* show neurons at different stages of neuronal maturation. A primitive sympathetic cell (PSC) has a very dense, patchy nucleus and little cytoplasm while a neighboring neuroblast (NB) has a less dense nucleus and cytoplasm rich in organelles. Gilal investment (*) is more evident in the developing *in vivo* ganglia but is less extensive than in the adult. X 5,000

Figure 63. Neurons at different developmental stages from *in vivo* ganglion. X 5,000

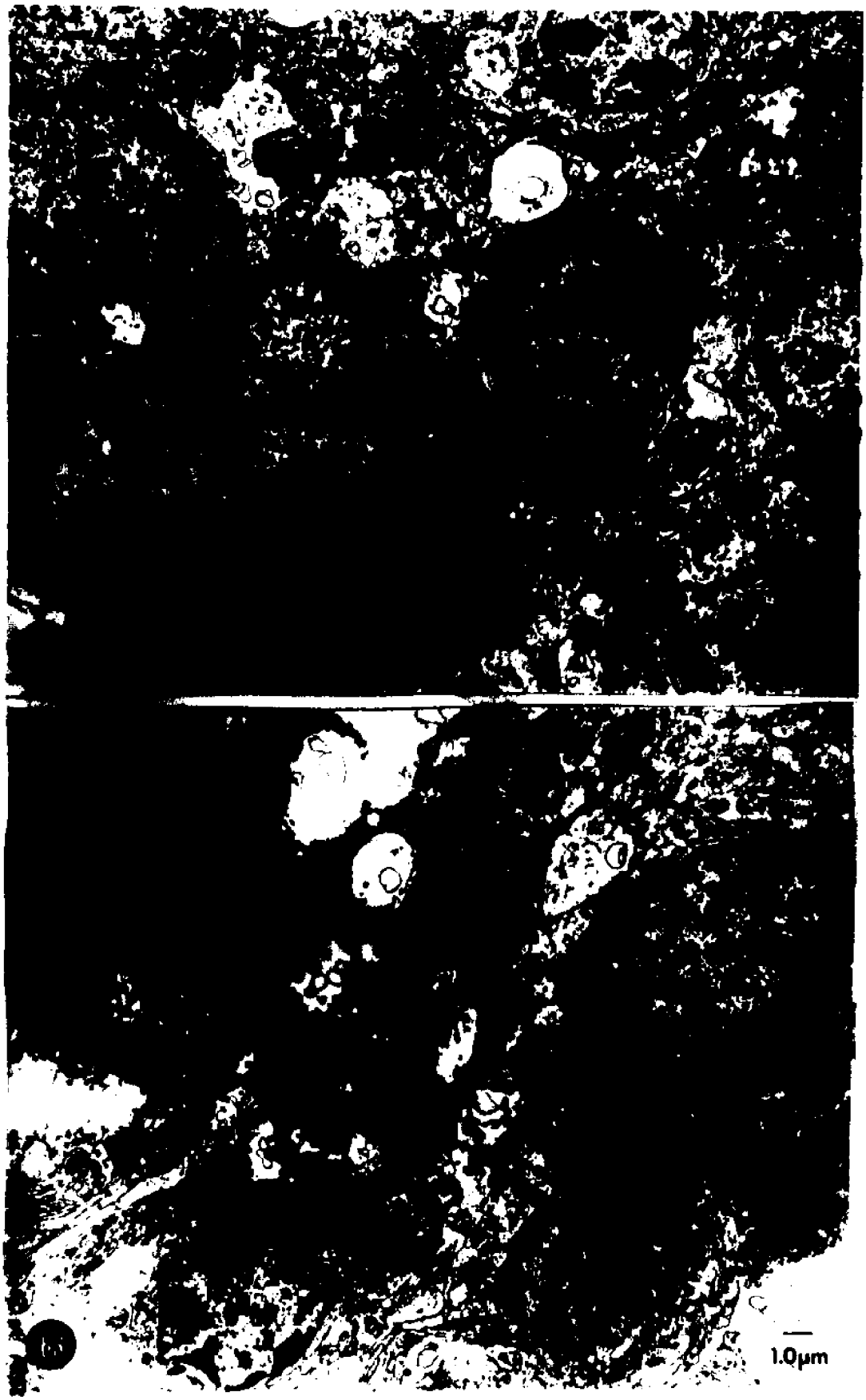

1.0μm

PLATE XXXII

Figure 64. Primitive sympathetic cell (PSC), from an in vivo ganglion. The nucleus (Nu) has condensed heterochromatin and is surrounded by a thin layer of cytoplasm. Few organelles other than free ribosomes and a few mitochondria are present. X 12,000

Figure 65. Neuroblast (NB) from an in vivo ganglion has a larger nucleus (Nu) with less condensed chromatin. The cytoplasm of this cell is more extensive and contains a few stacks of rough endoplasmic reticulum (rer), microtubules (mt), mitochondria and free ribosomes. A moderately developed Golgi complex (not seen in this section) is another feature of the neuroblast cytoplasm. X 12,000

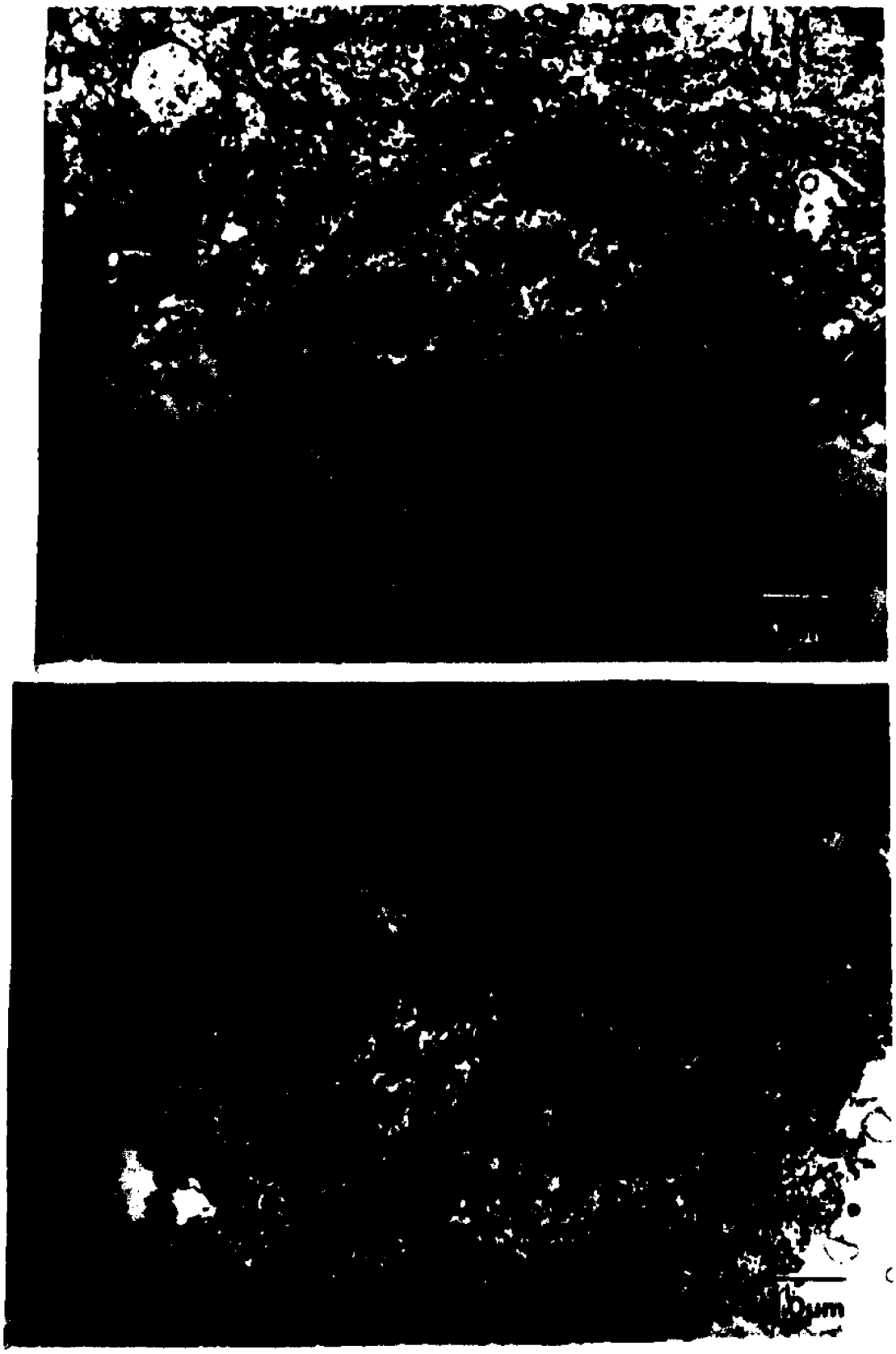

10μm

PLATE XXXIII

Figure 66. Neuroblast (NB) fixed *in vivo*. Nuclear material is still patchy in appearance. The cytoplasm has abundant microtubules (mt), mitochondria (m), and polysomal aggregates. Only a few scattered stacks of rer are present. Laminar bodies (lb) are sometimes seen *in vivo*, as in this cell, but occur most frequently in long term cultures of the ganglion cells. A pair of centrioles (c) with prominent microtubules is found near the nucleus. X 18,000

