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City University of New York PH.D. 1982

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PROCESS FORMATION IN THE HUMAN NEUROBLASTOMA CLONE SK-N-SH-SY5Y IN VITRO

by

DONALD W. BURMEISTER

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

1982

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.

January 22, 1982
Date

Chairman of Examining Committee
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May 10, 1982
Date

Executive Officer
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Professor William Cohen — Hunter College, Institution

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ABSTRACT

PROCESS FORMATION IN THE HUMAN NEUROBLASTOMA CLONE SK-N-SH-SY5Y IN VITRO

by

DONALD W. BURMEISTER

Adviser: Professor Katherine Lyser

The cultured cells of the human neuroblastoma clone SY5Y extend long, narrow, neurite-like processes. These processes appear under all of the culture conditions tested, but their length and number can be changed by nerve growth factor (NGF), dibutyryl cyclic adenosine monophosphate (dbcAMP), and fetal calf serum.

SY5Y cells and processes have an ultrastructure similar to that of developing sympathetic ganglion cells. The cell bodies exhibit eccentric nuclei, and dispersed, sparse, rough endoplasmic reticulum and golgi complexes. The neurites are dominated by microtubules with few intermediate filaments and they end in stuctures typical of neuronal growth cones. The fine structure is not appreciably altered by agents which alter neurite outgrowth.

NGF causes SY5Y cells in serum-containing medium to double the percentage of cells with processes greater than 100 μm. However, the population doubling time, the length, width and the area of the substrate covered by the cell, the distribution of cell shapes in the population, the number of processes longer that 25 μm and the average

number of processes of any size per cell remain the same as in non-NGF treated controls. This indicates that NGF acts not by inducing new processes, but by lengthening the processes that are normally on the cell.

When SY5Y cells are grown in a serum-free medium which supports population growth, there are some differences from serum fed cells, but the percentage of cells with long processes remains the same as in serum. Added NGF does not increase this number, but does reduce population growth and causes a pronounced clumping of the cells. The cells under this condition also show a pronounced aversion to areas bordering scratches made on the culture substrate, a property not seen under other conditions. An hypothesis is presented that NGF alters the adhesive properties of the cells and that serum modulates the expression of the adhesive change.

The effects of dbcAMP in serum and and serum-free medium are the opposite of NGF. Long process extension is not influenced by dbcAMP in serum but is greatly stimulated in serum-free medium. Although both dbcAMP and NGF can stimulate process formation they probably act through different mechanisms.

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The work of this thesis was done in the laboratory of Dr. Katherine Lyser and I gratefully acknowledge her kind guidance and support over the last five years.

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I especially want to acknowledge the patient sacrifices of my wife, Susanna, and sons Abraham and Tobias, who had far too many 0.5 day weekends, so that this thesis could be completed.

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INTRODUCTION

Cells of the human neuroblastoma clone SK-N-SH-SY5Y will extend long processes when they are exposed to nerve growth factor (NGF) in vitro [Perez-Polo et al., 1979]. This thesis is an examination of this phenomenon.

The SY5Y neuroblastoma cells possess a number of neuronal properties, including electrically excitable membranes [Kuramoto et al., 1981] and the production of the neurotransmitter synthesizing enzymes dopamine-B-hydroxylase and tyrosine hydroxylase [Ross et al., 1980; Carroll, 1982]. The production of long processes is thought to be another neuronal characteristic of these cells.

Nerve growth factor is a protein hormone required by developing sympathetic neurons for survival [reviews by Greene and Shooter, 1980; Vinores and Guroff, 1980] and it has also been implicated in directing and speeding neurite outgrowth [Levi-Montalcini, 1976; Letourneau, 1978]. The dissociation of the two effects has been difficult because the NGF requirement for cell survival by sympathetic neurons precludes the establishment of controls not receiving NGF.

The SY5Y clone does not require NGF for survival, but it does give a morphological response to it. Therefore the study of this cell line offers an opportunity to examine the morphological effects of NGF without the complications brought about by the survival requirement.

I have made a detailed examination of the growth of processes

from SY5Y cells, quantitating the response and documenting the changes in the cell fine structure.

There were three general problems I addressed. 1. Are the processes of SY5Y cells legitimately comparable to the processes of neuronal cells? 2. How does NGF enhance the production of processes? 3. How do the responses to NGF compare with the morphological changes brought about by other agents, particularly agents which raise the intracellular levels of cyclic adenosine monophosphate {cAMP}?

HISTORICAL BACKGROUND

Any study concerned with cellular morphology has its roots in the work of the great 19th century microscopists. In the study of neuronal morphogenesis our current ideas stem largely from the work of Wilhelm His (1831-1904) and Santiago Ramón y Cajal (1852-1934). Ramón y Cajal in particular saw that early neurons would extend individual processes out from the cell body to make contact with other cells in distant areas [Ramón y Cajal, 1937].

The observations of Cajal and His were based upon the static images of fixed and sectioned embryos and the observations were disputed by many eminent scientists of the time [Peters et al., 1976, pp 1-4]. The American embryologist Ross Harrison (1870-1959) [1907;1910] reasoned that only if neuronal precursor cells were isolated from the complex multicellular environment of the embryo would it be possible to determine the origins and method of production of nerve fibers. To this end he was successful in isolating and cultivating early neural tubes from frogs. The neural tubes were from stages before any axons were visible, but in culture the cells extended long axon-like processes. With this series of experiments Harrison fully validated the reconstructions of Cajal and also established both a method and an experimental perspective that has been carried on for seventy years.

"The possibility becomes apparent of applying

> the above method to the study of the influences which act upon a growing nerve. While at present it seems certain that the mere outgrowth of the fibers is largely independent of external stimuli, it is, of course, probable that in the body of the embryo there are many influences which guide the moving end and bring about contact with the proper end structure."
>
> [Harrison, 1907]

ADHESION AS A FORCE IN MORPHOGENESIS

The role of the physical environment surrounding the growing neurite has been the most intensively studied factor affecting neuronal morphogenisis. Wilhelm His first postulated the role of the surrounding tissues on axon growth, directing axons along `the path of least resistance' [see Ramón y Cajal, 1960; Jacobson, 1978]. This was picked up by Harrison [1910], who observed that growing neurites required a solid substrate to advance and proposed that `paths of predilection' may exist in the developing embryo along which nerve fibers elongate.

The role of the substrate in directing neuronal outgrowth was examined in detail by Paul Weiss [1934]. Weiss had previously shown that non-neuronal cells grown in culture on plasma films, stretched in various ways, aligned themselves along the lines of stress. Previously stretched plasma films and plasma clots oriented by repeated stroking with a fine brush also caused cells to elongate. Weiss proposed that the `ultrastructural organization' of the clot, the fibrin strands, became oriented in one direction and that this in turn leads to orientation of the individual cells. Working in Harrison's lab, Weiss showed similar effects on the outgrowth of chick dorsal root ganglion cell fibers [Weiss, 1934], an effect he termed `contact guidance' [Weiss, 1941].

The underlying force in contact guidance is adhesion. In analyzing the orienting behavior of dissociated non-neuronal cells Weiss saw that the shape of the cell was determined by the number of points of close contact with the substrate. On substrates composed of

long parallel lines (such as the collagen fibers seen on the inside surface of fish scales [Weiss and Taylor, 1956], or parallel scratches made on glass slides [Weiss, 1958]), cells assume long bipolar morphologies. When grown on an amorphous substrate the same cells assume a more spread, `multiform' character. This, Weiss concluded, was due to differential adhesion of the cell and the substrate. On the aligned substrates, cells are more adherent to the collagen fibers or the scratches than to the surrounding regions and extend along the preferred substrate assuming a bipolar shape.

The growing nerve tip, the growth cone, was thought to be analogous to the spreading periphery of cells in culture, and likewise was assumed to grow out along paths by `selective adhesivity' [Weiss, 1941].

The analysis of adhesiveness of cells has since then been very productive [see review, Grinnell, 1978], but work on the adhesion of neuronal processes has not been quite as fast. For one thing the aligned substrates of Weiss do not alone align the outgrowth of neurites. In the fiber clot experiments with dorsal root ganglia an outgrowth of non-neuronal cells is apparently aligned first; this then aligns outgrowing neurites. Neural tube explants which have little non-neuronal outgrowth do not have aligned neurite outgrowths [Weiss, 1934]. Fish scales do not align neurite outgrowth [Weiss and Taylor, 1956], and "similar results" where reported with lines etched on glass [Weiss, 1958].

In 1965, Carter reported that fibroblastic "L" cells in culture move up a density gradient of palladium placed on the substrate, moving from areas of less adhesivity (less palladium), to areas of higher adhesivity. Ludueña [1973] was unable to get chick dorsal root ganglion neurites to do this. Ludueña did report, however, that the rate of outgrowth of axons is altered by changes in the adhesion of the growth cone to the substrate. Neurites grow faster on more

adhesive substrates.

The first direct demonstration that neurite outgrowth could be influenced by differences between substrates was provided by Letourneau [1975a,b]. Dissociated dorsal root ganglion cells were grown on several different substrates and the adhesion of the growth cones was measured. After a hierarchy of substrate adhesiveness was established composite plates were made by shadow coating palladium on the various other substrates. Small areas of the original substrate were protected by electron microscope grids. When the grids were removed grid patterns on the dish consisting of islands of palladium bounded by paths of the underlying substrate were obtained. When dissociated dorsal root ganglion cells were plated on this surface the cells regularly remained on the substrate with higher adhesiveness and sent out neurites which also stayed on the more adhesive areas. This was particularly pronounced when the paths were of higher adhesiveness than the islands. In time lapse cinematographs the growth cones of neurons were seen moving down the paths and turning or branching only at the grid intersections, remaining on the more adhesive substrate. The axonal growth cones of chick dorsal root ganglia could distinguish between different substrates and the morphologies of the cells could be determined by it.

The restraints on growth cone movement seen on these patterned substrates is consistant with the mechanisms proposed by Weiss to explain the movement of fibroblasts in culture [Weiss, 1958]. The growth cones send out many filopodia, some landing in highly adhesive areas, some in less adhesive areas. The ones touching the more adhesive areas are stabilized and the growth cone follows this lead. In less adhesive areas the filopodia retract with no apparent effect on the growth cone.

Recently, dissociated parasympathetic ciliary ganglion cells were shown to have a similar behavior, but in response to a more naturally occuring substance [Collins and Garrett, 1980]. These cells will not

extend processes in culture unless they are exposed to heart conditioned medium, or to a substrate treated with conditioned medium. When only part of the substrate is exposed to the heart conditioned medium, processes grow from the treated area out to the border, grow along the border, but then more than 99% of the time do not cross onto the untreated side. This illustrates the importance of the specific molecular composition of the substrate in the promotion of neurite growth.

There are other factors which influence neurite growth; the tensions exerted on the growth cone by the neurite [Bray, 1979], various internal factors probably related to the structure of the cytoskeleton [Solomom, 1981], chemotropic factors [Coughlin et al., 1977] and electrical currents [Jaffe and Poo, 1979]. However, all of these factors ultimately act by altering or controlling the interaction of the neuronal growth cone and the physical substrate.

MODEL SYSTEMS OF NEURONAL DEVELOPMENT

As Ross Harrison foresaw in 1907, extra-embryonic culture of nervous tissue has allowed examination of many more factors in neuronal morphogenesis than would be possible with whole animals. Nevertheless, the culture of primary explants from embryos has several difficulties and limitations. Not the least of these is the availability of material. Because they are most easily isolated after they have left the mitotic cycle, generally only primary explants of neuronal cells are possible. Many of the cells which are most desirable for culture, because of homogeneity of cell type, or because they can survive well in culture are available only in limited amounts. Speed of dissection and the perseverance of the investigator become the limiting factors in any assay which requires large amounts of material. The heterogeneity of the explanted tissue is also a factor which must be considered; both the heterogeneity of neuronal cell types and the combination of neuronal, glial, endothelial and

connective cells present may influence the responses of the cells, or the resolution of the assay used. This is especially important in long term cultures where the neurons are the only cell type which is not proliferating. A more subtle, but equally important point is that depending on the time of explant from the embryo, observed axonal outgrowth may actually be axonal regeneration. This is an activity which may differ significantly from primary axon initiation [Johnson and Wessells, 1980; Burstein and Greene, 1978].

For these reasons, many investigators have used tumorigenic cell lines which express (or which can be made to express) neuronal characteristics in culture as models in the study of neuronal differentiation and morphogenesis. The clonal nature of the population, the ability to follow the cells through the cell cycle, and the ease of obtaining large numbers of cells are factors which outweigh the altered properties of the cells caused by their malignant nature. The most popular cell lines in the study of neuronal development are the neuroblastomas and the closely related pheochromocytomas.

NEUROBLASTOMA

Neuroblastoma was first differentiated from other nervous system tumors by Wright [1910]. He was the first to note its characteristic cell structure and to see the similarity between neuroblastoma and the cells of early sympathetic ganglia. Wright observed small cells with scant cytoplasm, many neuronal-like processes, many of which were observed attached to cell bodies, and small balls of cells with cell free central areas, rosettes. The tumors Wright observed were in or near areas of normal sympathetic ganglia, and he proposed that the sympathetic ganglia were the tissue of origin for the tumor.

The idea that this highly malignant tumor may at times mature into another tumor type, a ganglioneuroma, was forcefully presented by

TABLE NUMBER 1.
AGENTS OF MORPHOLOGICAL DIFFERENTIATION OF NEUROBLASTOMA

AGENT	CELL	CRITERION	REFERENCE
Acetylcholine	NB42B	N.E./5d.	Harkins et al. 1972
Actinomycin D	Neuro2a	1x diam.	Bear & Schneider, 1979
Adriamycin	NBP2	50µm/3d.	Prasad et al. 1980
Adriamycin + Vit E.	NBP2	50µm/3d.	Prasad et al. 1980
6-aminonicotinamide	N2A	N.E.	Zeitz et al. 1978
8-benzylthio-cAMP	NBA2	50µm/3d.	Prasad 1972b
Bleomycin + vit. E	NBP2	50µm/3d.	Prasad et al. 1980
8-bromo cAMP	NB-2	2x diam.	Waymire et al. 1978
8-bromo cGMP	M-1	N.E.	Zwiller et al. 1977
5-bromodeoxycytidine	C1300	40µm	Schubert & Jacob 1970
5-bromodeoxyuridine	C1300	40µm	Schubert & Jacob 1970
4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone {RO-20-1724}	C1300	50µm	Prasad & Sheppard 1972
Buturon	41A3	1x diam/2d	Erkell & Walum 1979
Carboxymethyl cellulose	N-18	1x diam/4d	Koike & Pfeiffer 1979
1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea+vit.E	NBP2	50µm/3d	Prasad et al. 1980
Chlorozotocin + vit. E	NBP2	50µm/3d	Prasad et al. 1980
Cytosine arabanoside	NB42B	extensive	Kates et al. 1971
Dexamethasone	NBP2	2x diam	Sandquist et al. 1978
Dibutyryl cAMP	C1300	50µm/4d	Prasad & Hsie 1971
Dicoumarol	NE115	2x diam	Egilsson, 1977
4-(3,4-dimethoxybenzyl)-2-imidazolidinome	C1300	50µm/1d	Prasad & Sheppard 1972

AGENT	CELL	CRITERION	REFERENCE
Dimethyl sulfoxide	NIE-115	`long'/6d	Kimhi et al. 1976
Dinitrophenol	NE 115	2x diam/3d	Egilsson 1977
Diuron	41A3	1x diam/2d	Erkell & Walum 1979
Fluomethuron	41A3	1x diam/2d	Erkell & Walum 1979
Fluorodeoxyuridine	NB42B	`extensive'	Kates et al. 1971
Glioma conditioned medium	NB-2a	1x diam/3d	Monard et al. 1973
Hexamethylene bisacetamide	NIE-115	N.E.	Palfrey et al. 1977
Hydergine	C1300	2x diam/8d	Nandy & Schneider 1978
Hypertonic medium	C1300	`long'	Ross et al. 1973
Lactic acid	C-1	40µm/5d	Schubert et al. 1971
Linuron	41A3	1x diam/2d	Erkell & Walum 1979
6- mercaptopurine	NBE-	50µm	Prasad 1973
Methoxuron	41A3	1x diam	Erkell & Walum 1979
1-methyl-cyclohexane carboxylic acid	NIE-115	N.E.	Portier et al. 1980
Monouron	41A3	1x diam	Erkell & Walum 1979
Mutomycin + vit. E	NBP2	50µm/3d	Prasad et al. 1980
Neburon	41A3	1x diam/2d	Erkell & Walum 1979
Nerve growth factor	*SY5Y	N.E.	Prasad et al. 1979
Oxygen (80%)	41A3	N.E.	Erkell 1980
Papaverine	C1300	50µm/1d	Prasad & Sheppard 1972
pH (low-6.6)	C1300	1x diam	Bear & Schneider 1977
Plasminogen deficient medium	*SK-N-SH	50µm/4d	Becherer & Wachsman 1980

AGENT	CELL	CRITERION	REFERENCE
cis-Platinum + vit. E	NBP2	50µm/3d	Prasad et al. 1980
Prostaglandin E1	NBA2	50µm/2d	Prasad 1972
Prostaglandin E1 + vit E	NBP2	50µm/3d	Prasad et al. 1980
Prostaglandin E2	NBA2	50µm/2d	Prasad 1972
Quinidine sulfate	NE-115	2x diam/3d	Egilsson 1977
Serum, calf, agammaglobulin	NBP2	50µm/4d	Prasad et al. 1979
Serum, calf, dialized	NBP2	50µm/4d	Prasad et al. 1979
Serum, calf, heat inactivated	NBP2	50µm/4d	Prasad et al. 1979
Serum, fetal calf delipidated	NB2A	1x diam/2d	Monard et al. 1977
Serum withdrawal	N-18	25µm/90m	Seeds et al. 1970
Sodium butyrate	NBP2	50µm/4d	Prasad & Vernadakis 1972
Sodium isobutyrate	C1300	1x diam/2d	Schneider 1976
Sodium proprionate	C1300	1x diam/2d	Schneider 1976
Sodium valerate	C1300	1x diam/2d	Schneider 1976
Substance P	N18	1x diam/90m	Narumi & Maki 1978
6-thioguanine	NBA2	50µm/1d	Prasad 1973
Valinomycin	N18	1x diam/2h	Koike 1978
Vitamin E	NBP2	50µm/3d	Prasad et al. 1979
X-rays	C1300	50µm	Prasad 1971

Notes. All cells are mouse C1300 neuroblastoma or C1300 clones except those marked * which are of human origin.
Criteria refer to the measure used to determine morphological response and the time period when the measure was taken.. 1x diam/3d means all cells with processes longer than one cell diameter were scored on the third day of exposure. N.E. means not explicated.

Cushing and Wolhbach [1927]. Ganglioneuromas are benign tumors which, like neuroblastomas, are located in positions suggesting a sympathetic origin. Their cytology is similarly neuronal, but of mature sympathetic ganglion cells rather than the immature cells of the neuroblastomas. Cushing and Wolbach presented a case history in which a neuroblastoma tumor in a boy of three was removed ten years later and found to have all the features of a ganglioneuroma. Similar cases have been found repeatedly [Everson, 1964].

The idea that neuroblastoma may spontaneously mature or at least remit in a large number of cases is supported by several studies based on systematic observations of the adrenal glands of children dying from other causes. Incidences of neuoblastoma-like regions can be found in more than 2% of the necropsies of children under 3 months of age [Beckwith and Perrin, 1963; Guin et al., 1969].

Neuroblastoma tumors are quite variable in appearance and frequently exhibit areas of mature ganglioneuroma juxtaposed with immature neuroblastoma regions. A higher survival rate is correlated with the appearance of the more mature tumor [Bove and McAdams, 1981], but small areas of neuroblast-like cells can still be malignant and fatal [Alterman and Schueller, 1970].

Despite the frequent reports of maturation, these cases represent a small fraction of the total cases reported and neuroblastomas remain one of the commonest and most malignant of childhood cancers [Jones and Campbell, 1976; Ashley, 1978]. The mortality rate for all patients is over 50% in the first six months [Kemshead and Black, 1980].