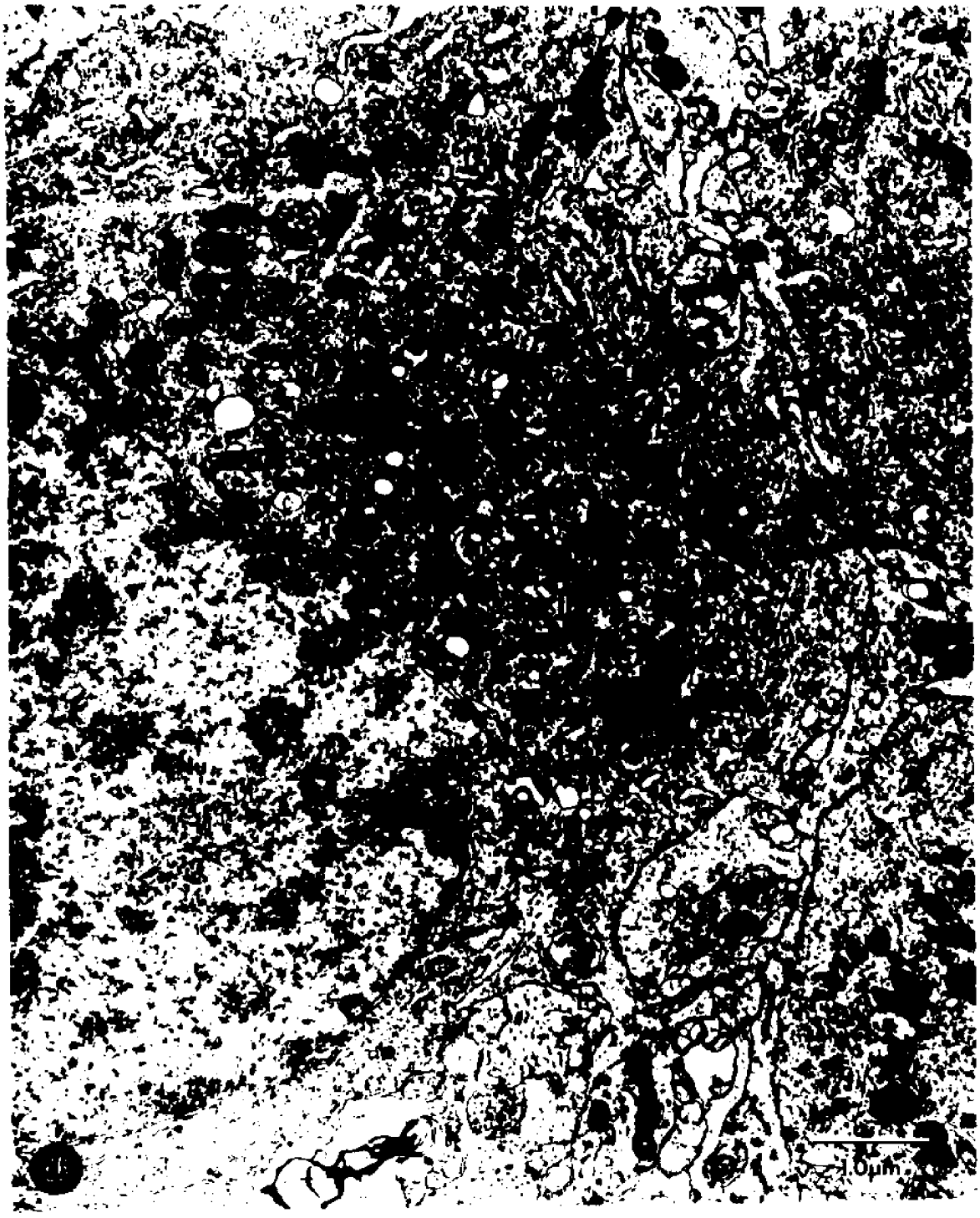

1.0μm

PLATE XXXIV

Figure 67. Several varicosities are seen in this micrograph from a ganglion fixed *in vivo*. The one at the lower left contains many 30 - 50 nm agranular vesicles (av) and two large dense core vesicles (dcv). At upper right is a synaptic profile (►) showing membrane densities. One side of the synapse (most likely the post-synaptic side) has a series of dense core vesicles in linear alignment. X 21,000

Figure 68. Synaptic profile (►) from a ganglion fixed *in vivo*. Agranular vesicles (av) and membrane densities are evident. Glial investment of a process is seen at (*). X 36,000

Figure 69. Processes from an *in vivo* ganglion showing another synapse at (►). X 20,000

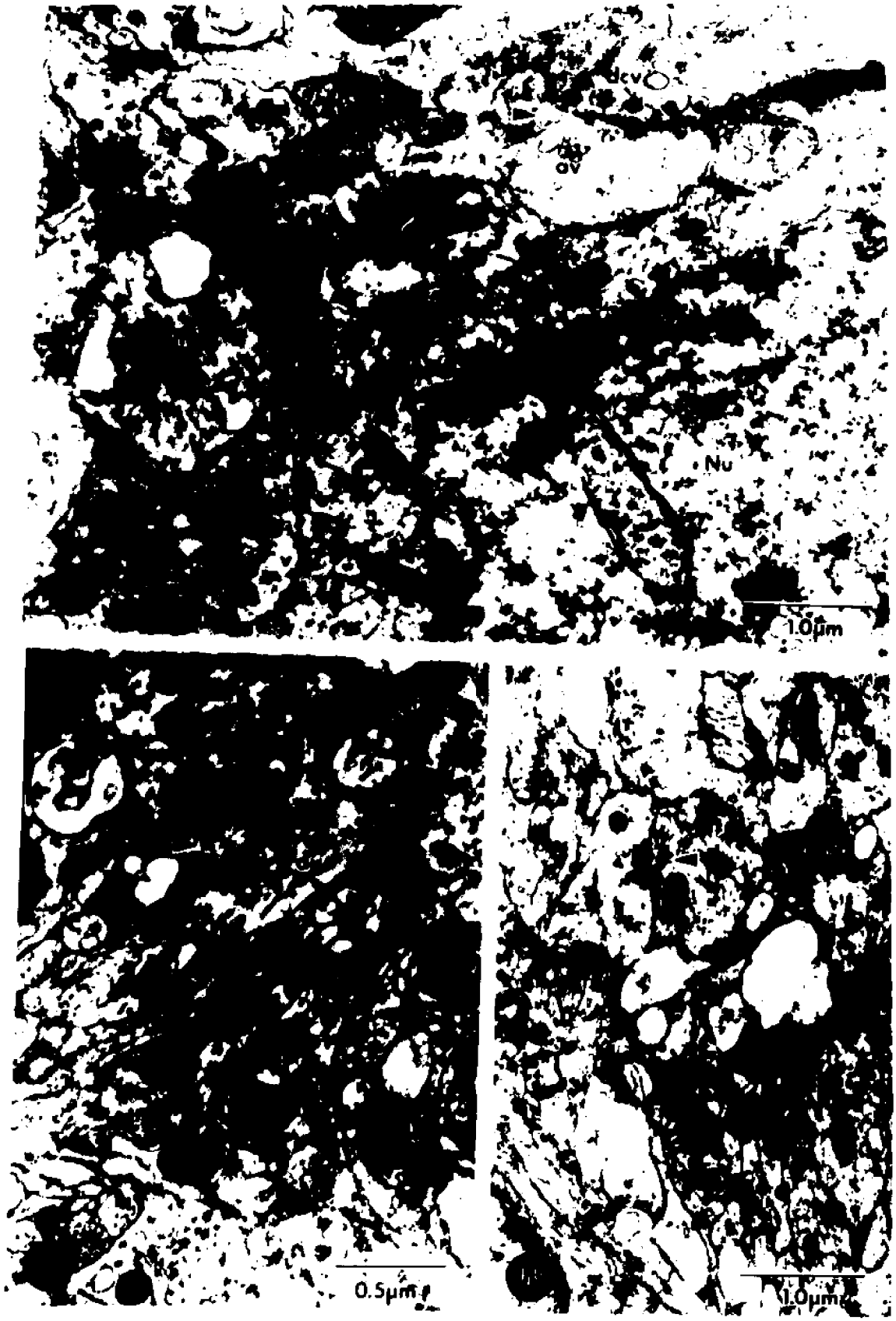
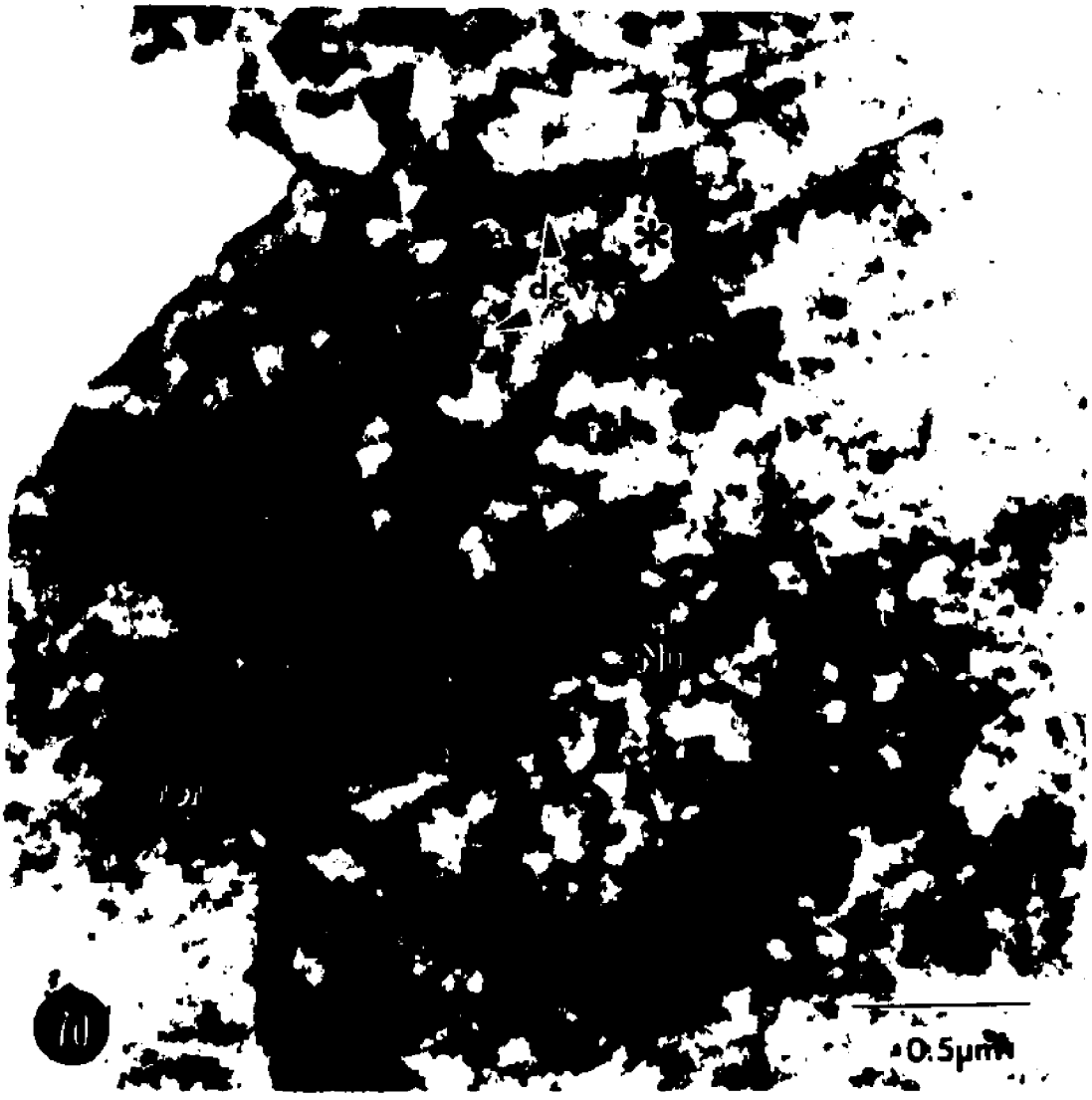

dcv
ov
Nu
1.0μm
0.5μm
1.0μm

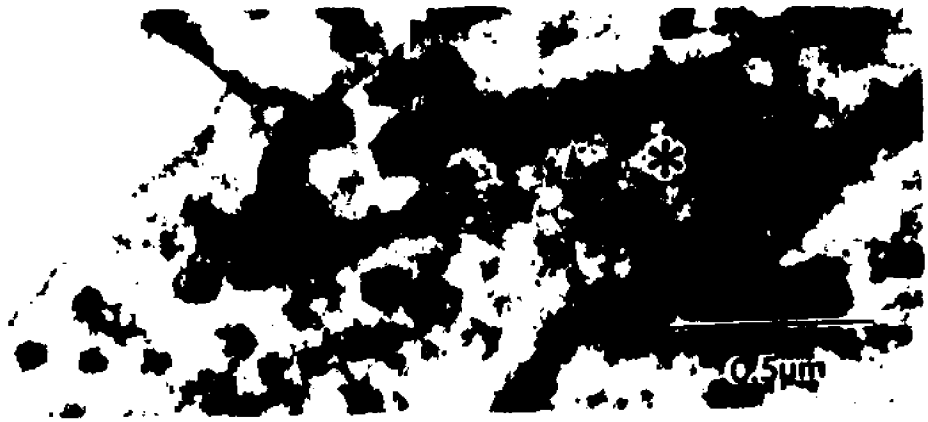
PLATE XXXV

Figure 70. Ganglion cell treated with chromate-dichromate, post-fixed in osmium, and viewed unstained, shows a cluster of electron-dense, membrane-bound vesicles filled to varying degrees (►). These clusters, although found in some cells from 6 day cultures, appear more frequently in the 25 day cultures. Defined medium culture, 25 days *in vitro*. X 48,000