When human neuroblastomas are explanted and grown in culture they spontaneously extend long neurite-like processes. This is such a consistant trait that it has been recommended as a diagnostic tool for cases where identification of the tumor by standard histological

methods is inconclusive [Murray and Stout, 1947; Reynolds et al. 1981]. This, combined with the findings on spontaneous transformation into benign ganglioneuromas, provides evidence that the information required for neuronal differentiation is not lost to the cell. Given the proper conditions the cells may be able to differentiate into ganglion cells, recapitulating the events occuring during normal differentiation.

The greatest stimulus to the use of neuroblastoma as a tool for the study of differentiation and morphogenesis came from the successful transfer of a mouse neuroblastoma, C 1300, from long term passage in mice [Klein, 1951] to tissue culture [Klebe and Ruddle, 1969; Augusti-Tocco and Sato, 1969; Schubert et al., 1969].

In culture the C 1300 cells exhibit several neuronal properties including the production of long neurite-like processes with microtubules and dense cored vesicles [Schubert et al., 1969], the neurotransmitter synthesizing enzymes cholineacetyl transferase and tyrosine hydroxylase, as well as acetylcholinesterase [Augusti-Tocco and Sato, 1969].

In the initial cultures of C 1300, cells were observed to extend processes spontaneously when they came in contact with 'tissue culture' plastic dishes. It soon became apparent that subpopulations could be grown continuously in culture without expressing processes, but could then be made to extend neurites by various perturbations of the medium. One agent capable of inducing processes is dibutyryl-cyclic-3':5' adenosine monophosphate {dbcAMP} [Prasad and Hsie, 1971]. Both dbcAMP and other factors which raise the level of cAMP within the cell such as prostaglandin E1 [Prasad, 1972] and various inhibiters of cyclic nucleotide phosphodiesterase (the enzyme which breaks down cAMP) lead to extensive process production [Prasad and Sheppard, 1972].

It quickly became obvious however that cAMP is not the only agent of `differentiation'. Table 1 lists more than 60 agents or conditions which induce processes in neuroblastoma cells. Both the production of processes and the expression of neurotransmitter enzymes could be induced by other agents. For example, bromodeoxyuridine [Schubert and Jacob, 1970], the removal of serum from the medium [Seeds et al., 1970], and X-rays [Prasad, 1971] all lead to the production of neurites, but do not significantly increase the cAMP levels of the cells [Prasad, 1975]. These and other morpological `differentiators' are not invariably coupled to the synthesis of neurotransmitter enzymes; some factors raise enzyme levels in some, but not all, of the C 1300 clones [Amano et al., 1972; Prasad et al., 1973].

The ability to induce `differentiation' of neuroblastomas at will has allowed numerous biochemical comparisons between `differentiated' and `undifferentiated' cells. Most such investigations have centered on the production of neurotransmitter enzymes [reviewed by Carroll, 1982], but a few have examined changes in the plasmalemma. The latter are of particular interest to studies of morphological change because of the importance that adhesion plays in the determination of cell shape. Unfortunately the investigations so far have been mainly descriptive with little attempt to correlate observed changes wirh morphologic mechanisms.

The most comprehensive examination of the membranes of neuroblastoma, and in particular the portion of the membrane adhering to the substrate, has been done by Culp et al. [1980]. They find that the pattern of proteins and glycoproteins left on the substrate by C 1300 neuroblastoma is remarkably similar to the pattern left by mouse fibroblasts. The material in both is enriched in high molecular weight glycoproteins, a 220,000 molecular weight glycoprotein which is probably fibronectin, and the cytoskeletal proteins actin and myosin. The patterns seen from cells with and without processes are similar

except for the appearance of one high molecular weight polysaccharide in cells which have processes. The close correspondence between neuroblastoma substrate associated material and fibroblastic material validates the comparison of fibroblast adhesion and movement with the adhesion and movement of neuroblastoma cells and processes.

Electron microscopy of mouse neuroblastoma reveals a cytoplasm rich in microtubules, microfilaments and 10 nm intermediate filaments [Ross et al., 1975]. However, the absolute levels of actin and tubulin do not change during induced morpological differentiation, so the changes in shape are probably due to the rearrangements of the available protein subunits [Morgan and Seeds, 1975; Schmitt, 1976; Rein et al., 1980]. There are changes in the levels of microtubule associated proteins however [Seeds and Maccioni, 1978; Olmstead and Lyon, 1981].

Although murine neuroblastomas are responsive to numerous agents, morphological responses to NGF have been difficult to obtain. It is known that C 1300 neuroblastomas have specific receptors for NGF [Revoltella et al., 1974], but it is only under very specific conditions, with specific clones, that an enhancement of morphological differentiation is observed [Revoltella and Butler, 1980].

The development of a cell culture model system that responds to NGF is of particular importance in studying its effects because the normal cells which respond to NGF, the sympathetic ganglion cells and some cells of the dorsal root ganglion, require NGF for survival during the time they are responsive. Comparisons between cells receiving NGF treatments and cells not receiving NGF are clouded by the imminent doom of the cells not receiving the factor.

Three cell lines have been shown to have a variety of responses to NGF, including a pronounced morphological change. One is a rat pheochromocytoma, PC12, a tumor of the adrenal gland [Greene and

Tischler, 1976], an other is the human neuroblastoma SK-N-SH clone SY5Y [Perez-Polo et al., 1979; Beidler et al., 1978]. Recently Reynolds and Perez-Polo [1981] have demonstrated an NGF effect in a third cell line, the human neuroblastoma IMR 32. None of these cell lines requires NGF for survival, they can be grown in continuous cell culture indefinitely without it, but all exhibit a variety of changes consistant with neuronal differentiation in its presence.

NERVE GROWTH FACTOR AND NEURITE EXTENSION

NGF is a protein hormone made of 118 amino acids with a molecular weight of 13,259. NGF is isolated as a larger complex (7S NGF) which consists of a dimer of the 118 amino acid molecule (2.5S NGF) plus two molecules each of an α subunit and a γ subunit. The overall molecular weight of the 7S form is 130,000. The functions of the and α subunits are not known, although the γ subunit is know to be a peptidase [Vinores and Guroff, 1980].

The original discovery of NGF activity was by Bueker [1948] who observed a massive outgrowth of fibers from dorsal root ganglia when mouse sarcoma 180 cells were implanted in chick embryos. The factor was isolated by Cohen [1960] who also discovered a rich, but anomalous source of the protein, the male mouse submaxillary gland.

NGF is required for the survival of developing sympathetic ganglia. When neonatal animals are injected with antibodies to NGF there is a nearly total atrophy of the sympathetic ganglia [Levi-Montalcini and Booker, 1960].

The NGF protein, its cellular receptors and its physiological effects have been extensively studied and the work extensively reviewed [Greene and Shooter, 1980; Thoenen and Barde, 1980; Vinores and Guroff, 1980].

In the 1970's, several investigators began to notice and study directive influences of tissue explants on neurite outgrowth, and to assign these effects to the action of NGF.

Chamley et al. [1973], and Chamley and Dowel [1975] demonstrated that rat sympathetic ganglia grown in vitro would preferentially send more fibers to tissues normally richly innervated by sympathetic neurons than to explants of tissues normally less richly innervated. They reported that neurite outgrowth is greatly stimulated on the sides of the ganglia facing explants of atrium or vas deferens, while kidney or lung explants do not show any clear effects. The fact that the stimulatory explants might release NGF was not overlooked by these authors, especially since non-NGF responsive spinal cord explants did not exhibit differental growth towards any target.

Ebendal and Jacobson [1977] demonstrated that NGF concentration gradients can orient sympathetic fibers. In a series of experiments similar to those of Chamly they observed directed outgrowth of chick sympathetic ganglion neurites toward selected tissue explants as well as towards capillary tubes loaded with NGF. They observed that the initial outgrowth of fibers from the ganglia is not influenced by explants but that further away many fibers bend towards the explanted tissue.

Letourneau [1978] brought the analysis of the NGF effect to the cellular level. He observed dissociated chick dorsal root ganglion neurons cultured in an agar matrix which had a source of NGF at one end. When the neurons sent out processes, about 60% of the fibers were seen growing towards the NGF source. In addition, the processes growing towards the NGF source were longer than those growing away. The site of process initiation on the cell body was not influenced by the NGF gradient and appeared to be random, but the rate and direction of growth was consistent with a positive chemotropic effect.

The analysis has been taken a step further recently with the report by Gunderson and Barrett [1980] that the tips of chick dorsal root ganglion neurites, the growth cones, can be made to change their direction of growth within 20 minutes, when presented with a steep NGF gradient. In this study a high level of NGF was delivered through a pressure driven micropipette to the side of individual growth cones. A medium perfusion system kept a constant gradient of NGF around the pipette. The growth cones sent out more filopodia on the side of the growth cone nearest the pipette and moved towards the pipette. By manipulating the micropipette, the growth cones were made to follow the pipette and to turn as many as 3.3 degrees per minute away from their original orientations. Similar results were obtained when dibutyryl cyclic adenosine monophosphate {dbcAMP} or elevated calcium ions in the presence of the calcium ionophore A23187 were applied.

Although the effectors of growth cone movement were themselves free floating molecules it is important to note that the expression of the effects was at the interface of the cell and the substrate.

In vivo, several experiments have purported to demonstrate that NGF acts as a chemotropic agent. Levi-Montalcini [1976] has shown that if a continuous source of NGF is injected into the rhombencephalon of a young rat, sympathetic fibers enter the spinal cord through the dorsal root and follow discreet pathways to the brain. The fibers are dependent on continued NGF injections to the brain to maintain this aberrant morphology. This experiment, like other experiments which show hyperinnervation of NGF sources in the body [Bueker, 1948], suffers from the complications brought on by the trophic actions of NGF. Sympathetic neurons require NGF for survival [Levi- Montalcini and Booker, 1960] and the NGF can be supplied from sources present only at the ends of neurites [Campenout, 1977]. Sympathetic fibers may reach an area rich in NGF by chance or through mechanisms other than chemotropic attraction and be selectively

maintained by NGF. Areas less richly endowed with NGF may allow similar exploratory fibers to wither. In these cases NGF may act to stabilize specific cell morphologies, but not necessarily to play a directive role in its formation.

The effects of NGF on process production have been extensively studied with the rat pheochromocytoma, PC12. PC12 cells in culture have rounded cell morphology with few cell extensions or processes. When they are exposed to NGF they elaborate long, branched, neurite-like processes [Greene and Tischler, 1976]. The production of these processes is RNA synthesis dependent, they will not form unless RNA synthesis is allowed [Burstein and Greene, 1978]. In this respect they are different from neuroblastoma cells in culture [Prasad, 1972; Schubert et al., 1971; Seeds et al., 1970] and explanted ganglion cells [Partlow and Larrabee, 1971; Mizel and Bamberg, 1976] both of which can extend processes in the presence of actinomycin D.

Whether there is an inductive role for NGF in the production of processes at all can be questioned. Mouse sympathetic ganglion cells from embryonic day 14 can survive for several days in culture without added NGF and in the presence of anti-NGF. During this time the cells extend processes much as later explants do in the presence of NGF [Coughlin et al., 1977]. The precursor cells of the sympathetic gangia, the neural crest cells [Seiber-Blum and Cohen, 1981], isolated in vitro can also produce processes without exogenously added NGF. It is not known whether these cells are exposed to NGF in the embryo and therefor may have already been 'primed' by NGF to express processes.

Three areas of investigation into the mechanisms of NGF action are pertinent to its effects on neuronal morphology: NGF's direct interaction with microtubules, the inter-relationship of NGF and cAMP, and the regulation of calcium ion concentration by NGF.

The addition of NGF to a rat brain homogenate causes a rapid,

specific precipitation of tubulin [Callissano and Cozzari, 1974]. This precipitate is caused by the polymerization of tubulin subunits into tubules [Levi et al., 1975]. Because NGF is thought to play a role in neurite extension, and can also be found within the cytoplasm of responsive cells [Marchisio et al., 1980], a direct role for NGF in the regulation of microtubule assembly was hypothesized [Callissano et al., 1978]. Recently however NGF has been introduced into the cytoplasm of PC12 cells by the fusion of PC12 cells with erythrocyte ghosts loaded with NGF. This technique eliminates the interaction of NGF with NGF receptors on the plasmalemma. This does not lead to morphological differentiation of the PC12 cells. The interaction of NGF and its receptors seem to be critical for mediating the effects of NGF, acting most probably through a `second messenger' within the cell [Heuman et al., 1981].

The role of cAMP as a second messenger mediating the actions of NGF has been investigated in a number of systems, but is still controversial. Some investigators have seen transient increases in cAMP levels after treatment of sympathetic neurons or PC12 cells with NGF [Hier et al.,1973; Narumi and Fujita, 1978; Nikodijevic et al. 1975; Schubert et al., 1978] while others have not [Lakshaman, 1978; Otten et al., 1978; Hatanaka et al., 1978].

All of the major structures of the neuronal cytoskeleton are sensitive to calcium ion concentrations, either directly or through the action of calcium activated phosphorylases and proteases [Bray and Fine, 1981]. The direct effect of NGF on calcium fluxes across the membrane is still uncertain. Schubert et al. [1978] report a small efflux of calcium with NGF application in PC12 cells, a finding questioned by Landreth et al. [1980].

Gunderson and Barrett [1980] in their excellent experiments demonstrating the tropic ability of NGF were able to show similar effects with gradients of calcium chloride in the presence of the

calcium ionophore A23187. The turning response to NGF does not change in very low calcium conditions, so the flux of calcium into the cell in and of itself is not necessary. The response to NGF and dbcAMP is blocked however in the presence of dantrolene, a drug which inhibits the release of intracellular stores of calcium. This indicates that intracellular fluxes of calcium may be the effectors of NGF action on cytoskeletal elements in the growth cone, leading to increased adhesion and a turning towards the the NGF source.

Local, transient effects of NGF have been observed in other situations. PC12 cells when first exposed to NGF exhibit a sequence of membrane morphological changes including ruffle formation and the production of filopodia [Connolly, 1979]. When chick dorsal root ganglia are grown in low levels of NGF in vitro, and then suddenly exposed to very high levels there is a fast retraction of the growth cone filopodia and some resorption of the neurite [Griffin and Letourneau, 1980]. One explanation for this effect is that there is a radid release of calcium in the growth cone which causes a depolymerization of filopodial microfilaments and a consequent loss of adhesion of the growth cone to the substrate.

The effects of NGF are multifaceted; some, such as neurite initiation in PC12, require transcriptional changes while others are transient and occur too fast to be mediated by RNA synthesis. Developing sympathetic ganglion neurons require NGF for survival and the relationship between this requirement and subsequent developmental changes is uncertain. NGF stops cell division in PC12 cells [Burstein and Greene, 1978], but sympathetic cells stop dividing before they become NGF dependent [Coughlin et al., 1977]. PC12 cells require NGF to initiate process formation, yet sympathetic neurons and some neural crest cells in vitro do not. The relationship between NGF and cAMP is also unclear, both can lead to process formation with some cells, but whether NGF causes changes in the internal cAMP levels within the cell is uncertain.

THE HUMAN NEUROBLASTOMA CLONE SY5Y

In 1979 Perez-Polo et al. reported that the human neuroblastoma cell line SY5Y would respond to NGF. The cells stopped dividing, there was a pronounced clumping of the cells, and they extended long neurite-like processes. This report made SY5Y unique in being a well established cell line of neuronal origin, which would respond to NGF.

SY5Y is a clone of the human neuroblastoma continuous cell line designated SK-N-SH. SK-N-SH was isolated in 1970 from a bone marrow biopsy of a four year old female suffering from a neuroblastoma tumor in the thoracic cavity [Beidler et al., 1973]. The clone SY5Y was isolated from a clonal population designated SY5, which in turn was isolated from a clonal population SY, which was isolated from the parental population SK-N-SH. The triple cloning was done to separate two distinctive morphological types [Beidler et al., 1978]. However morphological heterogeneity can still be observed in the SY5Y clone [Ross et al., 1980].

The SY5Y clone has been found to have a stable karyotype [Perez-Polo et al., 1979], and to exhibit a number of characteristics of neuronal cells. These include the production of the neurotransmitter synthesizing enzymes dopamine-β-hydroxylase and tyrosine hydroxylase and the ability to synthesize dopamine, acetylcholine and gamma amino butyric acid [Beidler et al., 1978; Ross et al., 1980]. When the cells are exposed to NGF in addition to sending out long processes they are protected from the sympathetic neurotoxin 6-hydroxy dopamine [Tiffany-Castiglioni and Perez-Polo, 1979] and they develop electrically excitable membranes [Kuramoto et al., 1981].

RESEARCH OBJECTIVES

Although the effects of NGF on the SY5Y clone were noted and documented by Perez-Polo et al. [1979] the morphlogic response was

neither quantitated nor examined in detail. Given the lack of knowledge on the specific morphologic responses of sympathetic cells to NGF, and the better characterized, but possibly misleading, knowledge of the NGF effects in PC 12 cells, a detailed examination of process formation in SY5Y was undertaken.

Because of its wide-spread use as an inducer of morphologic change in other neuroblastomas, the effects of serum-free medium were tested. For this reason and because of the possible interrelationship with NGF, dbcAMP with the cyclic phosphodiesterase inhibiter isobutyl methylxanthine {IBMX} was examined as well.

Several appoaches to understanding the effects on SY5Y morphology were employed. The correlation between population growth and morphological differentiation was examined. The changes in morphology visible with the light microscope were quantitated, and a detailed examination of the ultrastructure of the cell body, process and growth cone was made.

The cells were measured after exposure to NGF and dbcAMP in serum supplemented medium and after culture in serum-free medium. In addition the cells were exposed to NGF and dbcAMP in the recently developed defined medium of Bottenstein and Sato [1979]. This medium has several hormone and mineral supplements which allow the growth of rat neuroblastoma without serum. The SY5Y clone will also grow in this medium [Lyser et al., 1980].

In characterizing the morphologic responses, the first priority was to establish whether, indeed, the SY5Y cells had a response to these agents. Preliminary examinations of the cells on a light microscope level revealed that although there were obvious differences under different culture conditions, the cells gave a stochastic response. There was a great deal of variability in the level of process formation from area to area in the dish. In order to validate

any observations on cell morphology a detailed morphometric analysis of the population was called for.

The most obvious characteristic to measure was neurite-like process extension. This is the characteristic which is most often used to examine 'differentiation', and is usually expressed in terms of the percentage of cells in the population which display processes longer than a critical length. Most investigators have used a criterion of twice the diameter of the cell body or about 50 μm (see table 1). SY5Y produced a number of processes spontaneously under all conditions tested and a more stringent criterion for 'differentiation', 100 μm, was used. A second assay was made of processes greater than 25 μm in length (including those also longer than 100μm) to moniter the total number of processes on the cell. This was done to determine if increases in long processes were due to increases in the total number of processes on the cell or to a lengthening of already present processes.

Once a response had been validated several questions were posed. The first was whether the processes of the SY5Y cells were comparable to the processes of normal neurons. For this an ultrastructural examination of the soma, processes and growth cones under each condition tested was made. Comparisons were made with the known ultrastructure of sympathetic ganglion neurons grown both in culture and <u>in vivo</u>.

The second question was, how is process formation related to population growth? Does NGF lead to a decrease in population growth, as was reported with PC 12 cells [Tischler and Greene, 1976] and by other workers with SY5Y [Perez-Polo et al., 1979], or was the elaboration of processes independent of growth control? Growth curves under all conditions were generated.

The third question was are the different conditions leading to

morphologic change inter-related, and, what can there inter-relatedness add to our knowledge of how processes form?

The SY5Y clone offered an opportunity to observe the effects of NGF and dbcAMP on a well defined, non-NGF dependent population of cells. By carefully detailing the responses of these cells I was able to clarify the actions of NGF leading to process growth and the inter-relationship between NGF and other morphogenic agents.