Figure 71. Enlargement of area (*) from Figure 70 shows some vesicles to be filled to capacity, while others contain only a small dense core of material (►). X 54,500



70



71

PLATE XXXVI

Figure 72. Chromate-dichromate treated culture, post-fixed in osmium and unstained, shows electron-dense material in a membrane-bound vesicle (►) within a varicosity. Defined medium culture, 6 days *in vitro*. X 30,000

Figure 73. Dense core vesicles (►) occur randomly throughout the cytoplasm, either singly as in this cell, or in small clusters (Figure 78). Chromate-dichromate fixation, osmium post-fixation, no stain, 6 day defined medium culture. X 30,000

Figure 74. Dense core vesicles (►) in a process from a 6 day defined medium culture treated with chromate-dichromate, post-fixed in osmium and unstained. X 30,000

Figure 75. Chromate-dichromate treated culture, post-fixed in osmium and stained with uranyl acetate and bismuth subnitrate, appears very coarse. Optimal conditions for staining were difficult to achieve and variable. Defined medium culture, 6 days *in vitro*. X 30,000

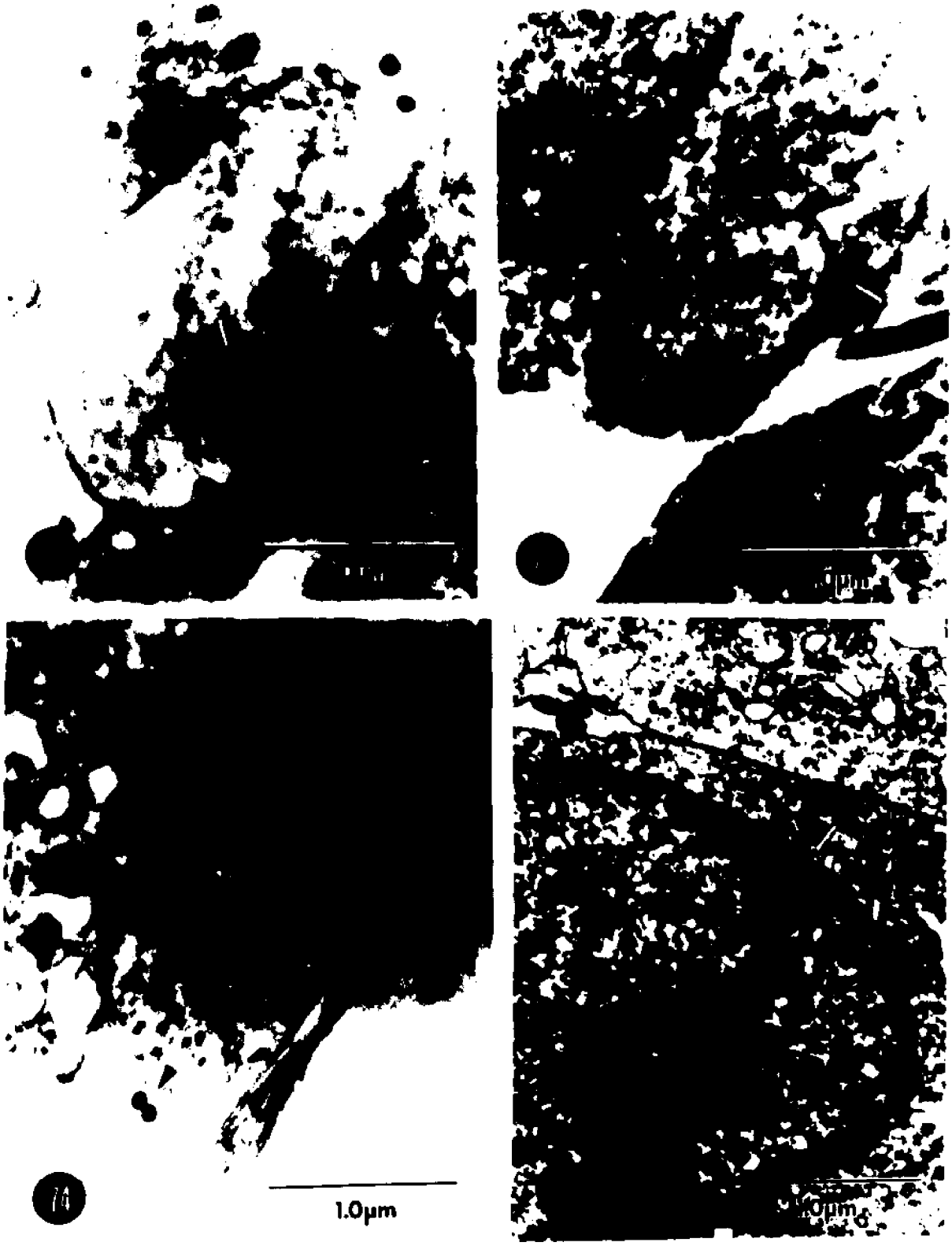

1.0μm

PLATE XXXVII

Figure 76. Two adjacent neurons showing the faint outline of their nuclei (Nu) contain a few electron-dense granules. Chromate-dichromate fixation, no osmium, 6 days in defined medium. X 23,000

Figure 77. Enlargement of area (*) from Figure 76. The electron densities (►) measure 50 to 94 nm. Mitochondria (m) can be seen in the background. X 46,000

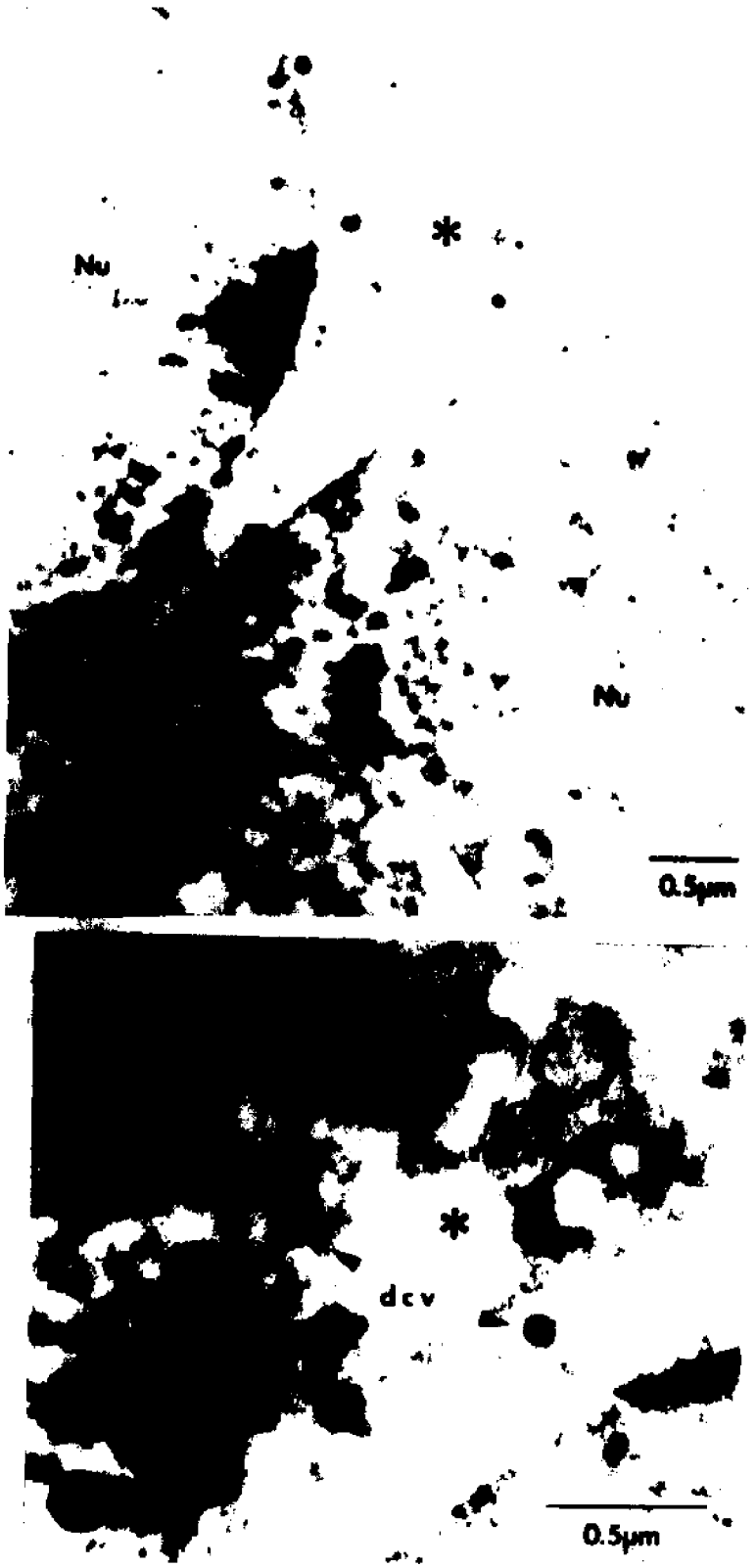
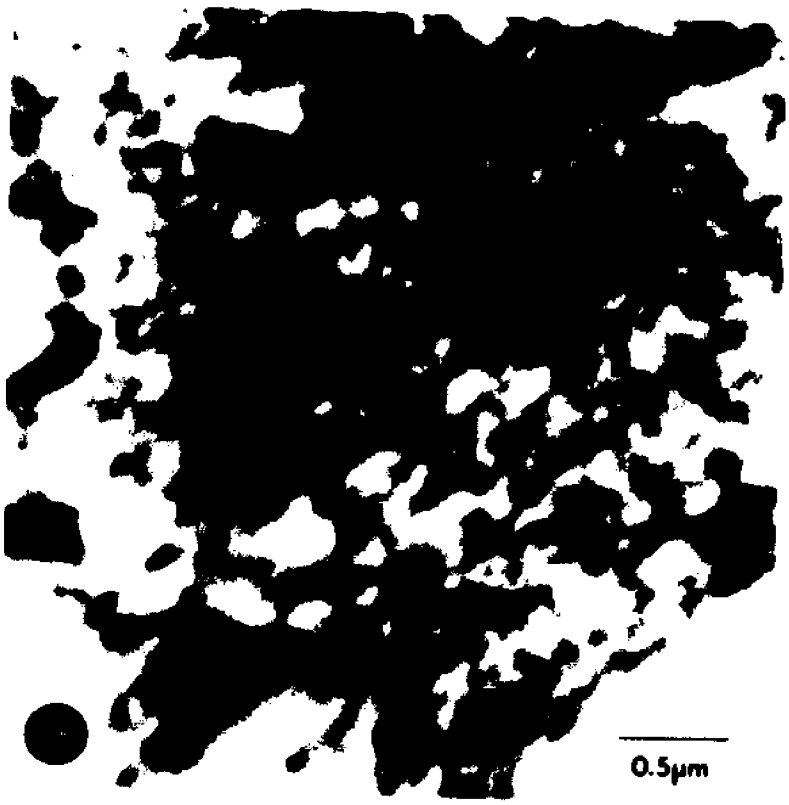

Nu
Nu
0.5μm
dcv
0.5μm

PLATE XXXVIII

Figure 78. Electron-dense reaction product (►) appears in a small cluster in the cell soma of this ganglion cell. Chromate-dichromate fixation, no osmium, 25 days in defined medium. X 28,000

Figure 79. Cores of electron-dense material (►) are located in what appears to be a varicosity of a process (p). Chromate-dichromate fixation, no osmium, 25 days in defined medium. X 29,000


0.5μm

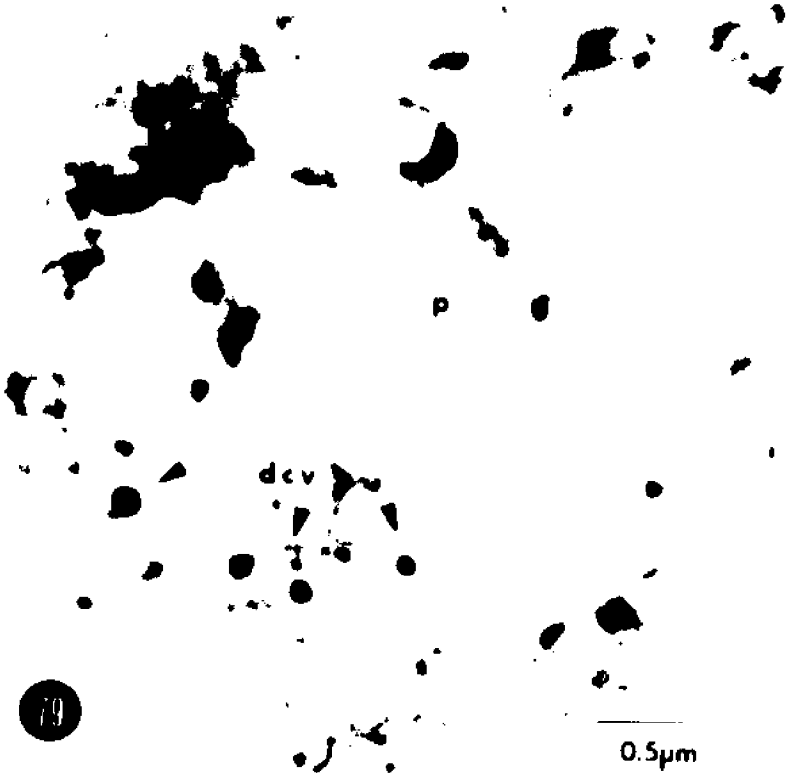

p
dcv
0.5μm

PLATE XXXIX

Figure 80. Clusters of electron dense granules (►) measuring 50 - 94 nm appear in the soma of this cell from a 25 day defined medium culture. Chromate-dichromate treated, unosmicated and unstained. X 25,000

Figure 81. Staining of the unosmicated material does not permit visualization of membranes. Chromate-dicromate treated, unosmicated, stained with uranyl acetate and bismuth subnitrate. 6 days *in vitro*. X 15,500; Inset X 46,000

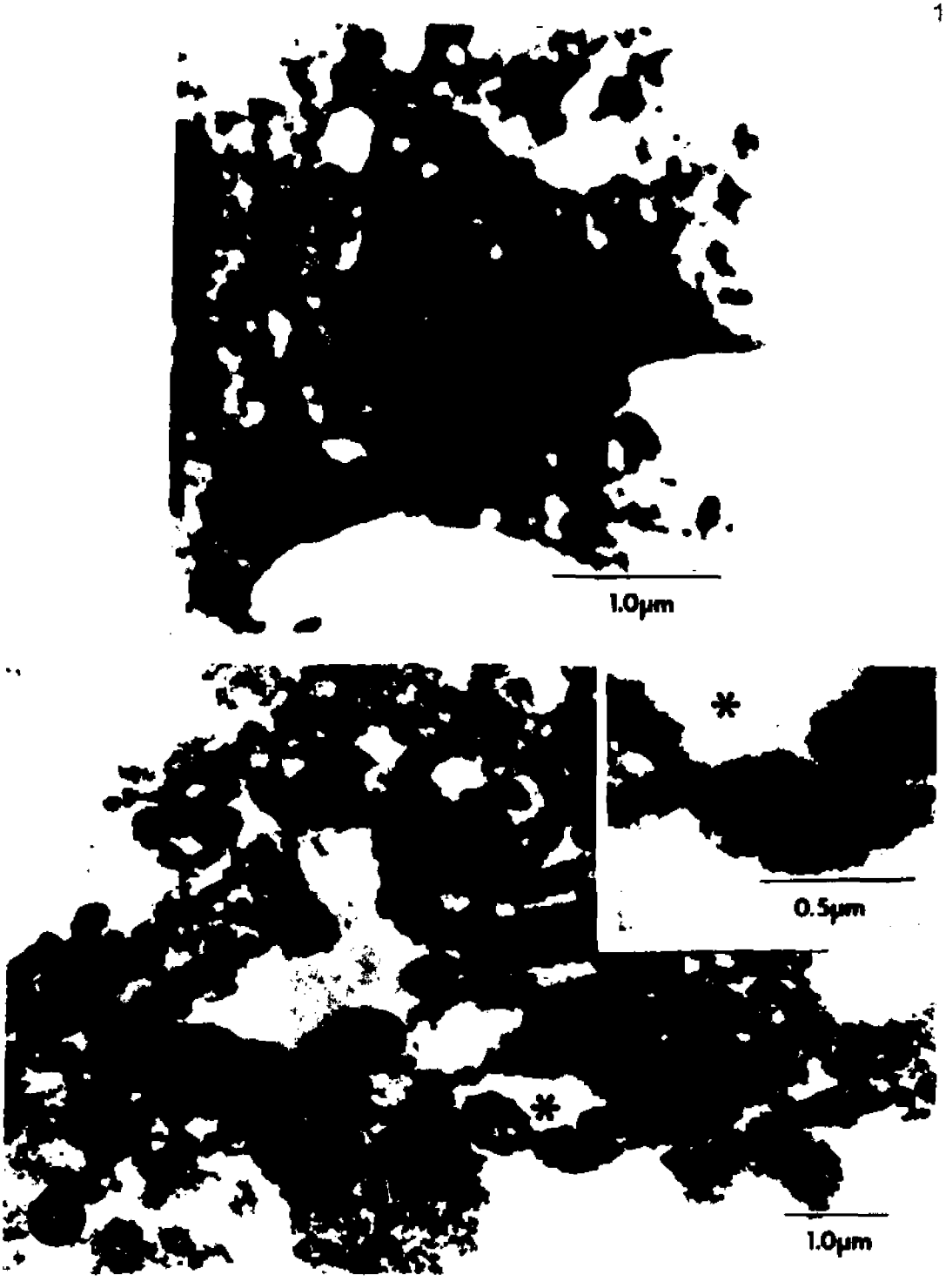

1.0μm
0.5μm
1.0μm

PLATE XL

Figure 82. Large dense core vesicle (dcv) from a 6 day potassium permanganate fixed culture. X 30,000

Figure 83. Potassium permanganate fixed culture of a ganglion cell, 6 days *in vitro*. A single positive dense core vesicle (dcv) is found in the cell cytoplasm. The somal structures such as Golgi apparatus (g), mitochondria (m) and nucleus (Nu) are also readily visualized. X 23,000

Figure 84. Positive dense core vesicle (dcv) from a 6 day potassium permanganate fixed cell. X 30,000

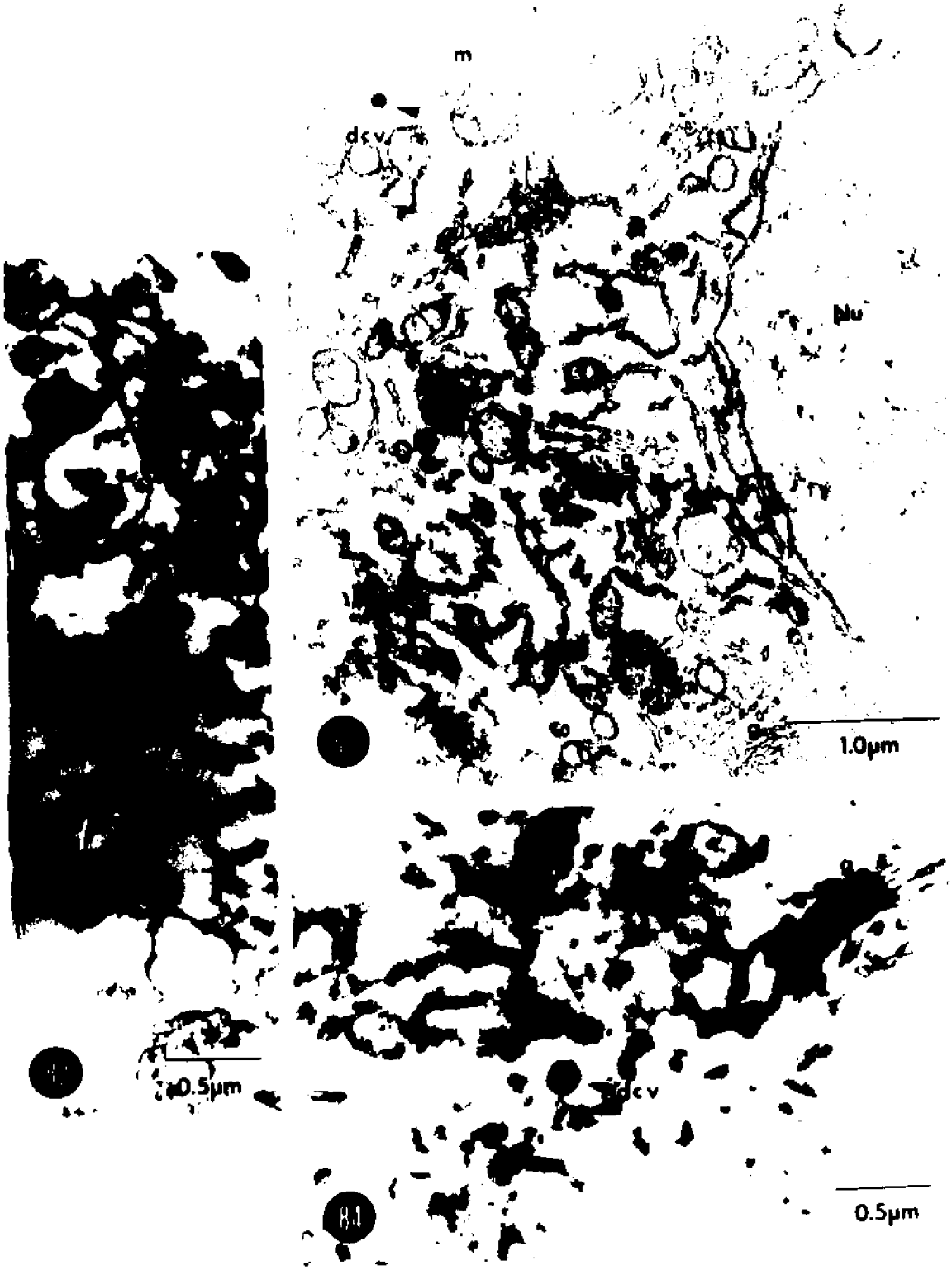

m
dcv
Nu
1.0μm
0.5μm
dcv
0.5μm

PLATE XLI

Figure 85. Transverse section through a varicosity (V) from a 25 day potassium permanganate fixed culture has numerous agranular vesicles (av) but is lacking in dense core vesicles. X 54,000