MATERIALS AND METHODS

CELLS AND CULTURE CONDITIONS

The NGF responsive SK-N-SH clone SY5Y neuroblastoma cells were obtained from Dr. Douglas Ishii (Columbia University College of Physicians and Surgeons) and were used exclusively in the experiments presented in this thesis. The SY5Y cells used in this lab and reported on previously [Lyser et al., 1980; Burmeister et al., 1980] were not responsive to NGF treatments and were not used.

Stock cultures of SY5Y were grown in 25 cm^2 plastic tissue culture flasks (Falcon and Costar) in a medium consisting of 45% Ham's F12 medium {F12} [Ham, 1965], 45% Dulbeccos modified Eagle's medium {DME} [Dulbecco and Freeman, 1959] (both from Gibco) pnd 10% fetal calf serum (Flow) {Stock medium}. The cells were kept in a humidified atmosphere of 95% air, 5% CO_2 at 37° C. The cultures were transferred weekly. The cells were dissociated in 1 ml per flask of 0.02% ethylenedinitrilo tetraacetic acid {EDTA} in a calcium-magnesium free buffered saline solution (8 gm/l NaCl, 1.15 gm/l Na_2HPO_4, 0.2 gm/l KCL, 0.2 gm/l KH_2PO_4) [Merchant et al., 1964]. Dissociation was ended by adding 9 ml of stock medium to each flask. One ml aliquots were then seeded in new 25 cm^2 flasks. The cultures were fed once midweekly by replacing 70% of the culture medium with fresh stock medium.

The cells used in the experiments described in this thesis were from the 26th to the 39th transfer from cloning and were grown continuously from December 1980 until May 1981.

No contamination of the cultures by Mycoplasm sp. was

found using the uridine phosphorylase assay [Levine, 1972]. The tests were generously performed by Dr. James Donegan.

EXPERIMENTAL MEDIA

All experimental media were based on the medium {N2} developed by Bottenstein and Sato [1979] for the serum-free propagation of rat neuroblastoma. N2 consists of 50% DME, 50% F12 and five additional ingredients, 5 µg/ml insulin, 100 µg/ml transferrin, 6.3 µg/ml progesterone, 160 µg/ml putresine, and 5.2 µg/ml sodium selenite.

The growth and morphology of SY5Y was examined using seven different media.

{FCS}: Stock medium with the 5 additives of Bottenstein and Sato's [1979] N2 medium added.

{N2}: Bottenstein and Sato's [1979] N2 medium.

{F12/DME}: 50% F12, 50% DME with no other additives.

{FCS NGF}: FCS medium containing 100 ng/ml 2.5S NGF or 500 ng/ml 7S NGF.

{N2 NGF}: N2 medium containing 100 ng/ml 2.5S NGF or 500 ng/ml 7S NGF.

{FCS. CAMP}: FCS medium containing 1mM $N^6,O^{2'}$-dibutyryladenosine 3':5'-cyclic monophosphate {dbcAMP} and 0.25mM isobutyl methylxanthine {IBMX}.

{N2 CAMP}: N2 medium containing 1mM dbcAMP and 0.25nm IBMX.

NGF was obtained from several sources: from Collaborative Research, and as the generous gifts of Dr. Mark Bothwell, Dr. Douglas Ishii and Kenneth Sonnenfelt, and Dr. J. Reginio Perez-Polo. No differences in population growth or morpholgical response were noticed between them. All NGFs were stored at -70°C as concentrated stocks (10 µg/ml 2.5S NGF,100 µg/ml 7S NGF) in phosphate buffered saline containing 1% bovine serum albumin.

All other additives were kept as refrigerated stocks at 100x the final concentration in F12/DME except progesterone, kept in 10% 95% ethanol, 90% F12/DME, and IBMX, kept in 50% F12/DME, 50% dimethyl sulfoxide. All the additives were purchased from Sigma except insulin (Iliten U-100R, Eli Lilly) and sodium selenite (Ventron). The experimental media were made from the concentrated solutions immediately before use.

Media and additives were sterilized by ultrafiltration.

CULTURE PROTOCOL

In all experiments the cells were treated according to the same protocol. Stock cultures of SY5Y were dissociated with 0.02% EDTA in calcium-magnesium free buffer. They were dispersed in stock medium and kept in suspension with a magnetic stirrer. The cells were counted with a hemocytometer and the cell concentration was adjusted to 2 to 4 x 10^4 cells per ml. Two ml of the cell suspension were pipetted into 35 mm diameter plastic tissue culture petri plates, or 5 ml were added to 60 mm diameter plates (Falcon and Corning). The cells were placed in the incubator and allowed to grow for about 24 hours. After 24 hours the cells were gently rinsed with 2 washes (of either 2 or 5 ml each) of F12/DME. The washes were aspirated and the experimental medium was added. Because there was a large loss of cells from the 35 mm dishes during refeeding operations the cells were not refed during the four to six day course of each experiment.

GROWTH CURVE PROTOCOL

The SY5Y cells used for growth curves were grown in 35mm diameter culture plates. Population growth was measured by counting the cells with a hemocytometer. Cells attached to the substrate were dispersed in 2 to 6 ml of 0.02% EDTA in calcium-magnesium free buffer containing 0.02% trypan blue (Sigma). Cells floating in the culture medium were collected by centrifugation of the aspirated medium and were added to the substrate bound cells. The cells were triturated with a capillary pipette. For each dish six samples of 4×10^{-4} ml each were counted. In the first count from each dish both trypan blue excluding cells (viable cells) and trypan blue stained cells were enumerated. For the last five samples all cells were counted without discrimination. Usually three dishes for each time point for each condition were counted.

The viability was determined by dividing the number of trypan blue excluding cells by the total number of cells from the first sample from each dish, then averaging the values from all three dishes. This average value was then used to determine cell number from the samples where only the total cell count was made. This convoluted procedure was neccesary because direct counts of viable cells alone showed that after trituration in trypan blue (but not after gentle incubation) the viability dropped sharply with time, giving rise to very low counts in the later samples. Trituration was neccesary because the SY5Y cells proved to be extremely sticky and strong measures were neccesary to maintain a single cell suspension. The compromise of counting viability shortly after trituration and total cell count thereafter was reached.

Growth curves were generated by averaging the viable cell count of all the dishes counted at each time point, usually three dishes. The values were converted to logarithms so that they could be analysed

as straight lines on a plot of the logarithm of the cell number per plate vs. time. The y intercept, the number of cells at time 0 was determined by regression analysis. Different experiments with slightly different initial population densities were normalized by subtracting the logarithm of the cell number at time 0 as determined by the regression line from each time point. A growth curve normalized to an initial plating density of 1×10^5 cells was then generated by combining all the experiments. The slopes of the normalized growth curves for each condition were compared using the two sided Student's t test [Chapman and Schaufele, 1970].

ELECTRON MICROSCOPY

Cells grown for electron microscopy were plated in 60 mm dishes with small, aproximately 1 mm^2 boxes inscribed on the growth surface with a carborundum marker. After four days in experimental medium the cells were fixed. The medium was gently aspirated and 5ml of freshly diluted 2% gluterahdehyde in 0.1M cacodylate buffer, pH 7.2, were added. The cells were kept in glutaraldehyde for 30 to 45 minutes while phase contrast micrographs were made of the cells located within the boxes on the culture dish. These pictures were then used to identify cells and processes seen in electron micrographs. After rinsing the cells once with 0.1M cacodylate buffer the cells were stained and post-fixed with 1% osmium tetroxide in 0.1M cacodylate buffer, pH 7.2, for 30 min. The osmium was removed and the cells rinsed with distilled water followed by a graded ethanol series through 70% ethanol. The cells were stained with 1% uranyl acetate in 70% ethanol followed by a rinse in 70% ethanol and continued dehydration through 100% ethanol. The cells were infiltrated with 50% ethanol, 50% Epon 812 resin [Luft, 1961] for one hour, with 100% Epon for 3 hours followed by a final infiltration with Epon 812 for two hours. The Epon was polymerized at 60^0 C for 36 to 48 hours.

During the final Epon infiltration the small squares on the inside surface of the culture dish were each covered by an upright embedding capsule opened at each end. After 18 to 24 hours of polymerization the Epon on the culture dish surface had hardened enough to allow additional Epon to be added to the capsule without escaping from the bottom. Each capsule was then filled with Epon and the polymerization allowed to continue. After curing, the Epon capsules were snapped off of the dish and a stub with the outlines of the inscribed square was obtained.

Thin sections cut parallel to the substrate from both photographed and unphotographed regions of the culture dish were obtained, as were sections cut perpendicular to the substrate from unphotographed areas. Both glass and diamond knives were used on a Porter-Blum MT-2 ultra-microtome. The best sections were silvery-gold in the knife trough. Sections were generally post-stained with lead citrate [Reynolds, 1963] for five minutes and rinsed once with slightly basic distilled water and twice with distilled water.

Electron micrographs were made on a RCA EMU-3H electron microscope with a 40 μm objective aperture operating at 100kv.

LIGHT MICROSCOPY.

All cells for light microscopy were grown in 60mm dishes. After four days in experimental medium the cells were fixed with 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 6 to 48 hours. After fixing, the cells were gently rinsed with distilled water followed by a graded ethanol series through 95% ethanol. They were then allowed to air dry. At the magnifications used there was no discernable shrinkage of the cell body. It was not necessary to stain the cells to veiw them with phase contrast or differential interference microscopy.

All plates from each experiment were coded with randomly generated numbers before they were viewed, and all the assays were scored in single blind tests.

PROCESS ASSAYS

SY5Y cells were assayed under each growth condition for the presence of processes longer than 100 μm or 25 μm. The cells were viewed through an eye piece graticule engraved with a 5 square by 5 square grid. With a 10x objective lens the squares of the grid measured 104 μm on a side. With a 40x objective lens each square was 26 μm x 26 μm. All the cells with nuclei within the overall grid were then scored as either having or not having a process longer than one square. The areas viewed were choosen by moving arbitrary distances (generally about 1 mm) in a straight path across the dish. Several paths were required for each dish and the assay was performed a several different times to minimize variables such as observer fatigue.

A process was defined as any cylindrical protrusion from the cell soma. Its length was measured from the point on the cell soma where the process diameter was twice the diameter of the cylindrical portion of the process to its farthest tip , including any growth cone-like regions. Processes which were bent or crooked were mentally straightened and scored. Areas with very high cell densities were difficult to score accurately, so if a part of an arbitarily chosen area was too dense to count no cells in that grid were scored.

In addition to process length the cells were separately scored for the number of processes emerging from the cell soma. Arbitrarily chosen areas of coded plates were again used, with all the cells within the grid being scored a having from 0 to 5 For this assay no length requirement was imposed for the process. It was evident however that in order to develop the necessary cylindricality

to be called a process cell protrusions had to be at least 5 μm long.

CELL SHAPE ASSAYS

The distribution of cell shapes in SY5Y was analysed from coded photographs of arbitarily chosen areas of coded plates. The photographs each represented an area 350 μm by 500 μm and showed an average of 120 cells.

All the clearly visible cells in each photograph were categorized into one of six distinctive shapes illustrated in table 7. The six categories were:

Round: Cells with no visible processes or extensions.

Unipolar: Rounded cells with only one process.

Bipolar: Extended cells with two processes or cell extensions at opposite poles on the cell

Triangular: Cells with three clearly demarked sides, the corners being either processes or other wider extensions. Some cells in this category appeared similar to bipolar cells except one end of the cell was extensively spread on the substrate.

Equilateral: Triangular cells with three equal sides usually with processes emerging from each corner.

Polymorphous: Cells with more that three corners or sides often with large areas spread on the substrate.

A seventh category corresponding to the flat substrate adherent cells described by Ross et al., [1980] was assayed, but there were too

few of these cells to be of statistical significance. These cells were added to the polymorphous category.

The same photographs were used to measure the physical dimensions of each cell. An acetate sheet with randomly spaced points was placed over the photograph and a predetermined number of polymorpic cells (6,7 or 8) on each photo were measured with calipers. Length was measured as a line crossing the nucleus from the two farthest points on the cell excluding processes. Width was the shortest line across the nucleus. The ratio of the length to the width was used as an assay of the cell shape. The area of the substrate covered by the cell was measured using a polar planimeter.

STATISTICAL TESTS

Statistical tests were made using a Texas Instrument TI 58C with statistical testing module. Two sided Student's t tests were used for testing the growth curve slopes, the process length assays and the average area of the substrate covered by the cell. Contingency tables using a two sided Chi square test were used in the other assays. Because of the variability encountered in some of the assays a $p < 0.01$ was considered significant.

OBSERVATIONS

The human neuroblastoma clone SY5Y was grown under seven different conditions. For each condition measurements were made of population growth and survival, the percentage of cells with processes greater than 100 μm and 25 μm in length, the number of processes per cell, and changes in the somal size and shape. In addition, for each condition a detailed examination of the cell ultrastructure, particularly of the processes, was made. Growth curves were run for six days and all morphological assays were performed on cells fixed after four days exposure to the experimental condition.

FIXATION

As noted in Materials and Methods, many cells became dislodged from the substrate during refeeding procedures. For this reason the cells were not refed during the experimental period. There was concern that a similar dislodgement might take place during the fixation procedures. For this reason pictures were taken before and after fixation to insure that there was no generalized disruption of the culture dish. As illustrated in figures 4 to 7 the overall pattern of cells and processes remains stable after fixation.

The cells grown for light and electron microscopy were grown in 60 mm diameter dishes; examination of the dishes after fixation often revealed a small region that was devoid of cells. This was probably the area where the glutaraldehyde fixative was added to the dish. Most of the dish appeared undisturbed. The effects of added medium were much larger in the smaller 35 mm diameter dishes used for the growth curves. In these dishes eddies of medium would spread around

the entire dish. This disruption was minimized in the larger dishes and the remaining cells were felt to represent the entire population of cells in the dish accurately.

FCS MEDIUM

In fetal calf serum supplemented medium containing the five additives of Bottenstein and Sato's [1979] N2 medium SY5Y grew and survived much as under stock conditions. Population growth was logarithmic (correlation coefficient {r}= 0.9768). The doubling time was 42 hours, with no initial lag period, and with no slackening of growth over six days without refeeding. The viability, as measured by trypan blue exclusion, was relatively low, but constant throughout the six days (Table 2; fig. 1).

SY5Y grew as a pleomorphic population that consisted mostly of small cells (ave. 31 µm x 16 µm) (figs. 8, 9; table 6). The cells exhibited numerous 'points', protrusions from the cell body which appeared to be making contact with the culture substratum. Many of the points extended into long roughly cylindrical processes. There were frequent areas, however, where the cell was widely spread on the substrate. There was a great deal of overlapping of both processes and cell bodies, although at the densities used in these experiments there were rarely areas where the nuclei of cells overlapped. Most of the cells were in direct contact with the substrate.

Sixteen hundred cells were sorted into one of six different shape categories (table 7). Nearly 1300 cells (75.9%) in the FCS condition fell into the category "polymorphic", that is, irregularly shaped, well adherent cells. Some cells exhibited morphologies which were decidedly different, with the largest category of these being cells which exhibited no extensions at all. This group, which constituted 7.3% of the population, was composed mostly of mitotic cells. Other morphologies were expressed by smaller numbers of cells.

The cell processes seen in FCS medium were relatively straight, with few major branches. There were however numerous areas where the process was swollen and where small extensions, which spread out over the substrate, were seen. Some of the processes had swellings at the tip which appeared to be growth cones (figs. 8, 9).

When the cells were scored for the presence or absence of processes longer than 100 μm (table 2), about 1 in 14 (7.2%) of the cells had long processes. When the same cultures were scored for 25 μm or longer processes (table 3), better than 1 in 2 (57.4%) were positive. And when all the cylindrical processes of any length were scored 93% of the cells were found to possess one or more (table 5).

The distribution of processes per cell (table 5), regardless of length, showed 34% of the cells with one process, 30% with two, 18% with three, 7% with four, and 4% of the cells with five or more processes. This produced a mean of 2.0 processes per cell (table 3).

The fine structure of SY5Y in FCS medium is illustrated in figures 10 to 24.

The cells were characterized by a large smooth nucleus, which was uniformly located eccentricly in the cytoplasm, separated from the plasmalemma by a thin layer of cytoplasm which excluded most other organelles. The nucleus usually had several nucleoli and was rimmed by a thin band of irregular, darkly staining material just inside the nuclear envelope (fig. 10).

The organelles of the cell were unevenly distributed. Some areas, usually adjacent to the nucleus were rich in mitochondria and golgi complexes, while other areas excluded these organelles, and were dominated by ribosomes and rough endoplasmic reticulum. In the

mitochondrion-rich regions near the nucleus a wide assortment of other organelles was seen, including, smooth endoplasmic reticulum, occasional profiles of rough endoplasmic reticulum, free ribosomes, microtubules, and scattered dense cored vesicles. The dense cored vesicles averaged about 100 μm in diameter and were similar to the dense cored vesicles encountered in developing sympathetic neurons [Eranko, 1972]. Centrioles were occasionally seen in the perinuclear region (fig. 11) but in no case were more than one pair observed per cell, although serial sections were not viewed. In sections cut parallel and close to the substrate (fig. 12) there was a higher concentration of microtubules and 10 nm filaments than in sections cut higher . Filopodia were seen extending directly from the cell soma, also close to the substrate. The filopodia were rich in a filamentous network which appeared to meld with a submembranous matrix of filaments in the cell body (fig. 13).

Cell processes measuring from 1 to 1.5 μm in cross section were frequently observed (figs. 15-21). Nearly all the processes encountered were rich in mictotubules, arranged roughly parallel to the process length. Occasional microtubules crossing from one side to the other, at an acute angle to the long axis of the process were seen. Microtubules within processes were never observed at right angles to the process. Interspersed with the microtubules were numerous membranous organelles. These were generally located centrally and often seemed to be arranged in groups (fig 17). The largest organelles were the mitochondria which were usually elongated along the length of the process. Dense cored vesicles ranging in size from 80 to 115 nm in diameter, with a central density covering 45 to 50% of the vesicle area, were seen in most processes, often near profiles of smooth ER. The frequency of occurence of the dense cored vesicles was highly variable (fig. 20, 21 vs. fig. 19). Lysosomal vesicles and multivesicular bodies were also seen in some processes (fig 17).

In favorable sections a network of long sheaths of 7nm filaments was adjacent to the plasmalemma (fig. 16). Microfilaments were occasionally seen coursing through the center of a process, however, most of the filaments seen in the center or the process measured 10 nm across (figs. 17, 18). Except in the subplasmalemmal microfilament network, ribosomes, unconnected with ER and usually not forming polysomes, were frequent. The heaviest concentration was in areas with accumulations of membranous organelles (fig. 17).

Many processes displayed regions of increased diameter which usually contained an accumulation of vesicles and smooth ER and a decrease in microtubule number and alignment (figs. 15, 16, 18). Frequently at these areas the submembranous microfilamentous network was seen to extend out into a filopodium (fig. 15), probably forming a `guy wire' from the process to the substrate. In one section microfilamentous extensions making contact with a second process formed a membrane density at the point of juncture (fig. 19).

At the tips of processes structures similar to growth cones were seen (figs. 22, 23, 24). The processes expanded, with the process microtubules splaying into the varicosity. There were frequent profiles of smooth ER and mitochondria and 100 nm, dense cored vesicles. As in the processes, dense cored vesicle occurence varied (fig. 24 vs. figs. 22, 23). Clear vesicles reminiscent of the `mound' vesicles known to be glutaraldehyde artifacts [Nuttall and Wessells, 1979] were seen; however, they were different in not being exclusively clustered under the plasmalemma. They often appeared in small clusters and queues (figs. 23, 24). Profiles cut in the more distal regions showed areas of microfilamentous meshwork and filopodia. The filopodia were smaller than microtubule containing processes and the network of microfilaments generally excluded all other organelles with the frequent exception of ribosomes.