Figure 86. A single positive dense core vesicle (►) is seen in this varicosity (V) from a 25 day potassium permanganate fixed culture. Agranular vesicles (av) and smooth endoplasmic reticulum (ser) are also present. X 48,000

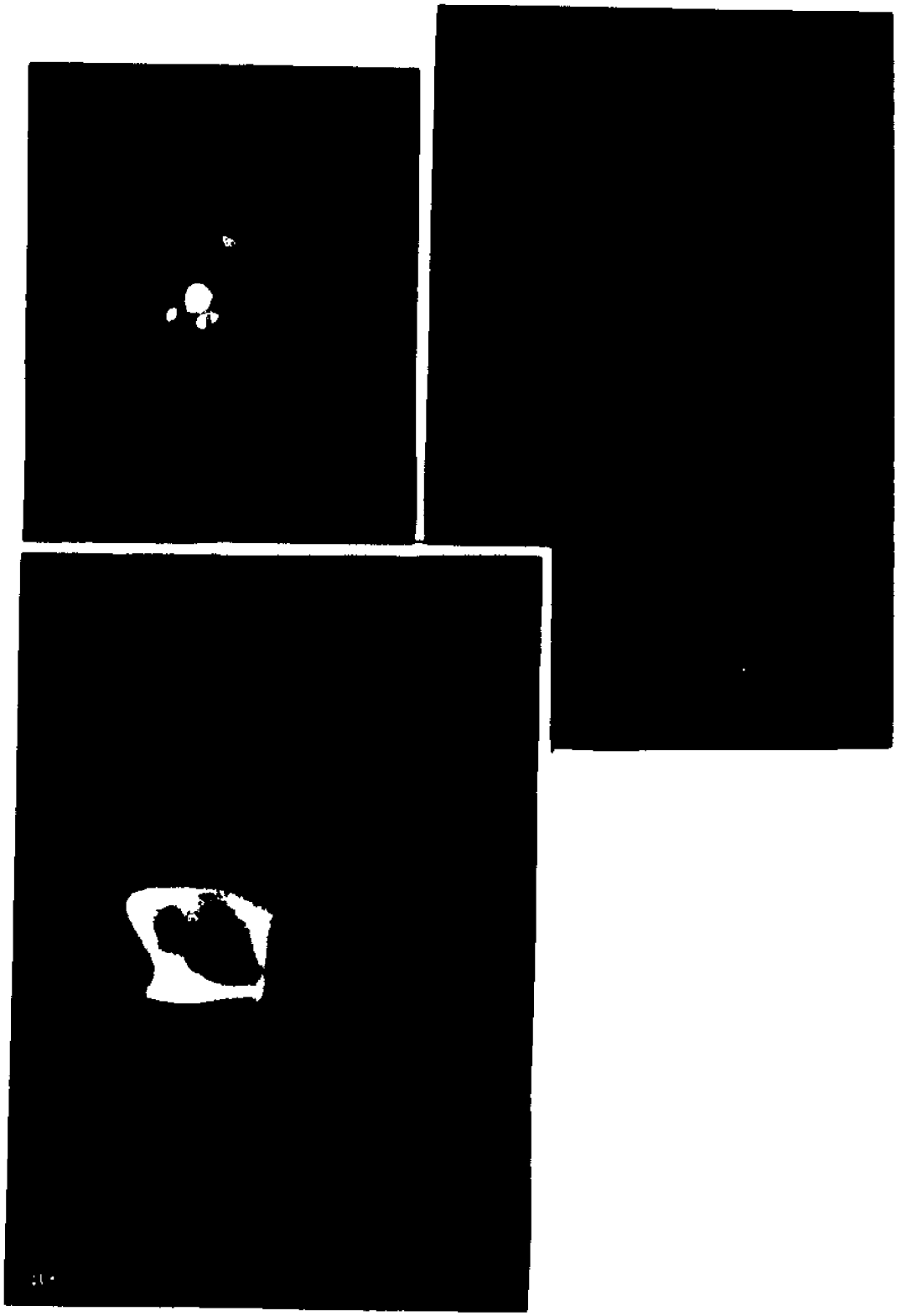

0.5μm
86
0.5μm

PLATE XLII

Figure 87. Three fluorescing neurons from a 5 day defined medium culture. Background nonneuronal cells show faint autofluorescence but are not evedent at this low magnification. X 400

Figure 88. Higher magnification of a ganglion cell (*) not exposed to paraformaldehyde vapors has only very dim fluorescence. X 1800

Figure 89. Formaldehyde-induced fluorescence of a neuronal cell cultured in defined medium for 5 days has moderatedly intense fluorescence of the cell soma. A process at the lower right edge of the cell also is seen to fluoresce. The (*) marks autofluorescence from background nonneuronal cells. X 1800



DISCUSSION

I. Defined Medium

The present work shows that human fetal sympathetic ganglion neurons can be maintained in hormone-supplemented serum-free medium. These cells have been successfully cultured in defined medium for up to 40 days. This is the first report to demonstrate the ability to culture developing human sympathetic cells in a culture medium free of serum. This culture system will permit a more controlled setting for subsequent manipulation, particularly in regard to investigation of growth and trophic factors that might influence development. The ultrastructural evaluation of these cells in defined medium in comparison with *in vitro* serum-supplemented and *in vivo* ganglia should contribute useful information regarding the extent of fine structural maturation and therefore, will provide a baseline for comparison for future studies.

A. Neurons: comparison with in vitro serum-supplemented and in vivo ganglia

The morphology, neurite extension and ultrastructure of cell somas and processes of the neurons grown in defined medium was not altered from that of sister cultures grown

in serum-supplemented medium. Cells grown under both conditions retained a rounded morphology and showed extensive neurite formation with many neurites joining together to form fiber bundles. Thin sections from both defined and serum-supplemented media revealed cells with ultrastructural features representative of primitive sympathetic cells, neuroblasts and young sympathetic neurons as described in Results. Characteristics unique to neuronal cells, such as neuritic processes, synapse formation and the appearance of DCVs, were found to the same extent in both media, demonstrating that certain neuronal features continue to be expressed in defined medium.

The ultrastructure of the cultured peripheral neurons was also similar to that of the _in vivo_ ganglia. There was, however, an apparent increase in the presence of laminar bodies in cultured neurons. The cytoplasm of cultured neurons in both serum-supplemented and defined media occasionally showed the presence of large vesicles containing swirls of membrane-like material. Similar structures have been described previously in rat and human sympathetic neurons and have been referred to as laminar bodies (DeLemos and Pick, 1966; Pick, 1967). These bodies were rare in neurons of uncultured ganglia and at 5 and 10 days _in vitro_, but were more apparent at 20 days. The cell somas of neurons from 40 day cultures frequently contained such bodies, which appeared more osmiophilic. The abnormal

accumulation of these vesicles over time in culture suggests that culture conditions are not yet optimal for very long term culture. This may be the result of a nutritional deficit. If this is the case then this deficit exists in both serum-supplemented and defined medium cultures since the appearance of laminar bodies and their increase over time in culture was apparent in both culture media. Another explanation is that disruption of the natural 3-dimensional environment and growth of these cells under partially dissociated conditions does not allow for necessary cell-cell interactions and communications to occur. This may in turn affect the cells' ability to carry out waste removal. An additional explanation is that culture conditions cause the accumulation of cytotoxic elements in the cell to occur at an increased rate which is beyond the normal capacity of the cell to handle. Studies using different components and proportions of medium additives would be useful in further evaluating this question.

Neuronal somas were seen to either attach directly to the culture dish or to be situated on top of fibroblast-like cells. The most probable explanation for this would be based on a combination of the random placement of cells relative to one another while settling and the differential time it takes for different cell types to attach. For example, it is known that fibroblasts attach to the culture dish more rapidly than neurons. If neurons and fibroblasts

are relatively close to one another in a particular area then fibroblasts would attach first. Neurons would then form attachments to the surface of the fibroblasts. In other areas where neurons are not in close proximity to nonneuronal cells, they would form attachments to the substratum of the culture dish.

B. Nonneuronal cells

One of the major drawbacks in doing long-term culture work on normal primary tissue is the rapid overgrowth of the culture by fibroblasts and other cell types not under investigation. The overgrowth of neuronal cultures by unwanted cell types has been checked in several laboratories by the addition of agents known to interfere with mitosis. These agents, however, may have an effect on the cells being studied and their avoidance would be advantageous. Serum-free supplemented medium offers an alternative that allows for the growth of an enriched population of neuronal cells. In the work reported here, phase microscopic observation of serum-supplemented versus serum-free cultures has shown a dramatic difference in the number of nonneuronal cells present under the two conditions.

1. Selection After 72 hours in culture, nonneuronal cells were present in about the same density in both defined and serum-supplemented media. By 12 days in

culture, however, nonneuronal cells had formed a confluent layer in the dishes containing serum-supplemented medium. The difference in the number of background nonneuronal cells in the 2 media at this time was dramatic (compare Figs. 9 and 14). The great reduction in the nonneuronal cell population in the serum-free cultures should make long term studies of these cells more feasible.

Scanning of defined medium culture dishes at 12 days suggested an actual reduction in the number of nonneuronal cells. This was supported by counts of nonneuronal cells in photomicrographs of areas of the dish where cells were present. Since many areas of the dish were lacking any cells, the counts do not represent average numbers of nonneuronal cells per unit area of dish but rather give an estimate of the density of their presence whenever encountered. It is uncertain whether the greatly reduced nonneuronal cell population seen in 12 and 20 day defined medium cultures as compared to 3 and 5 day cultures is due to a selection process alone or if a slowing of the cell cycle may contribute as well. At least some of the nonneuronal cells appear to divide over time in culture. One area containing a few nonneuronal cells on day 3 in culture was monitored until day 12 and showed an almost threefold increase in cell number. One possibility is that nonneuronal cells from adjacent areas may have migrated to this site and account for the increase in cell number. However, this

seems unlikely since there were few nonneuronal cells in the area surrounding the site on day 3. On day 12 the cells were concentrated in the same area with still no cells present for quite a distance in the surrounding circumference.

The basal medium used in the preparation of a defined medium can influence the survival of certain cell types. Recently, it was discovered that F12 does not maintain chick glial cells in culture (Bottenstein, personal communications). If the same is true for human glial cells then the reduction in nonneuronal cells may be due at least in part to the failure of many nonneuronal cells to survive in this medium. It seems likely that both survival and cell cycling play a role in the selection process seen here.

The great reduction in the nonneuronal cell population in the serum-free cultures as compared to serum-supplemented cultures makes long-term studies of human sympathetic neurons more feasible. In addition to the neurons being much more visible and thus much easier to observe and manipulate, effects of possible "factors" produced by nonneuronal cells are reduced and effects of added agents much more easily and reliably assessed. It can be envisioned that refinement of the culture conditions could completely eliminate the nonneuronal cells or eliminate selectively only fibroblasts or glial cells. The advantage of this

latter condition would be that different combinations of cells could be cultured and their influence on neuronal development assessed.

Although serum has been removed from the medium, the culture dishes were serum coated before plating the cells to promote cell attachment. Plating cells in defined medium without serum coating was attempted with rat and chick partially dissociated sympathetic ganglia and with a human line of neuroblastoma (SK-N-SH SY5Y). Cell attachment was drastically reduced and most cells were found floating in the medium after 24-48 hours. Due to the scarcity of the fetal tissue used in this study its plating onto uncoated dishes was not attempted.

Recent studies have shown that attachment-promoting factors such as polylysine or fibronectin can be used to coat the surface of the tissue culture dish to enhance cell adhesion (Yamada and Olden, 1978; Yavin and Yavin, 1974). These factors, however, may have additional effects on the cells. Polylysine and fibronectin, for example, increase proliferation in a neuroblastoma line (Bottenstein and Sato, 1980), while fibronectin coating of culture dishes was shown to promote adrenergic differentiation in quail cells (Sieber-Blum et. al., 1981). The role of the intercellular matrix on differentiation and mitosis is complex. Defined medium culture of cells may offer an impro-

ved culture system for approaching this problem. Because of the potential variables that might be introduced by the use of attachment-promoting factors, the protocol of Bottenstein and Sato was adhered to for this study. Further studies should be done to try to eliminate the need for serum coating.

2. Identity

Phase microscopy of early (72 hour) serum-supplemented cultures showed cells with a spindle-shaped morphology. At later time periods (5 days), it was evident that there were 2 distinct populations of nonneuronal cells. Beyond 10 days in culture, it was impossible to discern cellular morphology due to crowding. The first cell type was small in size, generally elongated, appearing bi-polar and very dense. This cell did not spread out over the surface of the dish and in intermediate age cultures (when crowding from overgrowth was not a problem) could be seen above the plane of and situated over the second nonneuronal cell type. Grouping of these smaller, bi-polar cells into ribbon or chain-like formations was common. The bi-polar shape, dense cytoplasm and palisade-like arrangement of cells has frequently been used to identify Schwann cells from peripheral nerve cultures of rat (Dubois-Dalcq et al., 1981; Wood 1966) and human (Murray and Stout, 1942; Askanas et al., 1980). The cells in the cultures from this study that

possess the same characteristics are therefore believed to be glial. More recent studies on rat peripheral nerve cultures have shown that only cells with this morphology show immunofluorescence staining with antisera to Ran-1, a Schwann cell specific surface antigen (Brockes et al., The glial cells were found intermixed with small groups of neuronal cells as well as isolated from the neuronal somas. Somas of these cells contained the usual array of organelles. They were distinguished from other cell types in the cultures by the high degree of electron density of their nucleus and cytoplasm, their size, shape and frequent association with neurites and neuronal somas. These cytoplasmic features are the same as described in ultrastructural studies of Schwann cells from in vivo mid-gestation human fetuses (Gamble et al., 1978), with the exception that the glial cells in vivo were surrounded by a basal lamina. Schwann cells, dissociated and placed in culture, have no basal lamina or associated collagen (Dubois-Dalcq et al., 1981; Odenwald and Askanas, 1981). However, if the integrity of the immediate environment is maintained as in explants of whole ganglia (Armati-Gulson, 1980) basal lamina will form.

The second type of nonneuronal cell was much larger in size, very flat and frequently found in multilayered arrangement, the bottom cell always lying in close association with the substrate. Other studies of cultured peri-

pheral nervous system (Armati-Gulson, 1980; Dubois-Dalcq et al., 1981; Askanas et al., 1980) showed large flat polymorphic cells which spread out over the surface of the culture dish. These cells frequently formed a paving sheet with Schwann cells lying across and interweaving between them (Armati-Gulson, 1980). These cells have been shown to stain with fibroblast-specific Thy 1.1 antisera (Brockes et al., 1979) and are identified as fibroblasts. The fine structure of these cells differs from the glial cell in that their nuclei and cytoplasm are less dense. In addition, the cytoplasm has extensive rer and numerous surface blebs, unlike glial cells (Dubois-Dalcq et al., 1981). There is also no evidence of cytoplasmic projections of these cells enveloping neurites or neuronal somas.

Defined medium cultures also showed 2 distinctly different nonneuronal cells. One, a glial-like cell, was small in size, with dense heterochromatin and cytoplasm, and frequently showed cytoplasmic envelopment of several neuritic profiles. As with the serum-supplemented cultures, these glial-like cells were found either in association with neurons or isolated from neuronal somas but in association with neuronal processes. The other nonneuronal cell was similar in ultrastructure to that seen in serum-containing cultures when viewed in sections cut perpendicular to the substrate, except that multilayers of this cell type were not found. Phase microscopy of the cell when

viewed parallel to the substrate, however, showed that the cell was very flattened but almost always circular rather than polymorphic. In spite of the difference in cell morphology, it is felt from their ultrastructural similarities that this represents the same fibroblast-like cell found in serum-supplemented cultures. Culture medium may affect cell shape. Perhaps the removal of serum or the addition of one or more of the defined medium ingredients accounts for this change. Testing for the presence of specific cell surface markers may offer insight regarding the exact identity of the nonneuronal cell population.

II. NGF Requirement

The human ganglion cells grown in the absence of NGF attached to the substrate but little neurite extension was noted. The addition of serum to the medium did not enhance neurite outgrowth. By comparison, neurite formation at 5 days was extensive in all cultures to which NGF was added. The NGF requirement by sympathetic ganglion cells for maintenance and neurite extension has been well documented (for review see Levi-Montalcini and Angelitti, 1968, and the Introduction). Other investigators (Hervonen et al., 1978) using human sympathetic ganglion cells found that the cells grown in serum-supplemented medium extended neurites and survived in culture for several weeks, although neurites were considerably shorter than those in cultures with

NGF. It is possible that endogenous sources of NGF might account for the survival and neurite outgrowth in this case. Glial cells as well as serum have been shown to contain small amounts of NGF (Ebendal and Jacobson, 1977; Varon et al., 1974). The amount of NGF present in serum may vary greatly from batch to batch and among commercial sources. The concentration of several different serum components has been measured and found to be extremely variable (Waymouth, 1972). The growth of normal cells in the absence of serum would therefore overcome this source of experimental variability.

III. Synapse Formation

The finding of synapses in long term cultures of the ganglion cells is consistent with their occurrence in cultured ganglia from chick (Teichberg and Holtzman, 1973), mouse (Kim and Muncasa, 1974), rat (Bunge et al., 1974; O'Lague et al., 1974; Olsen and Bunge, 1973; Rees and Bunge, 1974) and human (Hervonen et al., 1978). Electrophysiologic and pharmacologic studies (O'Lague et al., 1974) have indicated that some of the synapses made between principal neurons are excitatory cholinergic. This is unexpected since the only synapses found between the principal neurons in vivo are inhibitory and adrenergic (Kobayashi and Libet, 1970; Jacobowitz, 1970). The synapses found in culture are thought to be newly formed in vitro since

ganglionic connections are severed at the time of explantation and any existing synapses would have degenerated. The type of synapse found in cultures of the human neurons is not known. The ultrastructure of the synaptic region (small agranular vesicles and occasional LDCV) is consistent with some cholinergic endings in humans (Schulman, 1975), with immature adrenergic neurons, with adrenergic endings whose small dense core vesicles are not preserved by normal fixation procedures, or with cultured neurons expressing more than one transmitter phenotype (see Introduction--Plasticity in vivo and in vitro). Ultrastructure of synaptic endings in rat sympathetic ganglion cells over an 8 week period in culture shows a shift from predominantly adrenergic to predominantly cholinergic phenotype (Johnson et al., 1980). Sufficient time points were not done in this study to tell if there was an ultrastructural change in the synaptic profiles which would indicate such a shift. Synapses were not found at 5 and 10 days in culture but were found at 20 days in vitro. It is unlikely that adrenergic synapse formation and shift to cholinergic expression are occurring in the 10 day period of time. Cytochemical work performed during the second part of this thesis demonstrated that the neurons over short and long term culture retain an adrenergic phenotype. Preloading of the cells with 5' OHDA before fixation did not reveal the presence of a population of small dense core vesicles. It seems reasonable to assume then that these synapses

represent immature adrenergic endings. Alternatively, "dual transmitter" synthesis may be occurring in these neurons so that cholinergic endings may be present in spite of the demonstration of monoamines. Clearly more work on the developmental sequence of synapse formation in cultures of human sympathetic ganglion cells as well as electrophysiological, immunological and pharmacological studies must be done.

Examination of _in vivo_ ganglia occasionally showed the presence of synapses that were thought to be the cholinergic endings of preganglionic fibers. Synaptic connections are present _in vivo_ as early as 12 weeks (Hervonen and Kanerva, 1972) and are predominently of preganglionic origin.

IV. Developmental Staging

All time points examined (5, 10, 20 day defined medium and serum-supplemented cultures and 40 day defined medium cultures) showed cells that ranged in developmental maturation from the primitive sympathetic cell to the more mature young sympathetic neuron. This demonstrates that defined medium allows for the survival of all the cell types representing the different stages of neuronal maturation. Although different neuronal cell types were found in all cultures, there was a progression towards more mature appear-

ing neurons in later cultures (20 and 40 day). Examination of cultures grown in both serum-supplemented and defined media showed that the more mature neuroblasts and sympathetic neurons were the most common developmental stages found in the 20 day cultures (see Table III). Five and ten day cultures had neurons almost equally divided among the primitive sympathetic cells and neuroblasts while very few cells were labelled as young sympathetic neurons.

A study of progressive maturation in culture was not one of the initial objectives of this study. After extensive examination of the ultrastructure of the neurons from all time periods, it was felt that a greater number of neurons in the older cultures were more mature ultrastructurally. An analysis using the material available was done which supported the initial observations. Even though much was done to insure the randomness of the sample, the limited number of fetuses, the inherent bias in classifying neurons from micrographs already studied and the lack of information regarding the axial level of the ganglia from which the cultures were taken may affect this. Therefore, these results are offered as preliminary observations. In view of the findings from this analysis, several considerations are discussed below.