N 2 MEDIUM

The human neuroblastoma clone SY5Y survived and multiplied in the N2 medium of Bottenstein and Sato [1979]. The doubling time under experimental conditions was 74 hours, about 60% of the rate in FCS medium. Growth was logarithmic (r=0.9679) with no lag or plateau phase through 6 days. The viability was constant over the entire six day experimental period (Table 2; fig. 1).

In N2 medium SY5Y cells exhibited a different morphology from that of cells grown in serum (figs. 25, 26). There were fewer areas of cell spreading and more cells had a rounded appearance. The processes were similar to those seen in FCS, they were about the same size and were generally straight and unbranched, but they appeared to be adherent to the substrate in fewer locations. Many processes not ending on other cells had growth cones at their tips.

More than 5,500 cells were examined for long, 100 μm or longer processes, and about the same percentage of cells as in FCS had them, 7.2% in FCS, 8.4% in N2 (table 3). The subjective impression of more long processes in N2 could be accounted for by the decrease in the percentage of cells exhibiting 25 um or greater processes. In N2 44.8% of the cells had short processes while 57.4% of the cells in FCS did (table 4). Thus the longer processes were more accentuated in the N2 cultures. In N2 25% of the cells had no processes compared to 7% in FCS (table 5).

Analysis of the number of processes of any length emerging from cells in N2 (table 5) showed a significant decrease compared to FCS. Cells with one process represented 49.3% of the cells counted, cells with two processes 19.7% of the population, and those with three or more only 6.9% of the population. There was an average of 1.2 processes per cell, compared to 2.0 in FCS.

The ratio of cell shapes in the population was markedly altered

from FCS (table 7). Nearly 20% of the cells in N2 had rounded cell morphologies without any processes or spread areas, compared to 7% in FCS. Cells classified as polymorphous were reduced in number from 75.9% of the population in FCS to 52.1% in N2. Other cell categories also exhibited significant changes. It should be noted that the shape categories were generalized, so that many of the cells in the same category but under different conditions could still be distinguished one from the other (compare figs. 9 and 26). This was especially so in the polymorphous group, a category which was to some extent made up of cells which were too complex to fit into the other categories. Many cells in N2 polymorphous were rounder and more regular in outline than those in the FCS polymorphous category.

The physical measurements of polymorphic cells in N2 were slightly, but not too significantly (Chi square test $p=0.04$), smaller than FCS cells (table 6). In N2 the cells covered an area of 299 μm^2 while in FCS it was 349 μm^2. The ratio of width to length was 0.47 in N2, 0.46 in FCS.

Ultrastructurally the SY5Y clone in N2 medium looked quite similar to its FCS reared counterparts. The nucleus was located eccentricly, contained a few densly stained areas of chromatin and a rim of darkly stained material at the nuclear border. The cytoplasm contained the same mixture of components with the same tendency to form areas which exclude mitochondria and golgi complexes, but are rich in ribosomes (fig. 27). Dense cored vesicles were on the whole sparse in the cell body, although they were present.

Figures 27 and 28 show matched phase- and electron-micrographs of several cells grown in N2. Of particular interest is the structure which is isolated in figure 27 but which is seen to be the growth cone of a long process in figure 28. Figure 29 shows the same growth cone with increased magnification. This structure, which measures about 15 μm in length, is seen to consist of an initial microtubule rich

process which leads into an area rich in endoplasmic reticulum and other membranous organelles. Several filopodia of various sizes are seen (fig. 30, 31) which contain filamentous networks. One filopodium exhibits a large glutaraldehyde mound along its length (fig. 30). Figures 36 and 37 present other growth cone profiles.

Processes seen in N2 were similar in appearance to those seen in FCS (figs. 32 to 35). The same ultrastructural components were seen in short processes (< 25 μm, fig. 35) and longer processes (fig. 29).

F12/DME MEDIUM

The replacement of serum supplemented medium with a basal unsupplemented medium is a widely used method of inducing morphological differentiation in mouse neuroblastoma [Seeds et al., 1970]. The addition of a serum-free unsupplemented medium to SY5Y caused a number of changes, however, the production of processes was not one of them. Most of the effects could be attributed to physiological stress induced by starvation.

The SY5Y population stopped growing immediately (table 2, fig. 1). Out of three growth experiments, one showed no population growth from day 0 to 6, and two showed sharp decreases in population. The regression line generated from the three combined growth curves produced a doubling time of -54 hours, poorly correlated with logarithmic growth (r=-0.6570).

Light microscopy showed that the morphology in F12/DME was similar to that seen with N2 medium (figs. 38, 39). The cells were rounder on the whole, but many still had processes or small areas of cytoplasm spread on the substrate. Occasional long unbranched fibers ending in growth cones were seen but most cells with processes appeared to have stunted outgrowths. There were always more free floating, apparently

dead, cells in F12/DME medium than in the other conditons tested.

Nearly 4500 cells were screened for 100μm processes and only 52 were found (1.1%). There was a relatively low number of cells with 25 μm processes (19.3%), and about half of the cells had no processes at all of any size (table 3, 4, 5).

The cells which did have processes had predominantly one process (41.7% of the total population). About 10% had two processes, while only 1.3% had three or more. There was an average of only 0.7 processes per cell (table 5).

The distribution of shapes in the population was significantly different from that of N2 or FCS (table 7). There was a reduction in the number of cells classified as polymorphous; and the percentage of rounded cells was nearly the same in N2 and F12/DME but there were increases in the cells classified as unipolar, bipolar, triangular and equilateral with F12/DME.

The physical dimensions of the cells in F12/DME decreased significantly from N2 (table 6). The area of the substrate covered in F12/DME was 238 μm^2, in N2 299 μm^2. The ratio of width to length did not vary however, indicating that the basic shape of the polymorphic cells did not change.

The fine structure of SY5Y grown in F12/DME was generally similar to that seen with N2 medium (fig. 40 to 52). The same admixture of cytoplasmic components and nuclear structures was seen. In line with the overall decrease in cell size, the nucleus took up a larger volume of the cell, the eccentric position, with a thin buffer of cytoplasm between nucleus and plasmalemma, remained. There were areas of granular ER, but unbound ribosomes were far more conspicuous in the cytoplasm in F12/DME than in N2 (fig. 42).

Some of the processes encountered in F12/DME were indistinguishable from those seen under more favorable growth conditions (figs. 44, 49). Frequently however, processes were seen which seemed to have fewer microtubules and a more loosely arranged cytoplasm containing many membrane bound vesicles (figs. 47,48).

Growth cones with all the features seen in FCS or N2 medium were encountered (fig. 50). However, other, less robust looking profiles were also seen. In figure 52 a small growth cone identified from phase micrographs ends on a cell. Both the process and the growth cone are small and depleted looking, although the characteristic growth cone structure is still evident. Growth cones with and without dense cored vesicles were also seen (figs. 50, 51, 52).

FCS NGF MEDIUM

The addition of nerve growth factor to FCS medium did not significantly affect the population growth of SY5Y. Growth was logarithmic (r=0.9829) with a doubling time of 44 hours, virtually the same as the 42 hours seen in FCS (table 3).

Observation under the light microscope revealed a population nearly identical in morphology with that seen with FCS medium. Although no systematic counting was done on the formation of large clumps or "pseudo-ganglia" [Perez-Polo et al., 1979], few clumps were obvious with FCS NGF medium. Most of the cells appeared to be directly adherent to the substrate and the processes of the cells were similar to those seen in FCS medium.

Morphometric analysis of SY5Y in FCS NGF revealed that there was a highly significant increase in the number of processes longer that 100 um. In FCS NGF 15.3% of the cells had long processes, more than twice as many as in FCS (table 3).

Significant responses to NGF were not evident in the other morphological assays. The number of 25 µm processes (table 4), and the number of processes of any size (table 5), were no different in FCS NGF than in FCS. The number of processes per cell was the same (table 5), as was the distribution of cell shapes, the area of the substrate covered by the cell and the width to length ratio (tables 6, 7).

On an ultrastructural level no differences between cells maintained in FCS NGF and FCS media could be discerned (figs. 55 to 65).

The only effect of NGF in the presence of serum was to increase the number of long processes (table 8).

N2 NGF MEDIUM

In contrast to serum containing medium the addition of nerve growth factor to to serum-free supplemented medium caused a number of readily observable changes in SY5Y.

The growth rate was greatly reduced compared to N2 (table 2; fig. 2). The growth curves exhibited an unusual degree of variability. Seven separate growth curves were run, in each case N2 NGF was paired with another condition which showed a typical response, yet no two growth curves were identical. The reasons for this are unclear. However, all seven experiments were consistent in showing a clear decrease in the growth rate in response to NGF. In no case did the population increase more than 2.3 times over six days. The regression line generated by the combined growth curves produced a doubling time of 205 hours (r=0.5733). Viability was the same as seen in N2.

There were pronounced effects on the morphology of the cells. In

some areas cells which did not differ greatly from the cells in N2 were seen (fig. 66). However, there was a marked tendency for cells to form tight clusters. These clusters contained many cells which did not send processes or other cell extensions out onto the substrate (fig. 67). In extreme cases these clusters would attain a mound-like structure with few cells in contact with the culture dish (fig. 68).

Cells growing in areas near scratches made in the substrate with a carborundum point avoided an area about 50 um across bordering the scratch (figs. 69E,F). Under no condition were cells seen growing on the scratched surface itself, but only in N2 NGF did the cells avoid the adjacent area (fig. 69). The processes of the cells, however, did not discriminate this area from others and grew freely in it under all conditions.

Cells which were in clumps could not be analysed for process length and number. Because of this, morphometric measurements of process length and number in N2 NGF were performed on a subset of the population to a greater extent than in the other conditions. The cells which were measured were cells which to some extent did not show as dramatic a response to NGF as the clumped cells. However, significant differences from non-NGF supplemented conditions were seen.

One parameter which did not differ from that seen in N2 was the percentage of cells with 100 µm processes (table 3). With nearly 6000 cells measured only 9.1% of the cells displayed long processes. This was not significantly different from the percentage seen with N2. The number of 25 µm processes was increased in N2 NGF from 44.8% in N2 to 51.4%, and the percentage of cells with any size process rose from 76% in N2 to 85% in N2 NGF (table 5).

There was a significant increase in the number of processes per cell (table 5). This was accounted for by a decrease in the number of cells with no processes and an increase in the number of

cells with three or more processes compared it N2 medium. The average cell in N2 NGF displayed 1.4 processes.

The cell shape assay was less discriminatory against cell clumps than the process measurements and showed a significant difference from N2 because of it (table 6). In N2 NGF 33% of the cells were classified as rounded, with most of these cells in clumps. There was little difference between N2 NGF and N2 in the other categories except for a decrease in the polymorphic category which corresponded to the increase in rounded cells.

The physical measurements of cells were taken on polymorphs, so the clumped cells were mostly excluded. Some cells at the edges of clumps and occasionally within clumps did exhibit some spreading and these cells were measured (fig. 67). There was no difference in either the area of the substrate covered by the cell, or the width to length ratio between cells in N2 NGF and N2 (table 6).

The cell ultastructure in N2 NGF did not differ from that seen in N2 (fig. 70 to 77). No clumps, per se, were viewed because none formed in the predetermined squares cut for electron microscopy. Cells in dense areas of the dish were seen to posses processes identical to those seen in less populated regions, but whether the highly clumped cells had processes as well was not determined.

FCS CAMP MEDIUM

When 1mM dbcAMP and 0.25mM IBMX were added to FCS medium the population of SY5Y cells continued to grow for two days at a rate close to that seen with FCS (doubling time, 37 h), but then plateaued and grew at a very slow rate (doubling time, 192 h, table 1; fig. 3).

Despite the cessation of growth the morphology remained similar to FCS, although, the processes were smoother with fewer areas in contact

with the substrate (figs. 79, 80).

Measurements of processes gave values equal to those seen with FCS. About 8% of the cells had processes 100 μm or longer, 59.8% had 25 μm processes and 94% had some process like structure no matter how small (table 4, 5, 6).

The average cell in FCS CAMP had 2.0 processes, the same as in FCS (table 6), and the mixture of cell shapes in the populations were nearly identical (table 7).

The only feature exhibiting a significant difference between FCS CAMP and FCS, was cell size. The area of the substrate covered by the cell in FCS CAMP was 499 μm^2 compared with 349 μm^2 in FCS. This was accounted for by increases in both length and width as the ratio of the two were virtually the same for both (table 6).

The ultrastructure of SY5Y in FCS CAMP appeared identical in every way with that seen without dbcAMP and IBMX (figs. 55 to 65).

N2 CAMP MEDIUM

As in FCS CAMP, cells in N2 CAMP showed a dramatic halt in population growth. In N2 CAMP the doubling time for the cells was reduced to 290 hours, although it differed from FCS CAMP in showing an effect from the first day of culture (table 2; fig. 3).

The cells in N2 CAMP displayed the most spectacular outgrowth of processes of all the conditions tested (fig. 91, 92). The cell bodies seemed much the same as in N2 alone, rounded with few areas spread on the substrate. The processes however were quite striking. They were long, of various thicknesses, with few areas attached to the substrate.

Morphometric analysis confirmed the changes. Nearly three times as many cells (23.2%) had processes longer than 100 um than in N2 alone,(table 3). There was a significant increase in the number of 25 um processes with 64.4% of the cells exhibiting them (table 4) and cells with processes of any size comprised 90.6% of the population, compared with 76% in N2 (table 6).

Many more cells exhibited multiple processes in N2 CAMP. The average number of processes per cell jumped from 1.2 in N2 to 1.7 in N2 CAMP.

The mixture of shapes in the population (table 6) and the physical measurements of the cells (table 7) were not different from those seen in N2.

The ultrastructure of the cells was basically the same as in N2 medium, (fig. 93 to 102). The only unusual features were tangles of 10 nm filaments seen in two growth cones, a configuration not seen in other conditions (figs. 99, 100, 101).

TABLES OF RESULTS

TABLE 2.
SUMMARY OF SY5Y POPULATION GROWTH UNDER SEVEN CONDITIONS

Condition	Doubling Time	Cor.Coef. {r}	Avg. viability	Number expts.	p exp=cont
FCS	42h	0.9768	68.9	3	-------
N2	74h	0.9679	59.4	5	0.0073
F12/DME	-54h	-0.6567	48.6	3	<0.0001
FCS NGF	44h	0.9829	70.3	4	0.8938
N2 NGF	205h	0.5733	57.5	7	<0.0001
FCS CAMP	83h	0.8658	67.5	4	0.0038
N2 CAMP	290h	0.5133	52.8	5	<0.0001

Notes. p is the probability that the slopes of the combined, normalized growth curves of each condition and the appropriate control arise from the same population, determined by the two-sided Student's t test.
The appropriate controls in all the assays are:
FCS for N2, FCS NGF, and FCS CAMP;
N2 for F12/DME, N2 NGF and N2 CAMP.

TABLE 3.
100 MICROMETER PROCESS EXTENSION

Condition	Percent cells with processes >100μm	Number cells	Number plates	p exp=cont
FCS	7.3 ±2.67	4844	9	-------
N2	8.4 ±2.73	5535	7	0.4186
F12/DME	1.1 ±0.60	4485	9	<0.0001
FCSNGF	15.3 ±4.10	6403	9	0.0003
N2NGF	9.1 ±3.45	5891	9	0.6621
FCS CAMP	8.1 ±3.02	6261	9	0.6151
N2 CAMP	23.2 ±6.47	6710	9	<0.0001

Notes. p is the probability that the average of the response per plate in the experimental and the control arise from the same population, determined using the two sided Student's t test.

TABLE 4.
25 MICROMETER PROCESS EXTENSION

Condition	Percent cells with processes > 25μm	Number cells	Number plates	p exp=cont
FCS	57.4 ±8.76	2964	9	-------
N2	44.8 ±5.77	2980	7	0.0057
F12/DME	19.3 ±3.74	1466	9	<0.0001
FCS NGF	61.1 ±11.06	2538	9	0.4366
N2 NGF	51.4 ±3.31	2831	9	0.0137
FCS CAMP	59.8 ±7.92	2869	9	0.5434
N2 CAMP	64.4 ±6.44	2311	9	<0.0001

Notes. p is the probability that the average of the response per plate in the experimental and the control arise from the same population, determined using the two sided Student's t test.

TABLE 5.
PROCESSES PER CELL

Condition	Percent of cells with n processes						Total cells	Mean ±S.D.	p exp=cont
	0	1	2	3	4	5+			
FCS	7.2	34.1	29.7	18.1	6.9	3.8	1460	2.0±1.2	------
N2	24.0	49.3	19.7	5.3	1.4	0.2	1208	1.1±0.9	<0.0001
F12/DME	47.0	41.7	9.9	1.2	0.1	0.0	1029	0.7±0.1	<0.0001
FCS NGF	6.0	33.5	31.4	19.8	6.1	3.2	1344	2.0±1.2	0.6047
N2 NGF	15.2	47.3	24.3	8.6	2.9	1.6	1460	1.4±1.1	<0.0001
FCS CAMP	6.5	33.2	30.5	19.8	6.6	3.2	1442	2.0±1.2	0.8347
N2 CAMP	9.3	6.9	30.8	17.7	3.7	1.4	1337	1.7±1.1	<0.0001

Notes. p is the probability the distribution of processes through the population in the experimental and the appropriate control arise from the same population, determined by the Chi square test.

TABLE 6.
LENGTH WIDTH AND SUBSTRATE AREA OF SY5Y CELLS

Condition	Length μm ± S.D.	Width μm ± S.D.	Substrate area μm^2	W/L ratio	p area exp=cont
FCS	31.1 ±6.2	13.8 ±2.9	349 ±149	0.46	------
N2	30.1 ±6.6	13.3 ±2.4	299 ± 81	0.47	0.0365
F12/DME	23.3 ±4.6	11.5 ±2.7	238 ± 90	0.49	<0.0004
FCSNGF	30.7 ±6.3	14.0 ±3.1	371 ±117	0.47	0.4141
N2NGF	29.2 ±6.4	12.9 ±2.5	306 ±102	0.47	0.7110
FCS CAMP	34.3 ±6.5	15.6 ±3.3	499 ±167	0.47	<0.0001
N2 CAMP	28.5 ±5.8	13.8 ±2.5	330 ±111	0.50	0.1068

Notes. Substrate area is the average area of the substrate covered by the cell body, excluding processes. 104 cell were measured in each condition for the length and width measurements, 52 for the substrate area. p area is the probability that the average areas of the substrate covered in the experimental and the control condition arise from the same population, as determined by the two-sided Student's t test.

TABLE 7.

CELL SHAPE ANALYSIS

Condition	Round	Uni-polar	Bi-polar	Triang.	Equi-lateral	Poly-morph	Total Cells	p exp=cont
								
FCS	7.3	3.9	3.6	5.6	3.7	75.9	1682	-----
N2	20.6	7.8	6.4	9.0	4.1	52.1	2073	<0.0001
F12/DME	21.8	12.8	13.5	12.6	5.4	33.9	1705	<0.0001
FCS NGF	7.7	4.8	2.6	4.4	2.5	78.0	1931	0.0357
N2 NGF	32.5	8.4	5.8	7.4	2.6	43.4	1817	<0.0001
FCS CAMP	6.6	2.7	2.8	3.7	3.5	80.7	1441	0.0194
N2 CAMP	18.8	8.0	5.3	7.4	3.4	57.2	1896	0.0245

Notes. The cell shape categories are expressed in terms of the percent of the total population in each category. p is the probability that the distribution of shapes in the experimental and control arise from the same population, as determined by the chi square test.

TABLE 8.
SUMMARY OF RESULTS

Condition	Doubling Time	100 μm processes	25 μm processes	Process per cell	Area	Cell Shapes	DβH
FCS							
N2	**	--	**	**	--	**	**
F12/DME	**	**	**	**	**	**	na
FCS NGF	--	**	--	--	--	--	--
N2 NGF	**	--	--	**	--	**	--
FCS CAMP	**	--	--	--	**	--	**
N2 CAMP	**	**	**	**	--	**	**

notes: ** indicates the given condition was significantly different ($p < 0.01$) from the appropriate control
-- indicates no significant difference
na not available.

DβH dopamine-β-hydroxylase assays are from Carroll [1982]. Conditions FCS NGF and FCS CAMP for this assay differ from the conditions presented in this thesis in not including the 5 additives of Bottenstein and Sato's [1979] N2 medium.

PLATES

PLATE I.

Figure 1. Growth curves of SY5Y in FCS, N2 and F12/DME. Population growth or decline is charted as the number of viable cells normalized within each experimental condition to an initial density of $1x10^5$ cells per 35mm culture dish. The actual average plating density was $0.92x10^5$ for FCS, $1.1x10^5$ for N2 and $1.1x10^5$ for F12/DME. Each datum represents the average of all plates counted at that time within each experimental condition, usually 3 plates. The lines show the regression curves generated by the data. Note the high correlation of datum points with the regression curves in FCS and N2 media (r = 0.98 and 0.97), and the loose correlation with the F12/DME data (r= 0.66). Individual points at time 0 were not plotted for the sake of clarity although they were used in generating the regression line. Note the logarithmic scale of the y axis.

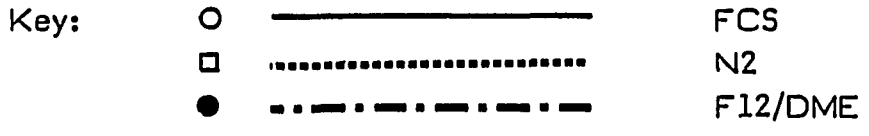


Figure 1
Growth Curves of SY5Y in FCS N2 and F12\DME

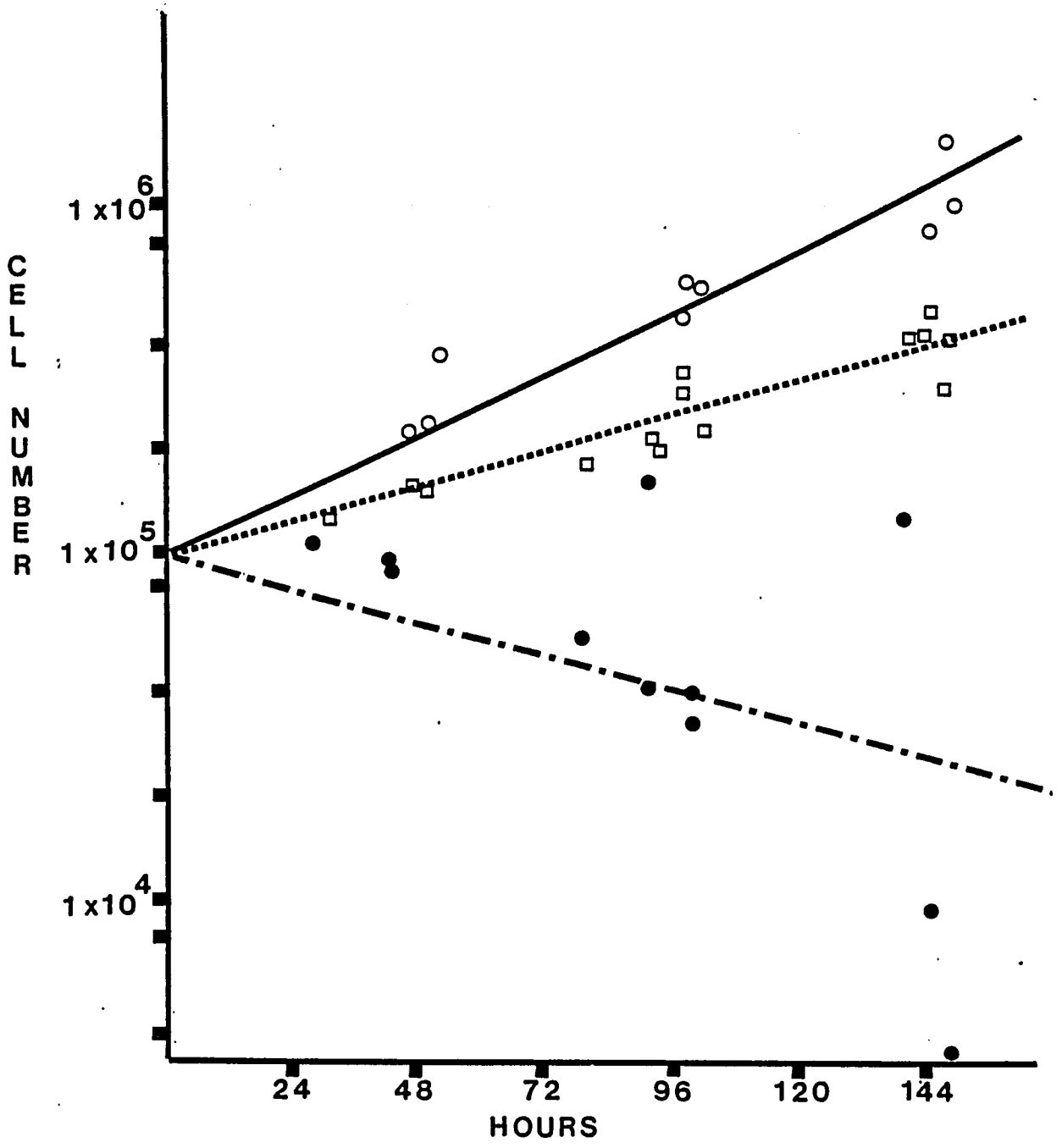


PLATE II.

Figure 2. Growth curves of SY5Y in FCS NGF and N2 NGF. The data are plotted as in figure 1, but the y axis is expanded. The actual average cell number at time 0 for FCS NGF was 0.97 x 10^5 and for N2 NGF 1.0x10^5 cells per 35mm dish. Note the high correlation in the regression curve with FCS NGF (r=0.98) and the loose correlation in N2 NGF (r=0.57).

Key: ● —·—·—·—·— FCS NGF

⊙ ····· N2 NGF

Figure 2
Growth Curves of SY 5Y in FCS NGF and N2 NGF

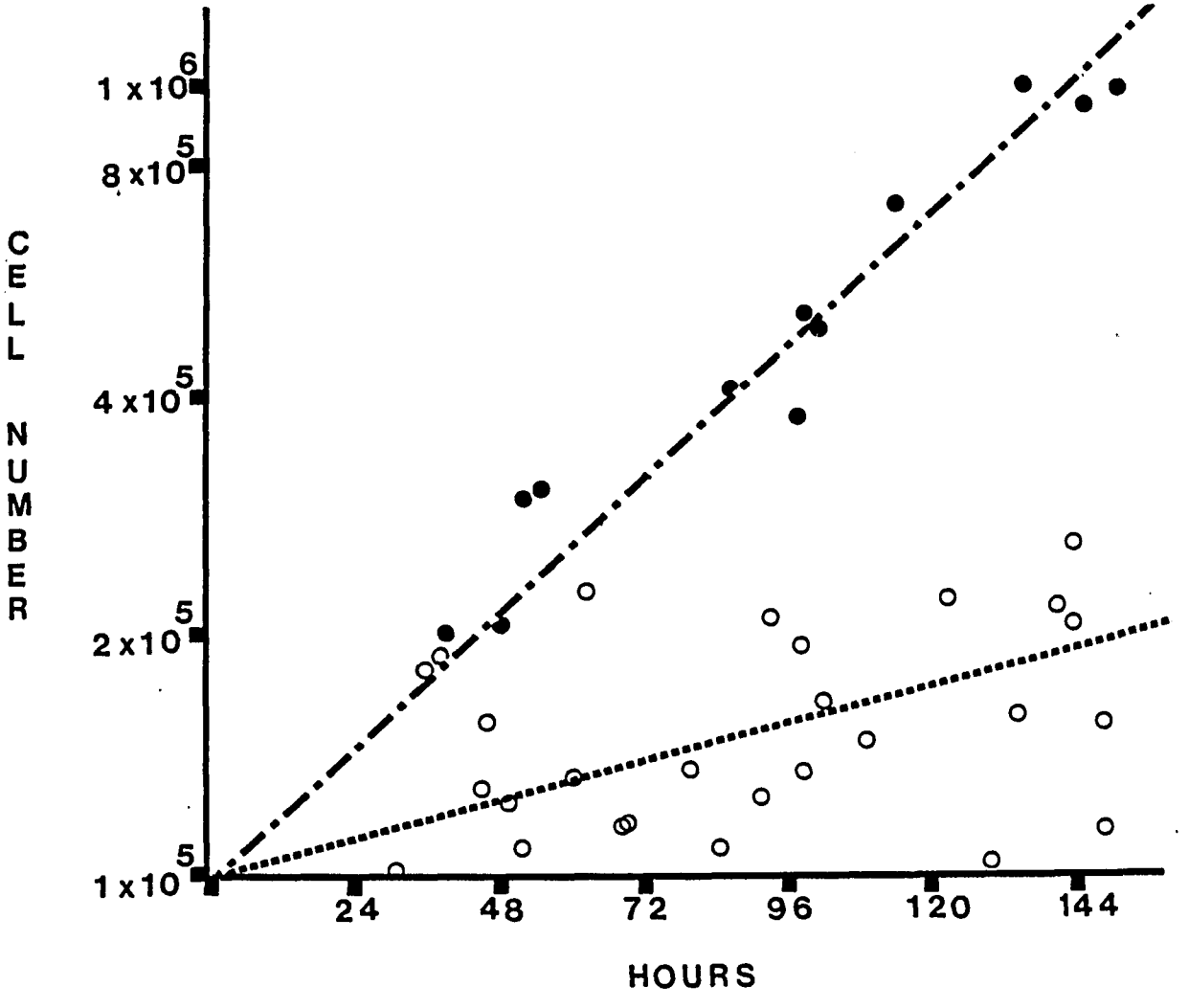


Plate III.

Figure 3. Growth curves of SY5Y in FCS CAMP and N2 CAMP. The data are plotted as in figure 2. The actual average cell number at time 0 was 0.97 x 10^5 cells per 35 mm dish for FCS CAMP and 1.1x10^5 in N2 CAMP. The data for FCS CAMP have a bi-phasic character. Points from time 0 through 63 hours are strongly correlated with logarithmic growth (r= 0.96) and are not significantly different from FCS growth characteristics (2-sided Student's t test, p=0.52). Points from 46 hours to 149 hours show very slow population increase. The overall regression curve used to establish the FCS CAMP doubling time (table 2.,83 hours) is not plotted. N2 CAMP shows slowed population growth with little correlation to logarithmic increase (r=0.51).

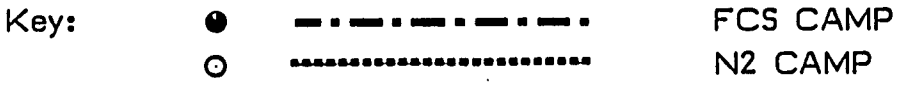


Figure 3

Growth Curves of SY5Y in FCS CAMP and N2CAMP

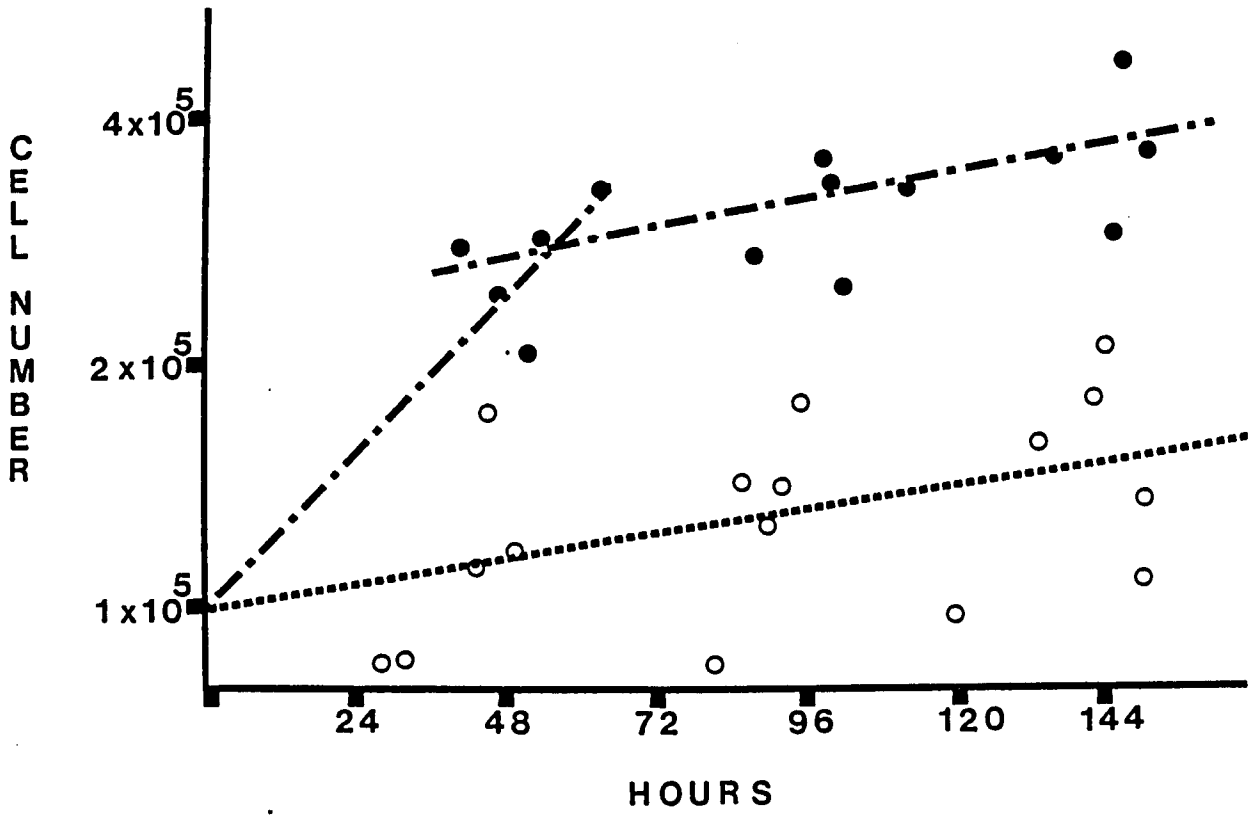


PLATE IV.

The same areas photographed before and after fixation with glutaraldehyde. In general fixation and washing has not disturbed the pattern of cells or processes. The arrowheads show areas of change. Some thin structures which appear phase lucent before fixation have a darker more spread appearance after fixation.

Figure 4. N2. Phase contrast, before fixation. x400, bar = 10 μm.

Figure 5. N2. Phase contrast, after fixation. x400, bar = 10 μm.

Figure 6. N2 CAMP. Phase contrast before fixation. Note the rounded cells at arrow. x400, bar = 10 μm.

Figure 7. N2 CAMP. Phase contrast after fixation. The rounded cells in figure 6 {arrow} have been dislodged. x400, bar = 10 μm.

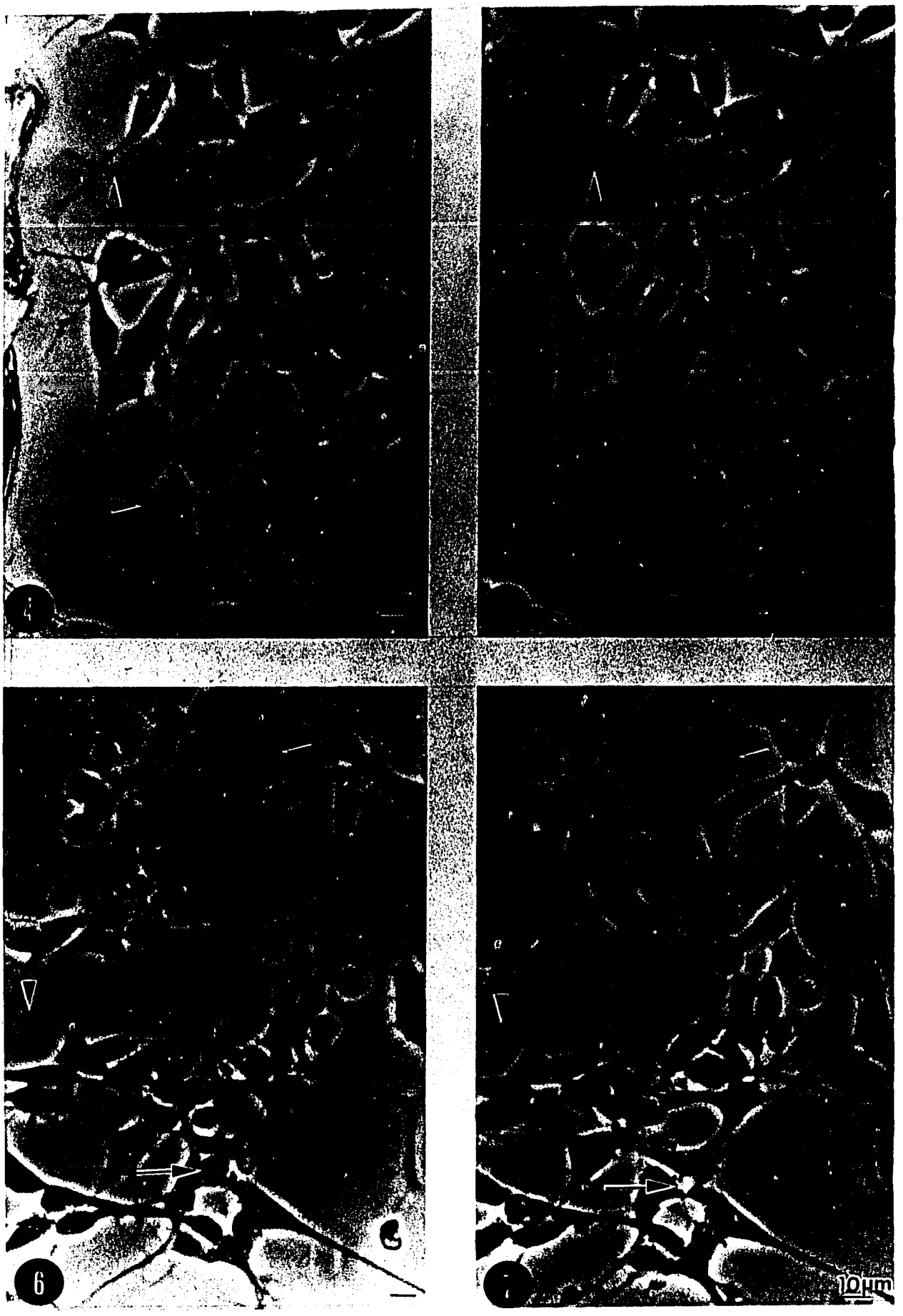
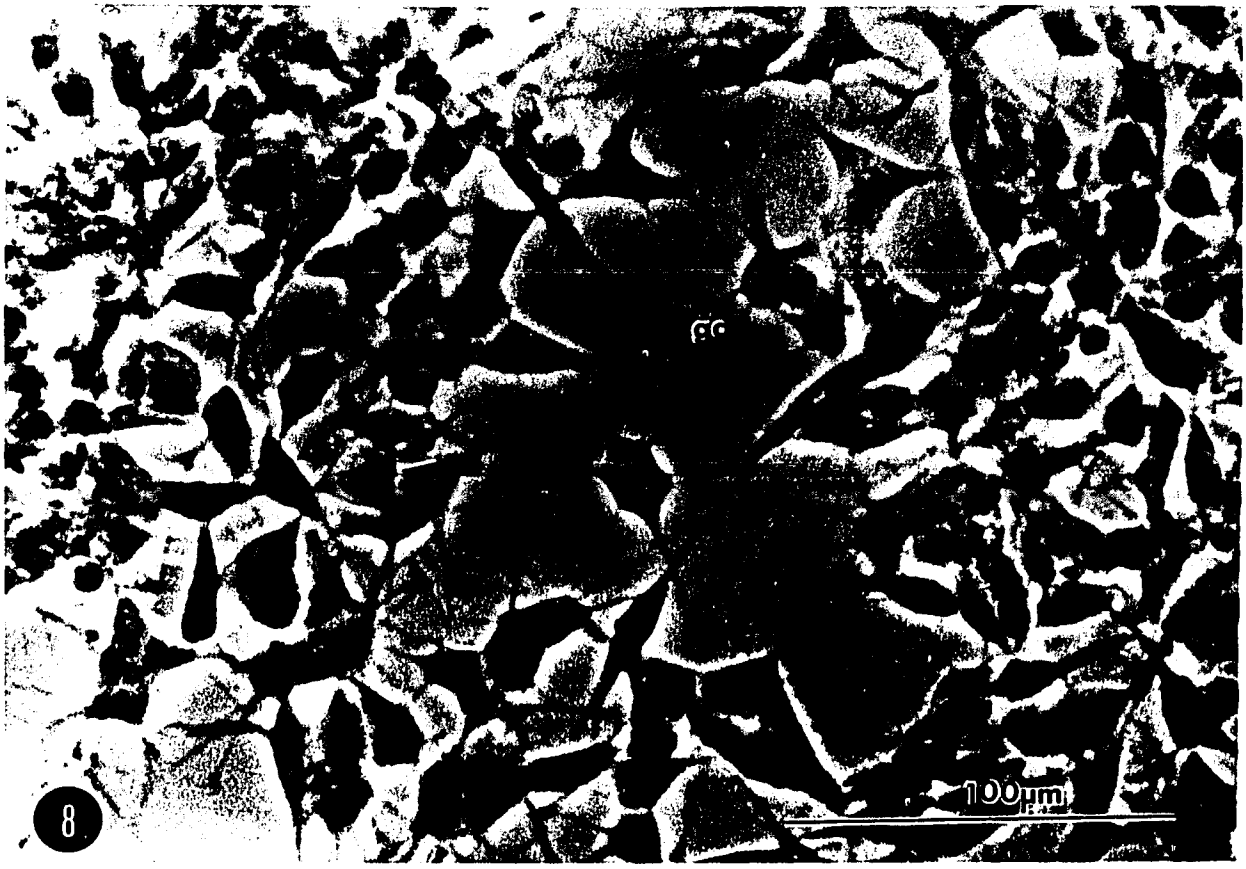

6
10μm

PLATE V.