The favored survival of neurons from a specific developmental stage would not be sufficient to account for the

data in Table III since all the different stages survived through 40 days in culture. While partial selection is still possible (that is, more immature cells dying over time in culture), phase and electron microscopic examination of cells from the different cultures did not show any evidence of cell necrosis at 5, 10, and 20 days. A very large percent of the immature neurons would have to die to account for the change in the young sympathetic neuron class from 2 to 50%. Examination of 40 day cultures, in general, showed less healthy appearing cells. Somas of many of the neurons contained large amounts of osmiophilic vacuoles. Therefore, cells from 40 day cultures were not included in the developmental analysis. Because of the necessity of a large number of the primitive neurons dying between 5 and 20 days to account for the developmental sequence seen and the lack of evidence of such death, the results of this analysis could not be accounted for by this means. Selection during preparation of the cultures also seems unlikely since 1-2 whole ganglia were used for each culture dish. In addition, cultures were derived from 3 fetuses. For cultures derived from a particular embryo, at least 2 specimens for each time point and for each medium condition were examined. Cells in 5 day cultures, therefore, had 10 and 20 day sister cultures established from the same embryo included in the analysis.

The number of neuronal cells did not appear to in-

crease in culture. Mitotic figures were never encountered in any phase or electron microscopic examination. Even if mitotic activity was still occurring, one would expect that this would influence results by increasing the number of younger stage neurons in the older cultures. Just the opposite was found.

One of the criteria used for classifying neurons was the ratio of cytoplasm to nucleus. The part of the cell examined and the angle of the section may produce a biased view of this relationship. It was felt, however, that the number of neurons viewed, the inclusion only of neurons whose sections represented cuts through approximately the center of the nucleus and the inclusion of 2 other separate criteria for evaluating maturation (nuclear and cytoplasmic differentiation) made this comparison a valid one.

From the above considerations, the finding of more mature neurons in older cultures of the ganglion cells is thought to represent a maturation process *in vitro* and is not the result of selection or survival in culture.

Examination and classification of *in vivo* ganglia was done to gain an appreciation for the distribution of cell types in the second trimester fetus. Neurons were predominently classified as primitive sympathetic, the youngest developmental stage. A direct comparison was not made

between the distribution of cells in developmental stages in *in vivo* ganglia and those in culture since ganglia used for *in vivo* examination were not from the same fetuses as those that were cultured. The possibility exists that *in vitro* cells were established from ganglia that showed a more advanced degree of development at the time of culture. However, the crown-rump lengths (an index of embryonic age) of the fetuses used for *in vivo* studies were approximately the same as those used for culture (10.5-16 cm *in vivo* versus 10.5-17 cm *in vitro*). If it is valid to assume that the developmental stages of the cultured ganglia at the time of explantation were the same as the *in vivo* ganglia studied, then the explanted neuronal cells continue the maturation process already begun *in vivo*. Since maturation is more advanced even at 5 and 10 days in culture as compared to *in vivo*, the maturation and aging process may be occurring at an accelerated pace. Careful studies examining the degree of maturation of ganglion cells throughout the second trimester and comparative ultrastructural studies between *in vivo* second trimester ganglion cells and cultures of ganglia from the same fetuses is needed for confirmation.

SIF cells were not found in the *in vivo* and *in vitro* material although they are known to be present in the human ganglia at this stage (Hervonen and Kanerva, 1972). This is most likely due to sampling since SIF cells are scarce

in number.

V. Neuroblastoma

The wide use of neuroblastoma as a model system for developmental studies had in part prompted this ultrastructural investigation. Several human lines, both parental (E. Barnes, 1980) and clones derived from these lines (Burmeister, 1982; Carroll, 1982), have been extensively studied in regard to their ultrastructure in serum-containing cultures. In addition, the fine structure of the clonal lines has been assessed during growth in N2 defined medium and in the presence of factors thought to promote differentiation. The extent of fine structural maturation can now be evaluated in comparison to their normal in vivo counterpart. Clonal lines SK-N-SH SY5Y and SK-N-BE(2) M17, consisted mainly of cells with immature-appearing cell somas, very much analogous to that of the developing neuroblast. No ultrastructual differences were found when cultured in either serum-supplemented or chemically defined medium. The cytoplasm of most of the cell somas in both clonal lines usually contained a few stacks of rer, ribosomes (found mainly as polysomal aggregates), a moderate to well developed Golgi complex and dense core vesicles found either as randomly distributed single vesicles or frequently in small clusters. The processes of the clonal neuroblastoma cells were found to contain the same variety of

organelles as the cytoplasm. Typical varicosities or terminal synaptic profiles were never found. The processes, however, did contain numerous longitudinally arranged microtubules and 10 nm filaments. Processes of normal human sympathetic cells, even at 5 days in culture, almost always exclude ribosomes and rer and show varicosities along their length. In contrast to the neurite elaboration seen in the human ganglion cells, processes from neuroblastoma cells showed a lack of association to form fiber bundles. It can be concluded that differentiation of the cell somas of the clonally derived neuroblastoma, although still immature, is further advanced developmentally than that of the primitive sympathetic cell. The content of the numerous dense core vesicles found in these cells has not yet been analyzed and must be before concluding that these vesicles represent the storage site for adrenergic neurotransmitter. On the other hand, the processes seen in the neuroblastoma cultures do not appear as typical axonal processes even under conditions which promote neurite extension. These processes may represent very early stages of process formation or simply be cytoplasmic extensions not representative of true neuronal differentiation.

The parental lines from which these clones were derived appeared more immature. Most cells resembled those of the primitive sympathetic cell. Many cell profiles showed cytoplasm containing only free ribosomes. Processes

were found mostly to contain ribosomes and were lacking in microtubules and 10 nm filaments. The reason for the difference in the degree of ultrastructural maturation betweem the clonal and parental lines is not well understood. Both parental lines showed some range of cell types. Cloning procedures may have isolated cells more capable of progressing developmentally.

VI. Dense Core Veiscles

Cytochemical observations made in this study indicate that large dense core vesicles are the storage sites for monoamines in developing human sympathetic ganglion cells. The location of monoamines in these cells was in doubt since potassium permanganate fixed cells failed to show any dense core vesicles (Hervonen et al., 1977). On the other hand, the presence of monoamines in human ganglia has been demonstrated with formaldehyde-induced fluorescence (Hervonen et al., 1977). A modification of the histochemical chromaffin reaction was employed to detect and localize monoamines on a fine structural level.

A. Cytochemistry

Model test tube experiments have shown that treatment of various amine solutions with glutaraldehyde followed by the addition of potassium dichromate results in the forma-

tion of an insoluble precipitate (Hokfelt, 1971). Only amines with unsubstituted amino groups, that is, norepinephrine, dopamine, and 5' hydroxy tryptamine, react. The false neurotransmitter, 5' hydroxy dopamine, also has been shown to be reactive (Tranzer and Thoenen, 1969). The postulated reaction involves 2 steps (Tramezzani et al., 1964). First, the formation of a Schiff monobase by the condensation of glutaraldehyde with the unsubstituted amino group. Second, the condensation product formed has reducing capabilities and reduces the dichromate solution to chromium dioxide which forms an insoluble brownish-black precipitate.

On a fine structural level, an electron dense precipitate is seen wherever reaction has occurred. Specificity of the cytochemical reaction for monoamines has been determined in a variety of rat tissues (iris, mesentery, heart, sympathetic ganglia, adrenal medulla, pineal body and brain) (Tranzer and Richards, 1976). Monoamine-containing tissues treated with chromate-dichromate and post-fixed in osmium have intensely electron dense granules contained in small and large vesicles. Other cellular constituents such as glycogen particles and mitochondrial granules are nearly as electron dense. However, monoamine-containing tissue treated with chromate-dichromate but unosmicated and unstained show only small and large electron dense cores. All other cellular components including the large dense

core vesicles occasionally seen in cholinergic endings are unreactive. Depletion of monoamines from the neurons by reserpine results in the absence of electron dense precipitate from most of the small dense core vesicles and only a faint density in the large dense core vesicles.

B. Monoamine Location

The dense precipitate seen in the chromate-dichromate treated cultures in this study demonstrates the presence of monoamines in the ganglion cells. That the electron dense precipitate found in these cultures is the result of chromate-dichromate treatment is shown by the appearance of precipitate in chromate-dichromate treated material that had no osmication or staining of the sections. Osmium is a strong oxidant that is known to react with a variety of cellular constituents (unsaturated fatty acids, amino acids and proteins) including biogenic amines (Hanker et al., 1967; Hake, 1965). Osmium is included as a post-fixative so that cellular membranes and organelles can be visualized. Viewing of sections without osmium fixation or staining is a tedious chore but is necessary to insure that the electron densities are not artifactual. Tissue fixation with glutaraldehyde alone does not lend electron density to any cellular components. Electron dense precipitate in unosmicated material appeared in cell somas and processes, either as a single density or in clusters.

The densities were generally rounded and measured 50-94 nm in diameter. The location, distribution, morphology and size range of the precipitate is compatible with storage of the monoamines within large dense core vesicles. In addition to the vesicular storage of monoamines, an avesicular location is also possible and would not be visualized by this technique.

Since chromate-dichromate reacts with several of the biogenic amines, it is not known which monoamine is present in these cells. Norepinephrine is known to be the neurotransmitter in adult sympathetic ganglion cells and it is likely that the reaction product seen in these cultures is due to this monoamine. Dopamine, however, is an intermediary in the formation of norepinephrine from tyrosine and is a neurotransmitter in the interneurons within the ganglia in several mammals (Kebabian, 1976). Reaction product might also signify this monoamine. Serotonin (5' hydroxy tryptamine) is a less likely candidate since it is not a neurotransmitter in these cells in vivo. The "false" neurotransmitter 5' hydroxy dopamine may account for dense granules in loaded cultures but obviously would not be responsible for chromate-dichromate reaction product in unloaded cells since it is not a naturally occurring substance.

Recent immunocytological studies on human sympathetic

ganglion cells (Hervonen et al., 1980) has shown the presence of tyrosine hydroxylase, dopamine-β -hydroxylase, substance P, and enkephalin.

In sections prepared so that cellular membranes are adequately visualized, vesicle membranes around the precipitate could be readily seen in many instances. Failure to see membranes limiting some particles in the same material is most likely due to precipitate completely filling the vesicles and thus obscuring the membrane.

C. Developmental Significance

Dense core vesicles were found readily throughout the cytoplasm and in the neuronal cell processes at all the time points examined. The vesicles were always of the large variety (70-130 nm). It is interesting to note that small dense core vesicles were never encountered.