Figure 8. FCS. Phase contrast micrograph of living SY5Y cells. The population is composed mostly of `polymorphous' cells which each extend several processes. Many of the processes end in branching expansions similar to growth cones of neurons. Although most of the cells appear well spread on the substrate, a few are rounded and more refractile {arrowhead}. x520.

Figure 9. FCS. Differential interference of fixed and air dried SY5Y cells. The range of cells classified as `polymorphous is illustrated in this micrograph. Also visible are a `round' cell and a `unipolar cell'. Notice that although the cells are found in clumps each cell appears to be directly in contact with the culture substrate. x260.

Key:	gc	growth cone
	p	`polymorphous' cell
	r	`round' cell
	u	`unipolar' cell


GG
100µm
8

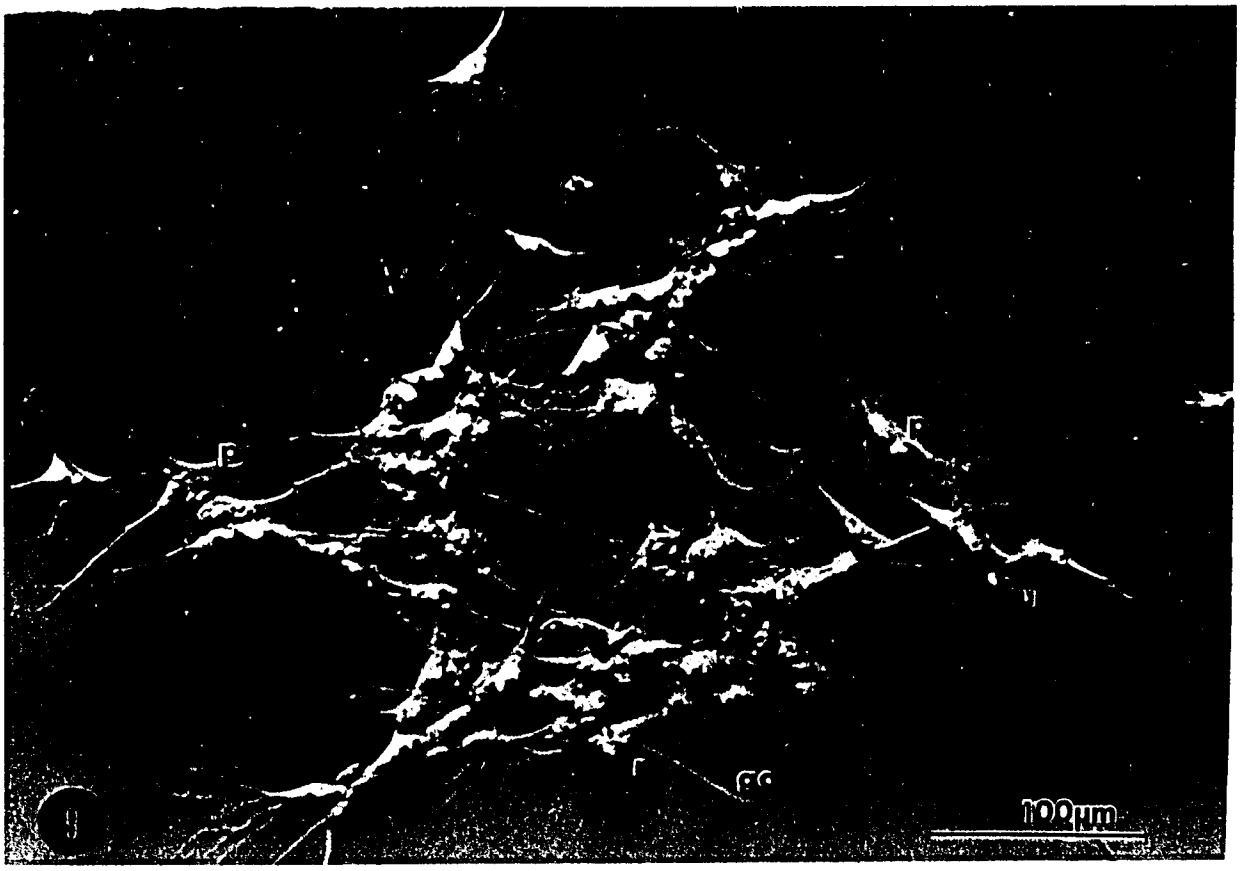

P
P
GG
100µm

PLATE VI.

Figure 10. FCS. A "typical" cell shown in the center of the micrograph is dominated by the granular eccentric nucleus. The nucleus exhibits several nucleoli and areas of electron dense material inside the nuclear envelope (arrowheads). The cytoplasm exhibits partitioning of cell contents with golgi and mitochondria predominating in the widest portion of the cell and ribosomes and rough endoplasmic reticulum predominating on the sides. x6,800.

Figure 11. FCS. The contents of a golgi rich region of cell cytoplasm. Although this region is predominantly composed of mitochondria and golgi other organelles are present, including many ribosomes and some rough endoplasmic reticulum. 23,500x.

Key:

c	centriole
dcv	dense cored vesicle(s)
er	endoplasmic reticulum
g	golgi complex(es)
mt	microtubule(s)
mv	membrane filled vesicle
Nu	nucleus
rer	rough endoplasmic reticulum

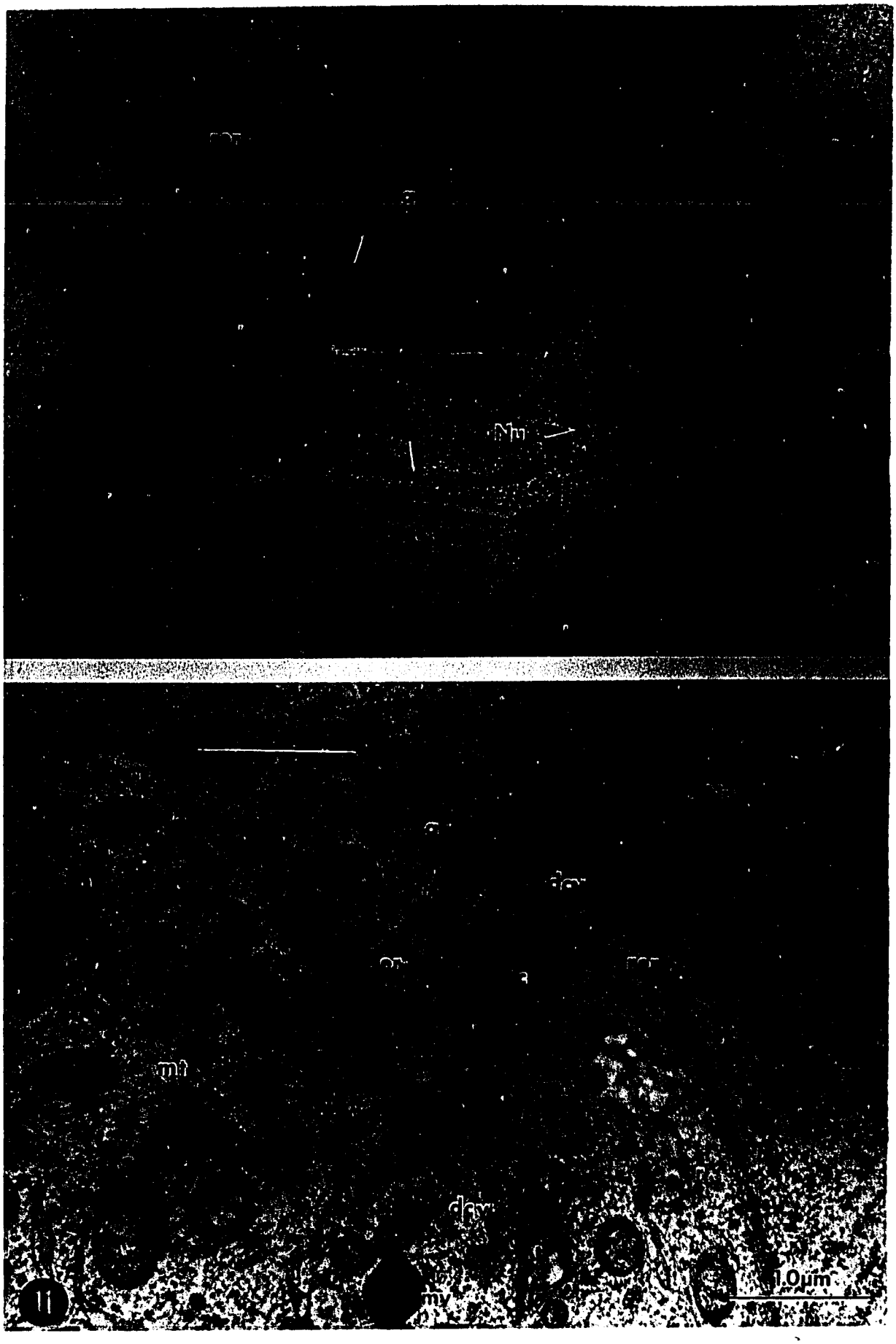

1.0µm

PLATE VII.

Figure 12. FCS. A cell cut very close to the culture substrate exhibits an extensive array of microtubules extending throughout the cell. The bottom of the nucleus exhibits darkly staining rim chromatin and numerous nuclear pores. Many filopodia can be seen leaving the cell body. The filopodium marked * is enlarged in figure 13. Note the compartmentalization of the cell, with the entire right side of the cell exhibiting no mitochondria. {P} is a microtubule and dense cored vesicle rich process of another cell. x6,500.

Figure 13. FCS. The filopodium marked * in figure 12 is enlarged and seen to be composed of numerous 7nm microfilaments, interspersed with ribosomes {arrowheads}. x33,100.

Figure 14. FCS. The ribosome rich region of cytoplasm. Sparse rough endoplasmic reticulum is surrounded by numerous clusters of ribosomes and polysomes. The nucleus exhibits several nuclear pores and condensed rim chromatin. x23,500.

Key:

mf	microfilament(s)
mt	microtubule(s)
np	nuclear pore(s)
P	process
ps	polysome
rer	rough endoplasmic reticulum

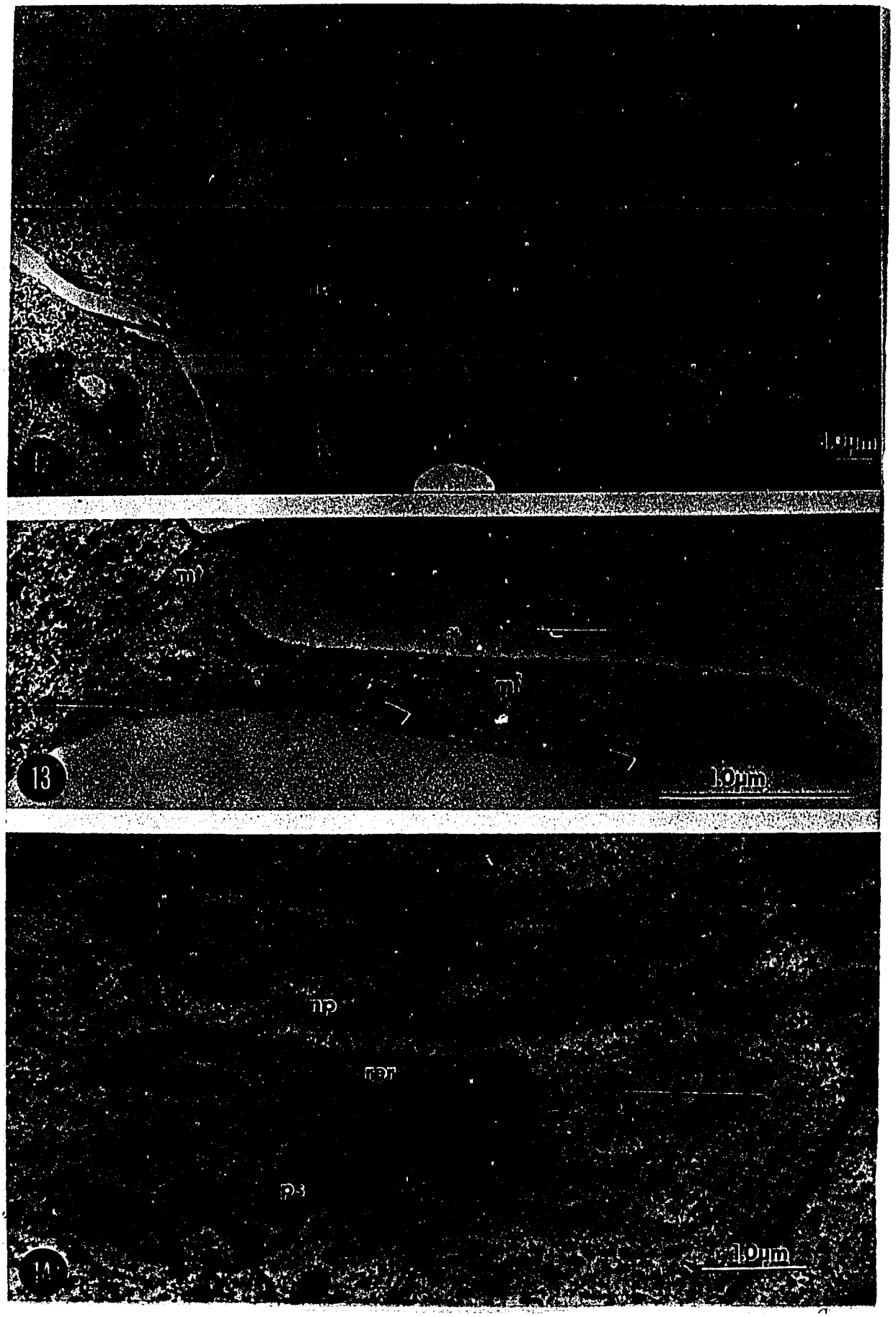

1.0μm
13
1.0μm
קה
1.0μm

PLATE VIII.

Figure 15. FCS. A long neurite-like process. Many parallel microtubules located primarily in the central portion and microfilaments located predominantly subjacent to the plasmalemma run along the length of the process. Several dense cored vesicles are dispersed through the process. A projection from the side of the process {arrowhead} contains several strands of microfilaments. This is probably extending out to the substrate forming a 'guy wire' from the process. Note the numerous ribosomes scattered in the cytoplasm. x23,500.

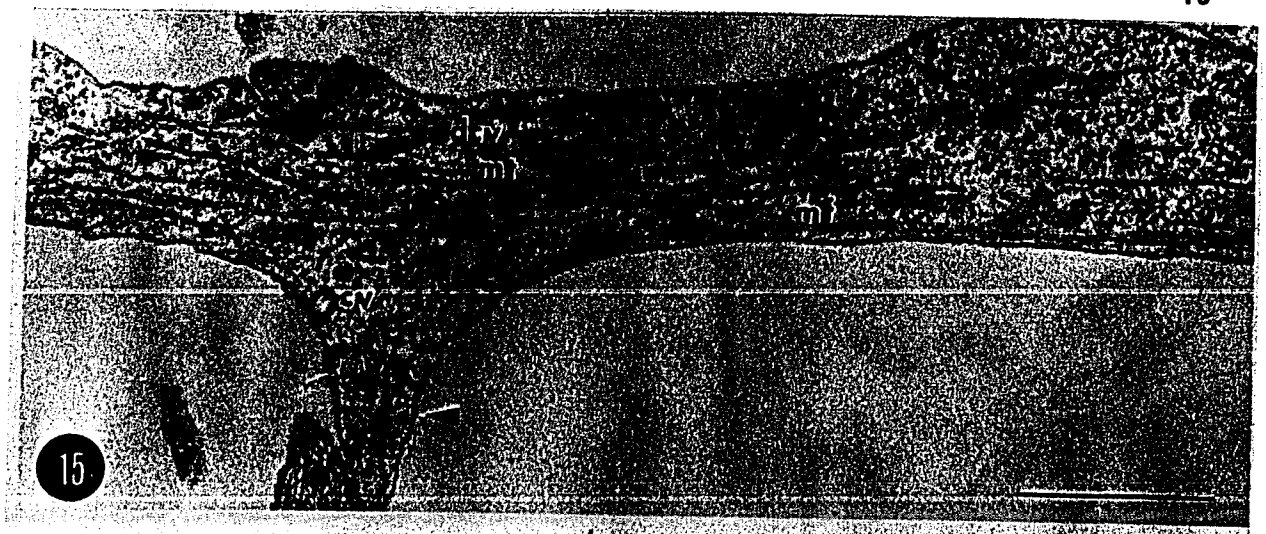
Figure 16. FCS. This process exhibits aligned 7nm filaments subjacent to the plasmalemma. Where the process swells microtubules are absent and there is an accumulation of endoplasmic reticulum. x23,500.

Figure 17. FCS. A microtubule-rich process shows a cluster of mitochondria and a multi-vesicular body {mvb} in the center. A 10 nm filament is seen coursing through the center of the process. x23,500.

Figure 18. FCS. A process exhibiting 7nm filaments near the plasmalemma and intermediate filaments in its center. x23,500.

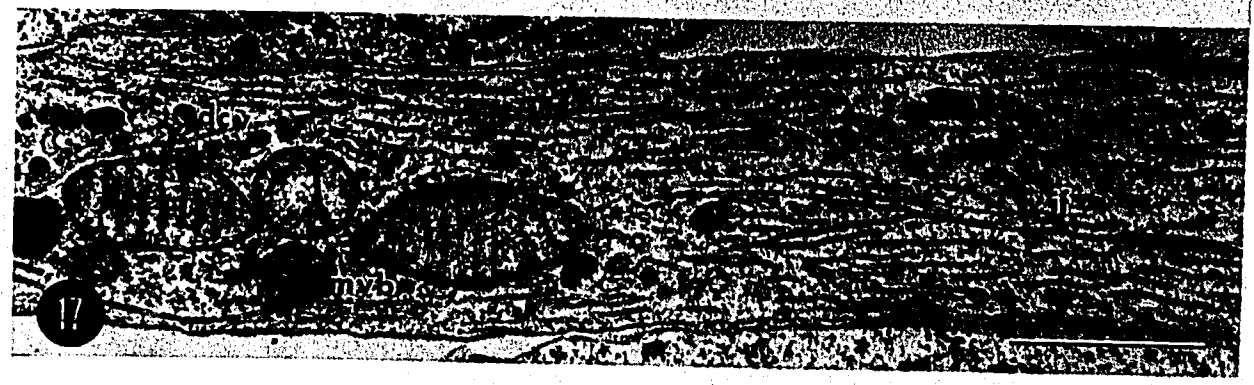
Key:

cv	coated vesicle
dcv	dense cored vesicle(s)
er	endoplasmic reticulum
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
mvb	multi-vesicular body

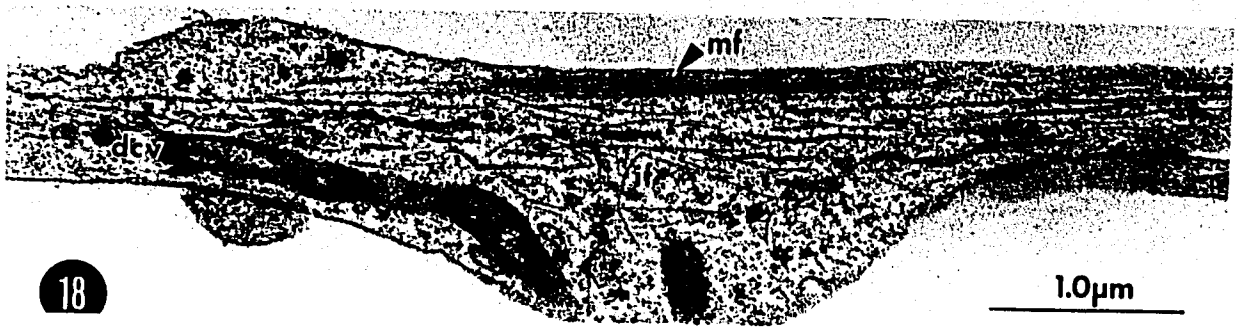


15

16



17



18

1.0μm

PLATE IX.

Figure 19. FCS. Two processes travelling next to each other here give off microfilamentous extensions which form membrane densities {arrowheads}. x23,500.

Figure 20. FCS. A microtubule rich process containing numerous dense cored vesicles. x23,500.

Figure 21. FCS. A process which is rich in membrane components but poor in microtubules. x23,500.

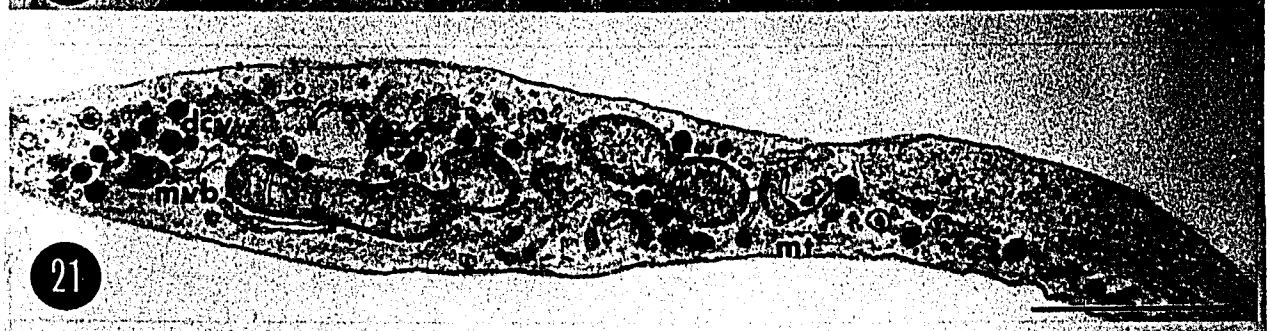
Figure 22. FCS. The tip of a process. A process {P} enters this area on the left and leads into an area rich in smooth endoplasmic reticulum, dense cored vesicles and mitochondria. On the right the arrowhead points to an area filled with tufts of filamentous material similar to the regions leading into filopodia in growth cones. This micrograph shows the most dense cored vesicles of any profile encountered in any condition. x23,500.

Key:

dcv	dense cored vesicle(s)
mf	microfilament(s)
mt	microtubule(s)
mvb	multi-vesiclular body
P	process
ser	smooth endoplasmic reticulum


mf
19
dry


dry
20


dcv
mvb
mf
21

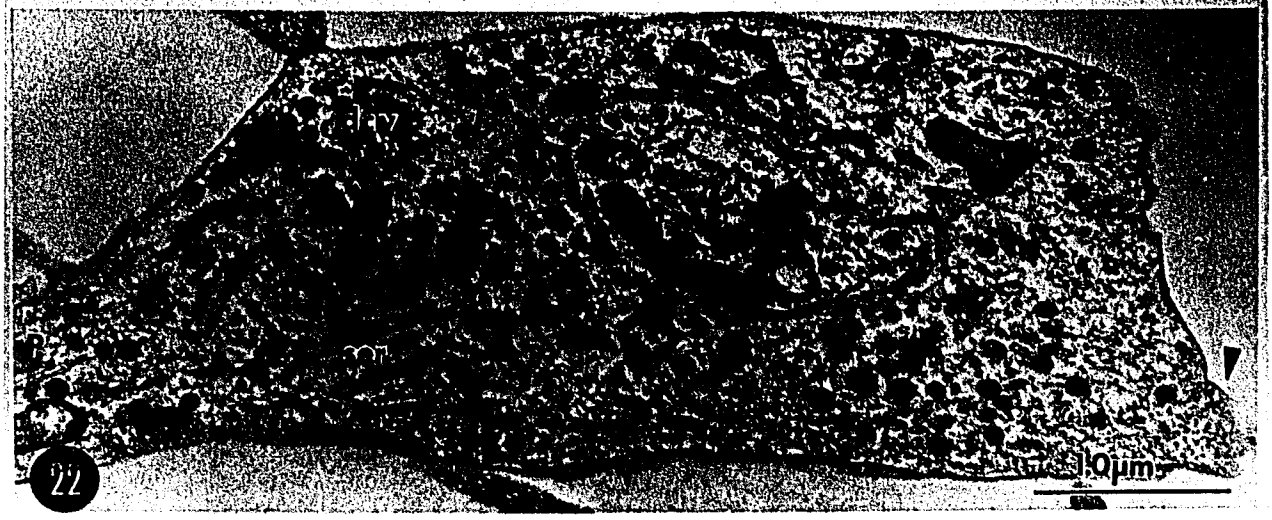

dry
mf
22
1.0μm

PLATE X.

Figure 23. FCS. The tip of a process. The process (a continuation of figure 20) enters into an expanded region rich in mitochondria and smooth endoplasmic reticulum which in turn enters an area free of most cytoplasmic organelles except for strings of 100 nm clear vesicles. The clear area ends in regions of filamentous material {arrowhead}. In this growth cone some microtubules continue to the end of the structure {*}. x20,600.

Figure 24. FCS. The tip of a process. The process {P} of this growth cone is rich in ribosomes which continue partially out into the expansion along with some microtubules. The adjacent region exhibits some endoplasmic reticulum and clear vesicles appear to be lined up. {f} may be a filopodium coming down into the section. x19,500.

Key:

clv	clear vesicle(s)
dcv	dense cored vesicle(s)
f	filopodium
if	intermediate filament(s)
mt	microtubule(s)
P	process
Q	lines of clear vesicles
ser	smooth endoplasmic reticulum


dev
1.0μm
23

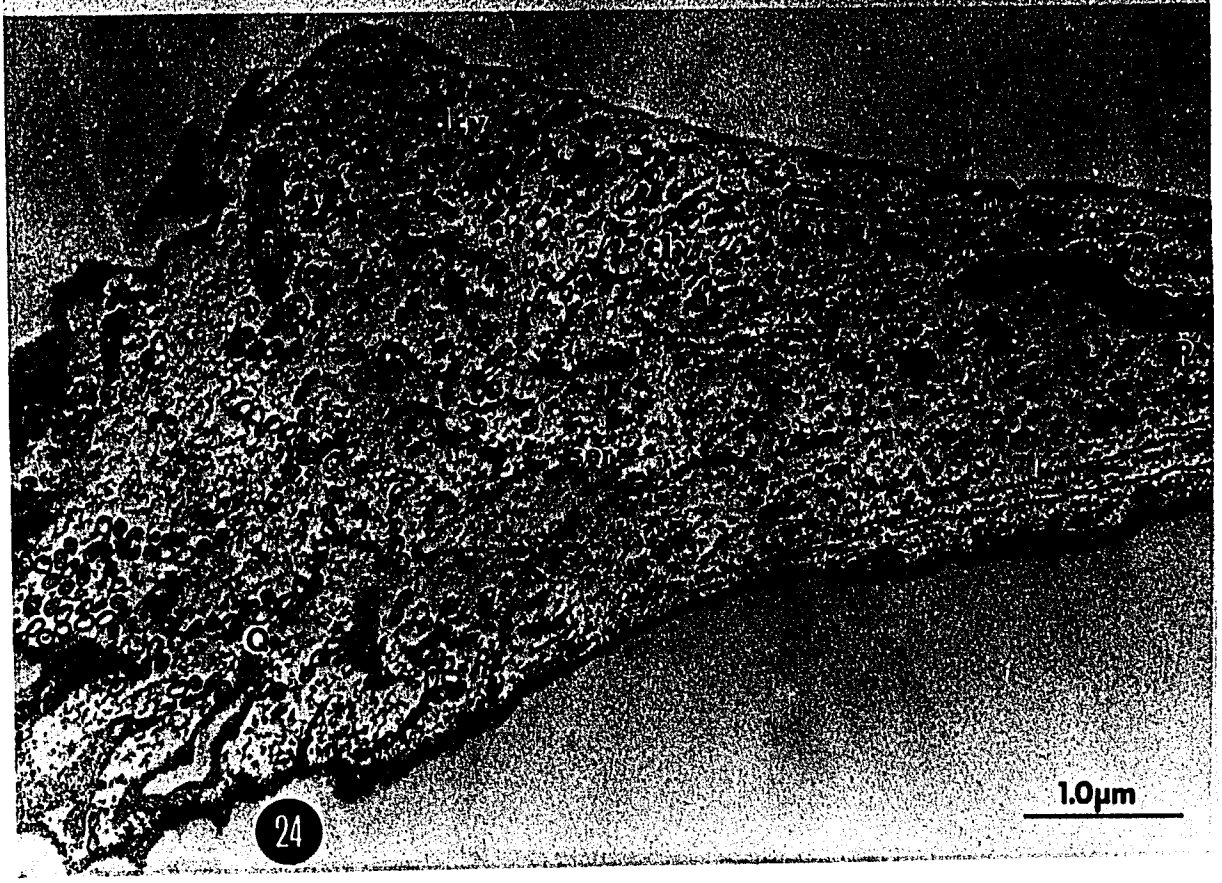

lev
ch
c
1.0μm
24

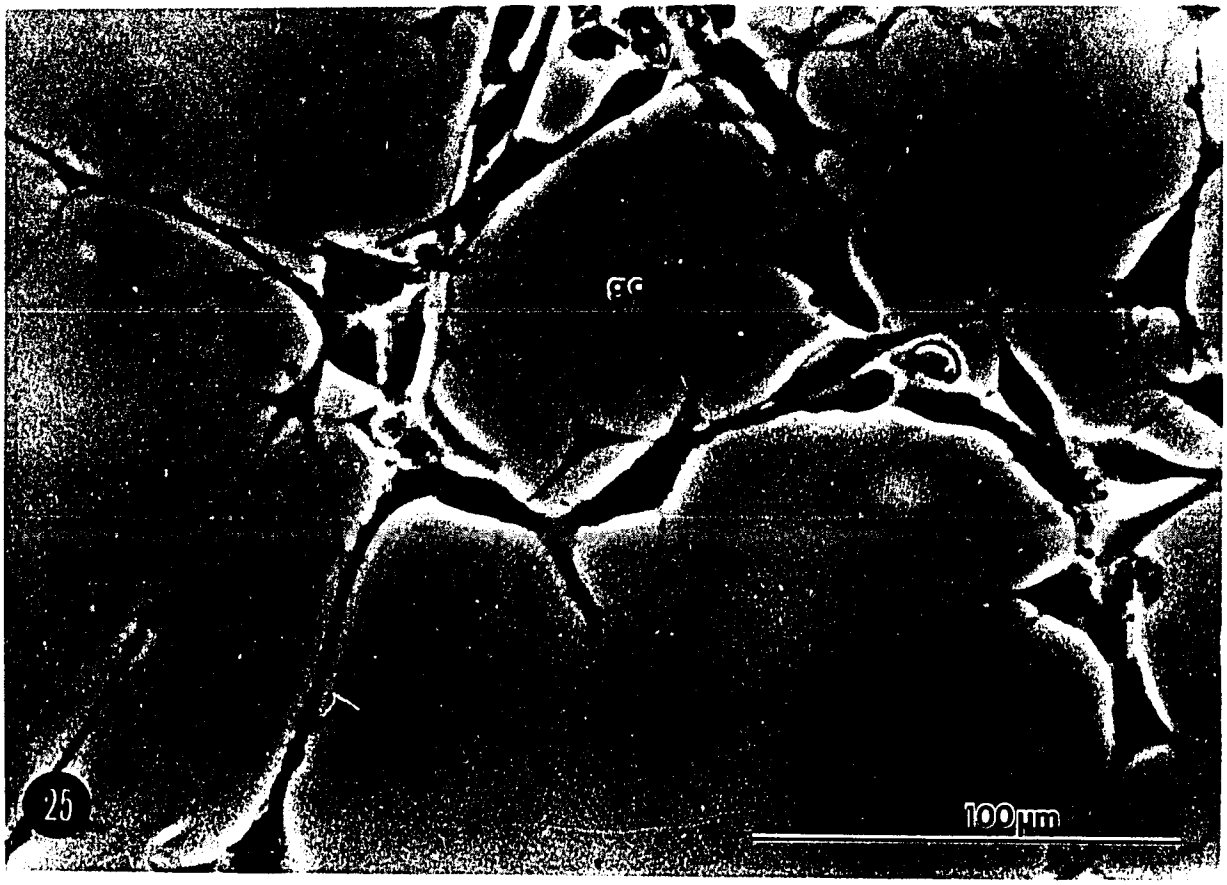
PLATE XI.

Figure 25. N2. Phase contrast micrograph of living SY5Y cells. The cells appear slightly rounder than in FCS with fewer processes. Some processes end in membrane expansions similar to growth cones. x520.

Figure 26. N2. Differential interference micrograph of fixed and air dried SY5Y cells. The majority of cells are `polymorphous', although `triangular' and `rounded' cells are also visible. x260.

Key:

gc	growth cone
p	`polymorphous' cell
r	`round' cell
t	`triangular' cell


GG
25
100µm

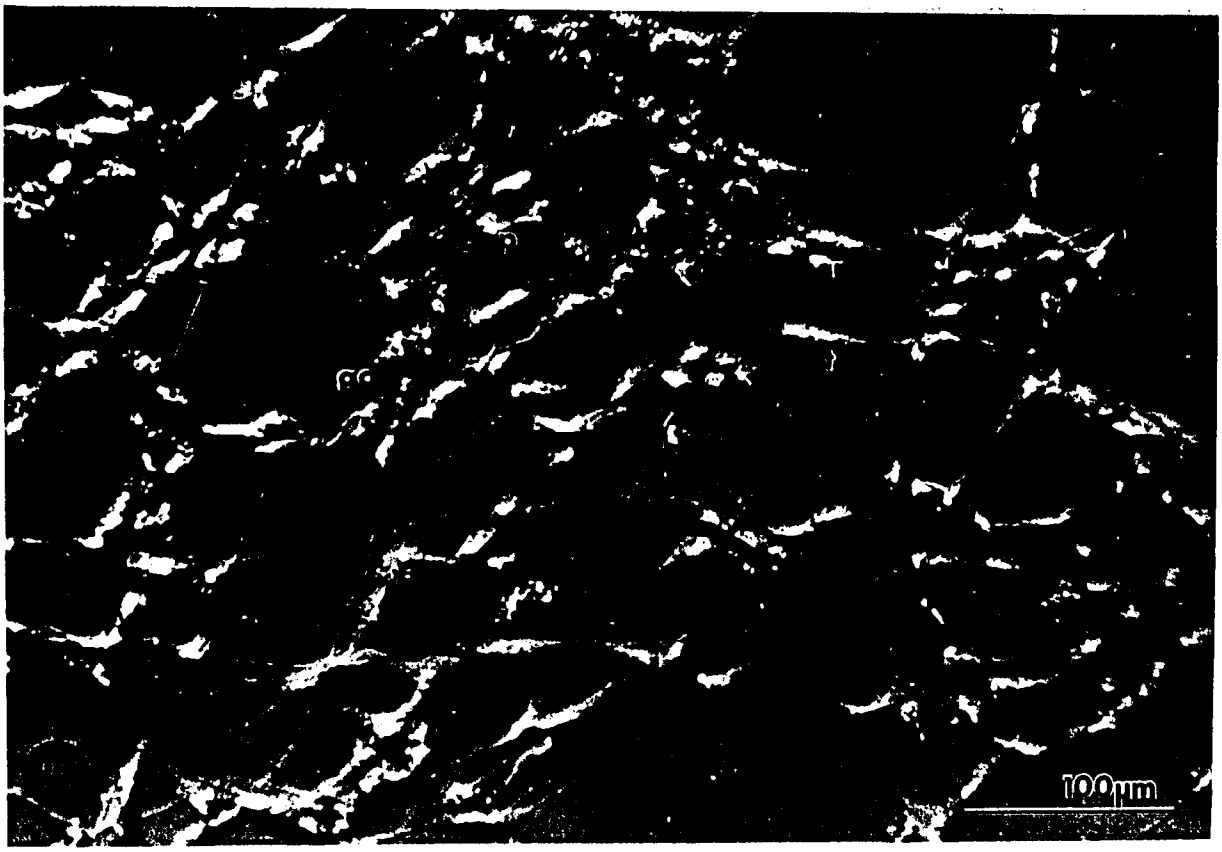

GG
100µm

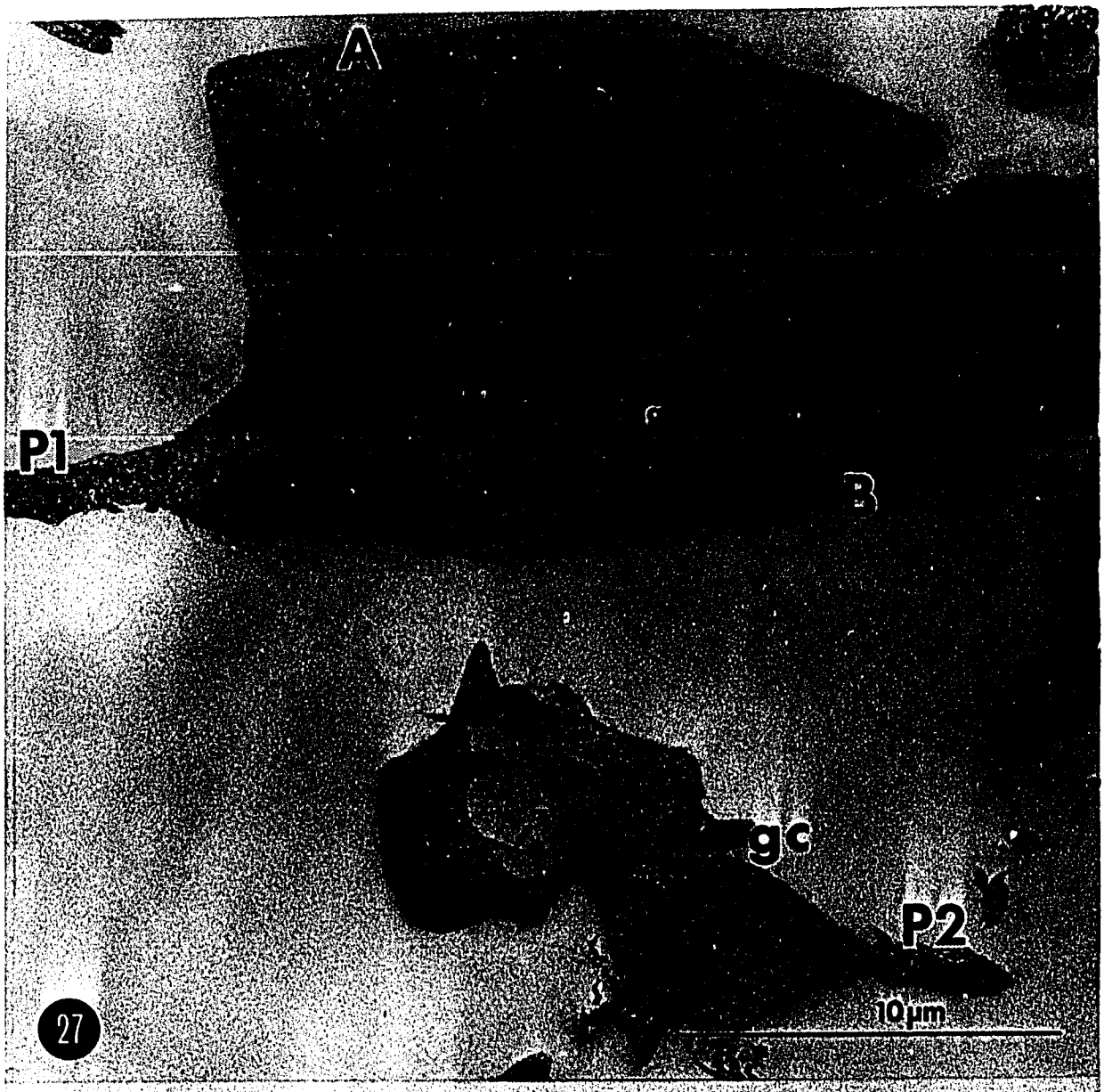
PLATE XII.