Several cultures were incubated with 5' OHDA before fixation in an effort to load adrenergic neurons with a biogenic amine that was reactive with chromate-dichromate. Cultures of sympathetic ganglion cells from rat (Rees and Bunge, 1974; O'Lague et al., 1974) continue to express the adrenergic property of monoamine uptake. The "false" neurotransmitter 5'OHDA is also taken up and stored by adrenergic endings (Tranzer and Thoenen, 1968). It was hoped that

a similar response by cultured human ganglion cells would result in a positive control and facilitate finding of chromate-dichromate positive granules. It was also felt that 5'OHDA treatment might reveal a population of small dense core vesicles not previously found. Examination of chromate-dichromate cultures with or without 5'OHDA incubation showed no difference in the apparent number or density of dense core vesicles. The size range of the electron dense granules (50-94 nm) in unosmicated cultures that were pretreated with 5'OHDA was not consistent with their presence in small dense core vesicles. Likewise, no small dense core vesicles were found in osmicated cells that were loaded with 5'OHDA. This is in agreement with ultrastructural studies of glutaraldehyde-osmium fixed ganglion cells (Zeevalk et al., 1982) cultured for up to 40 days and with other ultrastructural studies of human fetal sympathetic ganglia (Hervonen et al., 1978). Although small dense core vesicles are thought to be the major site of monoamine storage in most adrenergic neurons, the presence of monomines within large dense core vesicles has been demonstrated in the iris and vas deferens of the cat (Tranzer and Thoenen, 1968). The adrenergic uptake mechanism may not be functioning in these cultured cells; this would explain the lack of any significant differences in the extent of reaction product in loaded and unloaded cultures. On the other hand, the frequent finding of positive chromate-dichromate reaction product (particularly in the 25 day cultures) in

both 5'OHDA loaded and unloaded cultures suggests that monoamine storage may be maximal and preservation conditions optimal so that little difference was seen. A thorough quantitative examination was not done and may reveal subtle differences. Fine structural studies of adult human adrenergic endings in the myocardium (Kyosola et al., 1976), ureter (Schulman, 1975), oviduct (Ishii, 1972), and sympathetic ganglia (Helen and Hervonen, 1981) show the mature synaptic ending to contain both large and small dense core vesicles. The first appearance of small dense core vesicles within the endings of human sympathetic neurons is not known. In rat (Eranko, 1972), chick (Wechsler and Schmekel, 1967; Luckenbill-Edds and van Horn, 1980), and rabbit (Tennyson and Mytilineou, 1975), the first dense core vesicles found in the perikarya are of the large variety. As maturation proceeds, small dense core veiscles become evident. Some investigators have noted a decrease in the number of large dense core vesicles with a corresponding increase in the number of small dense core vesicles (Machado, 1971) during development of the neuron. Such observations have led to the proposal that small dense core vesicles are formed from large dense core vesicles and that the distribution and appearance of the 2 size classes may represent different stages of maturity. While the exact developmental sequence of the dense core vesicle population in humans has not been done, the presence of large dense core vesicles in the neurons fits a pattern seen in

other animals. The presence of large dense core vesicles but not small may reflect the immature developmental stage of these neurons. Other considerations which cannot be ruled out at present are a possible shift to cholinergic expression during culture (see Introduction), or to a labile storage of catecholamines within small dense core vesicles and the loss of these during fixation.

C. Potassium Permanganate

In view of the abundance of large dense core vesicles in glutaraldehyde-osmium and chromate-dichromate fixed cultures it is surprising to find so few positive vesicles in KMnO4-treated cells. This is, however, consistent with the work of Hervonen et al. (1977), who were not able to find KMnO4-positive vesicles in similar conditions. The appearance of occasional vesicles with a dense core in this material demonstrates the presence of monoamines. However, the low frequency of positive vesicles as compared to those found with the other fixation methods suggests that KMnO4 may not be an appropriate fixative for the demonstration of monoamines in this fetal tissue. The lack of positive findings with KMnO4 may be due to failure of KMnO4 to reach the storage sites in sufficient concentration or to too low an amine concentration at the storage site at the time KMnO4 reaches it (Hokfelt and Jonsson, 1968). The concentration of KMnO4 used in this study

(3%) is the same as that used in other mammalian ganglion cells (Eranko, 1972) and cultures of ganglion cells should not pose penetration problems for the fixative since the distance travelled is small. This would argue against a low concentration of KMnO4 reaching the storage sites.

It is more likely that there is a diffusion of monoamines from the vesicles though why this tissue would be more sensitive to KMnO4 fixation is not known. Other investigators also have noted the failure of KMnO4 to demonstrate dense core vesicles in cells known to contain monoamines. Dixon and Gosling (1976) were not able to demonstrate small granular vesicles in the vas deferens of perinatal rats. Likewise, rat adrenomedullary cells do not show electron dense vesicles when fixed with permanganate (Kanerva et al., 1977).

KMnO4 was first introduced by Richardson in 1966 as a fixative to demonstrate norepinephrine in small granular vesicles. The exact reaction involving KMnO4 is not known but it is believed that KMnO4 with its high redox potential (1.51) undergoes an oxidation-reduction reaction with the phenolic hydroxyl groups of the monoamines to form an insoluble precipitate of MnO2 (Holfelt, 1971). Model test tube experiments (Hokfelt and Jonsson, 1968) show the reactivity of KMnO4 with the biogenic amines norepinephrine, adrenalin, dopamine, and 5'-hydroxytryptamine as

well as with a variety of other compounds. Thus KMnO4, although frequently used to demonstrate the presence of monoamines, is not uniquely specific in its reaction with biogenic amines. The strong reaction with KMnO4 may often result in destruction of tissue components. KMnO4 solubilizes certain proteins and causes structural damage to others (Hake, 1965). Investigators also have noted shrinkage effects on many tissue components fixed with 3% KMnO4 (Wetzel, 1961; Hokfelt and Jonsson, 1968).

The generally poor preservation of KMnO4 was also noted in this study. Cells were frequently distorted and plasma membranes ruptured. This may have been the cause of the paucity of granular vesicles after KMnO4 fixation. It should be noted that adult human sympathetic ganglion cells show large and small dense core vesicles that are demonstrable by KMnO4 fixation (Helen and Hervonen, 1981). This suggests differences between the large dense core vesicles found in developing ganglion cells and the adult. These differences may be in the way monoamines are bound within the vesicles or in the vesicles themselves (in particular the vesicular membrane composition.)

VII. Conclusions

The completion of the experimental research as presented in this thesis has led to several conclusions. In ad-

dition, it has raised several questions which bear further investigation. Conclusions and queries are presented below.

1. Human fetal sympathetic ganglion neurons survive in a chemically defined medium for up to 40 days *in vitro*.

2. These cells retain their differentiated state with regard to neurite extension, fiber bundle formation and fine structural maturation. Ultrastructural features of the ganglion cells are similar to those in serum-containing cultures and in the more mature neuronal cells include a large vesicular nucleus, large dense core vesicles, Nissl bodies, mature Golgi complexes and numerous processes with varicosities and synaptic profiles.

3, The defined medium conditions used here permit the survival of cell types representing several successive stages of neuronal maturation.

4. The growth of these neurons in a serum-free medium makes this a more desirable culture environment for experimental cell research. Although serum coating of the culture dishes is at present necessary for the attachment of these cells (and may possibly provide other required factors), treatment of the culture dishes' surfaces with attachment-promoting factors such as polylysine or fibro-

nectin should eliminate this requirement.

5. The drastic reduction in nonneuronal cells in defined medium demonstrates the partial selectivity of this medium for neuronal cells. This medium would then be useful for long-term experiments since it would eliminate the problem of overgrowth by nonneuronal cells. Further manipulation of the medium may allow complete elimination of these cells. The ability to control the growth of nonneuronal cells in a graded fashion would facilitate experiments assessing the influence of nonneuronal cells on the development and differentiation of the neuronal phenotype.

6. Neurite extension _in vitro_ is dependent on the presence of NGF in the medium. When it is eliminated from the medium, neurons attach but fail to extend processes.

7. In addition to the cytological differentiation seen in the cell somas in 20 day cultures, synaptic profiles first become evident at this time. This is thought to represent _de novo_ synapse formation since the synaptic connections severed during explantation would have degenerated by 3 weeks in culture. This is confirmed by the lack of synaptic profiles at 5 and 10 days. The type of synapse present in these cultures (adrenergic, cholinergic, or both) and the function of such synapses remains to be elucidated.

8. The classification of neurons into different developmental stages suggested a maturation of the neuron over time in culture. It appears likely that neurons continue to differentiate in culture as reflected in the marked increase in the proportion of more mature neurons between 5 and 20 days.

9. Ganglion cells over long and short-term culture contain monoamines in cell somas and processes. These observations confirm the presence of monoamines in serum-supplemented cultures of the ganglion cells and for the first time demonstrate that monoamines are present in long-term cultures in the absence of serum. The most likely candidates represented by the positive reaction product in chromate-dichromate treated cultures are norepinephrine and dopamine.

10. Monoamines in the cultured fetal ganglion cells are stored within large dense core vesicles. The general morphology, location, distribution and size range of the electron dense cores are consistent with this interpretation. Although the mature adult ganglion cell is known to contain small dense core vesicles, no such vesicles were found in the cultures. This suggests that these ganglion cells, while expressing many neuronal differentiated features in culture, may still be developmentally immature. Another interesting question that merits further study is

whether cholinergic properties are being expressed in these cultures as they are in other mammalian ganglion cultures.

11. Potassium permanganate fixation does not adequately demonstrate the presence of dense core vesicles in human fetal sympathetic ganglia. The most likely cause for this is diffusion of the amine before it is fixed.

12. Monoamine storage in vesicles in human fetal ganglion cells may have differences from its storage in adult ganglion cells. This is suggested by the fact that monoamines are known to be present and stored in vesicles in cultures of the fetal ganglion cells but are not demonstrable by $KMnO_4$. On the other hand, $KMnO_4$ fixation adequately demonstrates small and large dense core vesicles in adult ganglion cells.

VIII. Future Considerations

The serum-free maintenance of human fetal sympathetic neurons provides a culture system that can be used as an important tool for future studies. This type of culture system is particularly applicable to studies of the developmental effects of various exogenous factors on the neurons. Baseline studies as reported here describe the extent of ultrastructural maturation of the neurons *in vitro*. A normal progression of this work would then

include the *in vitro* manipulation of the development of these neurons. Before conducting such studies, it is suggested that further refinement of the culture environment be done with respect to 1) the investigation of alternative methods of promoting attachment of the neurons to completely eliminate the need for serum [Some possible avenues of investigation might be the use of polylysine coating of the culture dish with fibronectin added to the medium (Bottenstein and Sato, 1980), addition of low concentrations of bovine serum albumin to the medium (Iacovitti, personal communications) and the use of high adhesive collagen as a substratum (Adler et al., 1979)], 2) the complete dissociation of the neurons and their plating as monolayer (single cell) cultures. This second refinement would minimize any possible cross-feeding events which may be masking additional nutritional requirements and permit quantitative evaluation of neuronal survival. Since some cell reaggregation seems likely, the use of high adhesive collagen might allow the maintenance of single cell cultures as well as promote attachment.

An interesting finding in these studies was the appearance of synapses with ultrastructural characteristics typical of cholingergic endings. The possible accrual of cholinergic properties by these neurons can be investigated by biochemical means through measurement of CAT activity or through electrophysiological means. If these cells do

indeed express both cholinergic and adrenergic properties, then investigation of which environmental cues permit or inhibit the expression of one or the other neurotransmitter would provide valuable information regarding developemnt of sympathetic neruons and would have clinical implications as well.

Important knowledge for any developmental biologist is the sequence of events occurring during development of a particular system. In the course of this research, I found little work describing the extent of ultrastructural development along a rostral-caudal gradient in sympathetic ganglia or the relative proportion of cells at different developmental stages during embryogenesis. Detailed work of this sort would be useful in determining whether *in vitro* studies using sympathetic ganglia from different axial levels but similar stage embryos are truly comparable and would provide analysis of whether the time sequence of developmental events *in vitro* is representative of development *in vivo*.

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