Figure 27. N2. Two cells and a growth cone. Cells {A} and {B} exhibit the typical ultrastructural characteristics of SY5Y cells: an eccentric nucleus with patches of condensed chromatin and densely staining nuclear rim and areas of the cell rich in membranous organelles. Other areas are rich in ribosomes and dispersed rough endoplasmic reticulum with mitochondria largely excluded. {P1} is an emergent process from the cell. The isolated structure at the bottom of the figure is a growth cone at the end of a long identified process {P2} (fig. 28). x5,100x.

Figure 28. N2. Phase micrograph of the region illustrated in figure 27. Process {P1} is a relatively short process ending in a varicosity. Process {P2} is a long process which ends in a growth cone. x900.

Key:

g	golgi complex(es)
gc	growth cone
P	process



27

28

PLATE XIII.

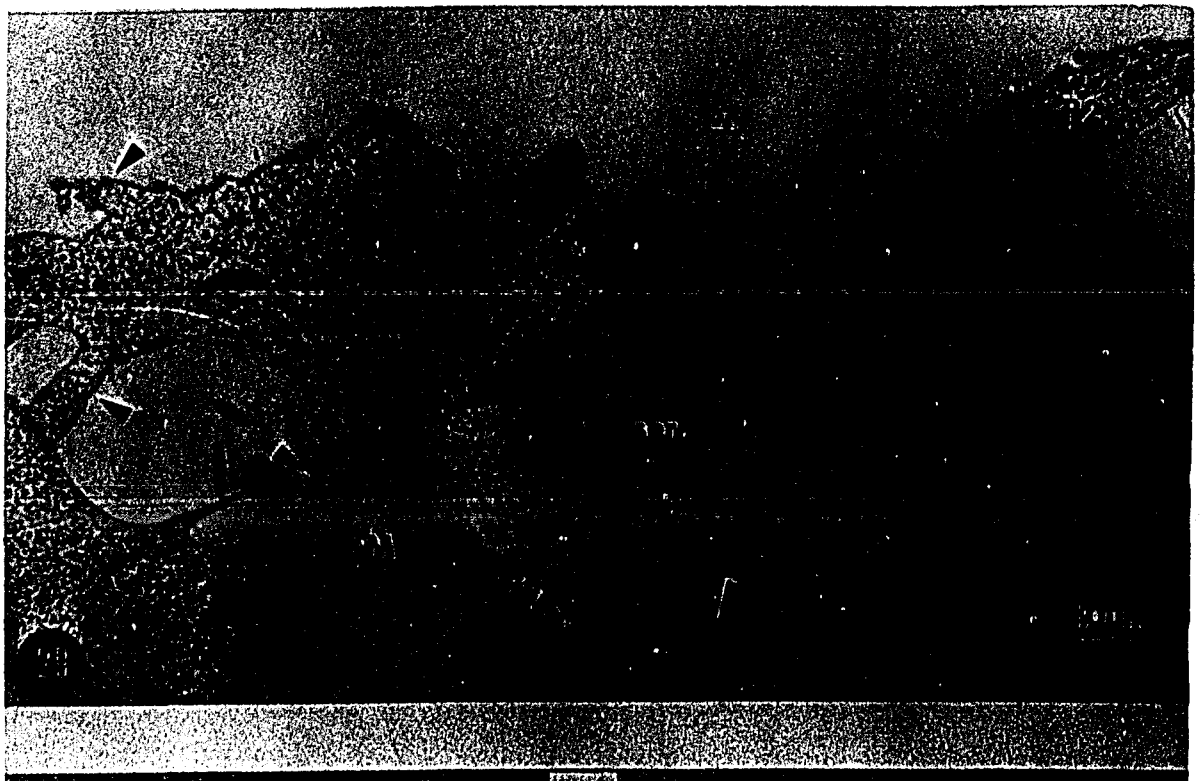
Figure 29. N2. Higher magnifcation of the growth cone of figures 27 and 28. The microtubules of P1 disappear or become disorganized as they enter the varicosity. The varicosity is predominantly smooth endoplasmic reticulum. At the periphery several microfilamentous filopodia can be seen projecting out {arrowheads}, on one a mound of clear vesicles is visible. x7,400.

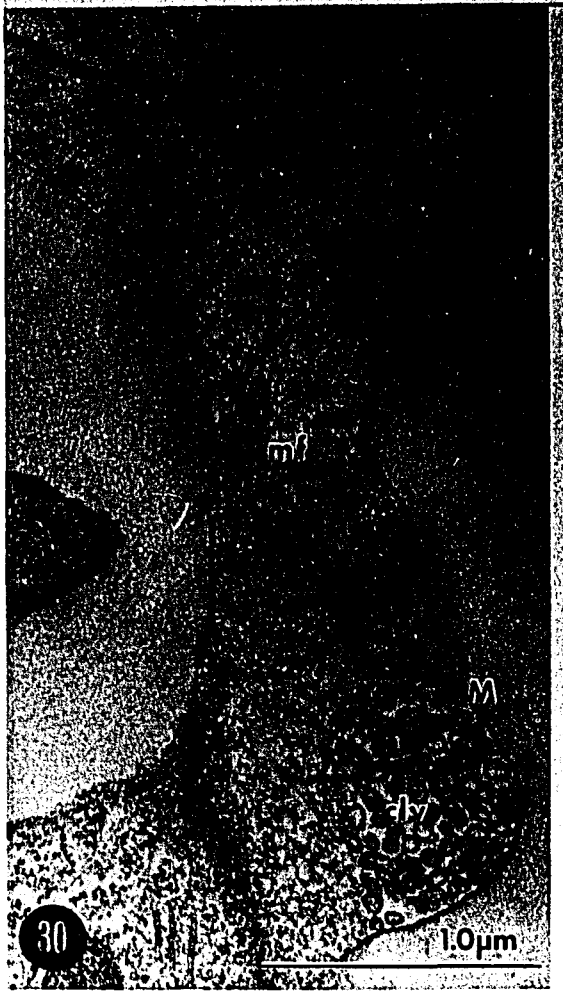
Figure 30. N2. Higher magnification of the large filamentous filopodium with a mound visible in figure 29. This is similar to the mounds known to be induced by gluteraldehyde fixation [Nuttall and Wessells, 1979]. 34,500x.

Figure 31. N2. Higher magnification of two filopodia visible in figure 29. x34,500.

Key:

clv	clear vesicle(s)
M	mound of clear vesicles
mf	microfilament(s)
P	process
ser	smooth endoplasmic reticulum




mf
M
dy
30
1.0μm

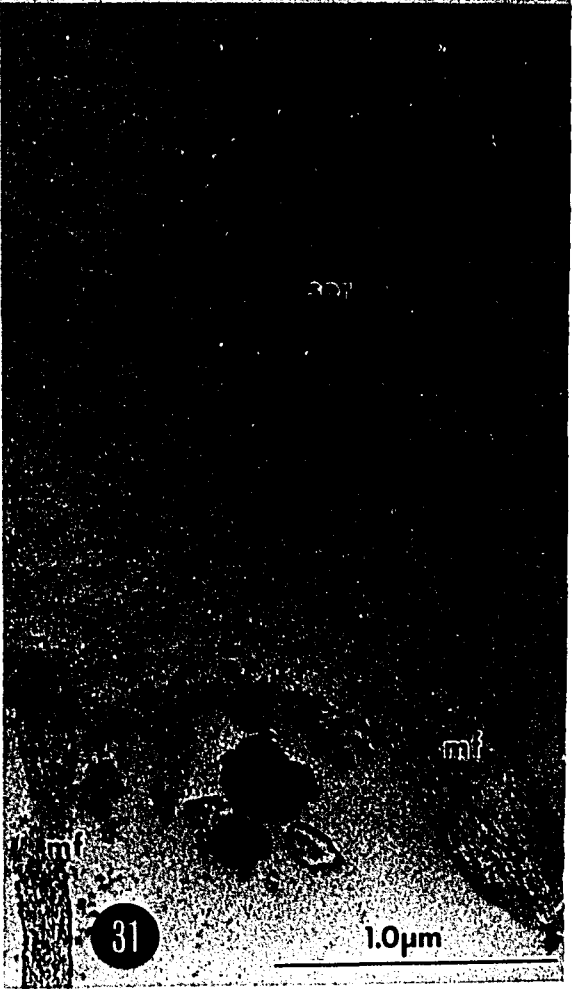

mf
mf
31
1.0μm

PLATE XIV.

Figure 32. N2. Two cell processes. This micrograph illustrates the variety among process profiles. The upper process has numerous microfilaments and some clear vesicles, possibly smooth endoplasmic reticulum, but no microtubules. The lower process displays several microtubules. x23,500.

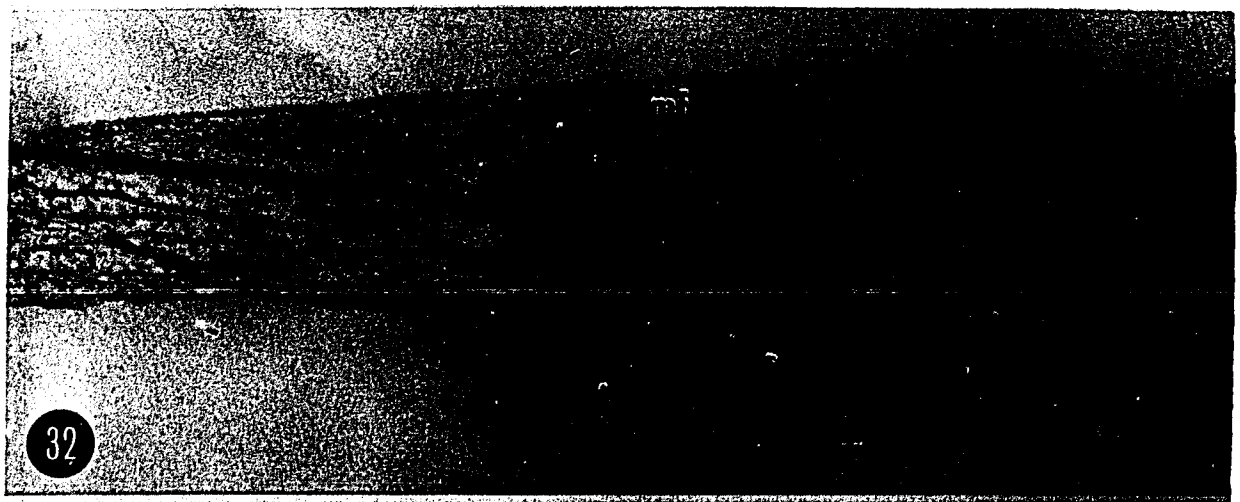
Figure 33. N2. This is a process with a varicosity filled with vesicles or smooth edoplasmic reticulum and some dense cored vesicles, but free of tubules or filaments. x23,500.

Figure 34. N2. A microtubule rich process displaying a long mitochondria in the central region. x23,500.

Figure 35. N2. A cell process. This is the process P1 of figures 27 and 28. Although it is relatively short extending only about 20 μm from the cell body (fig. 28) its ultrastruture is identical with longer processes such as process P2 of figure 29. The area on the right of the micrograph is the cell body. Note that although ribosomes are present throughout the process their number is greatly reduced in the more distal portions of the process in this section. x23,500.

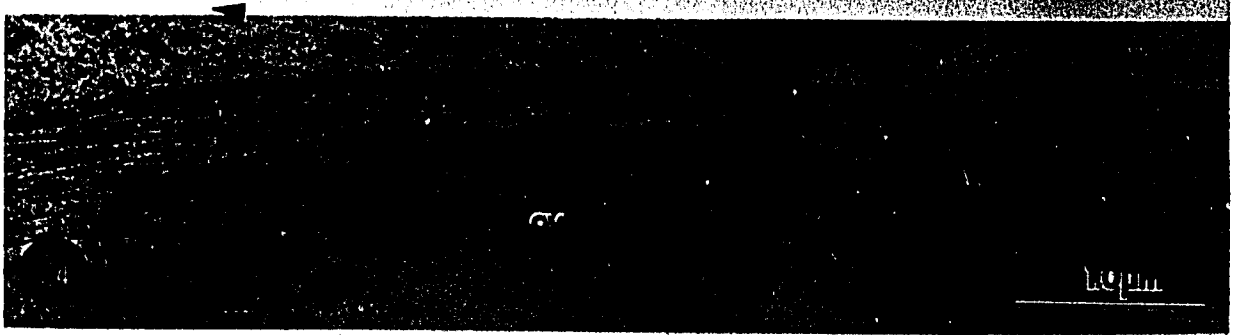
Key:

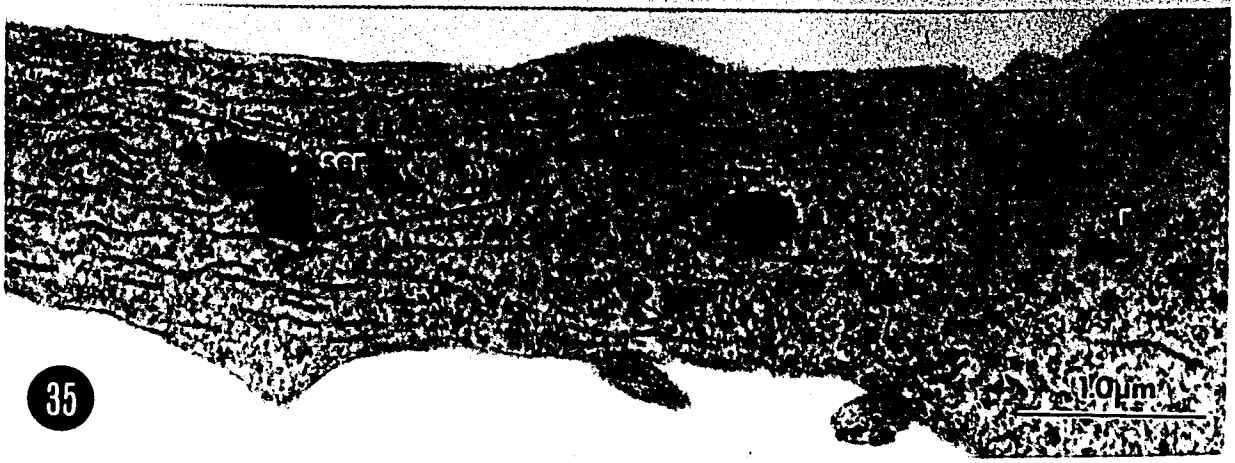
cv	coated vesicle
dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
r	ribosome(s)
ser	smooth endoplasmic reticulum



32

33





35

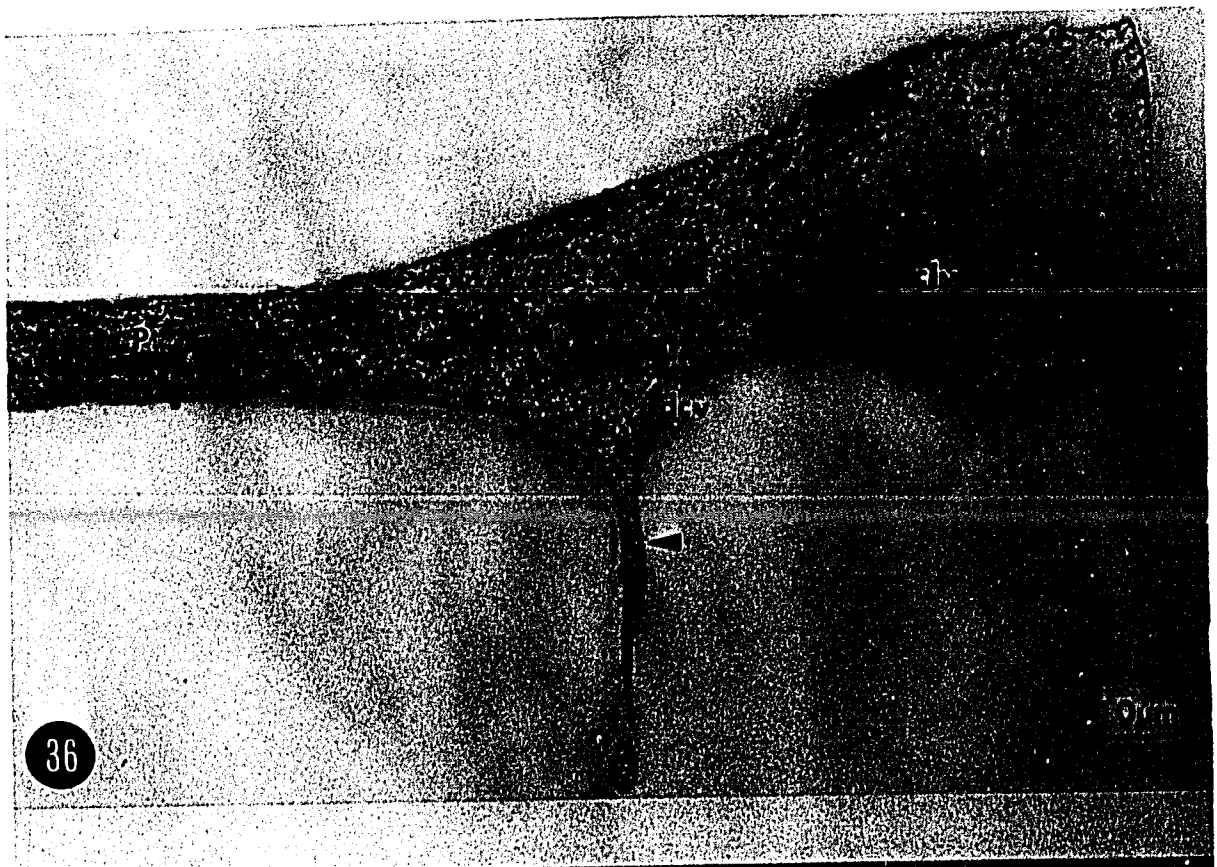
PLATE XV.

Figure 36. N2. The tip of a process. The tip of this process has a guy wire like extension (arrowhead) just before it ends in a clear expansion. x8,600.

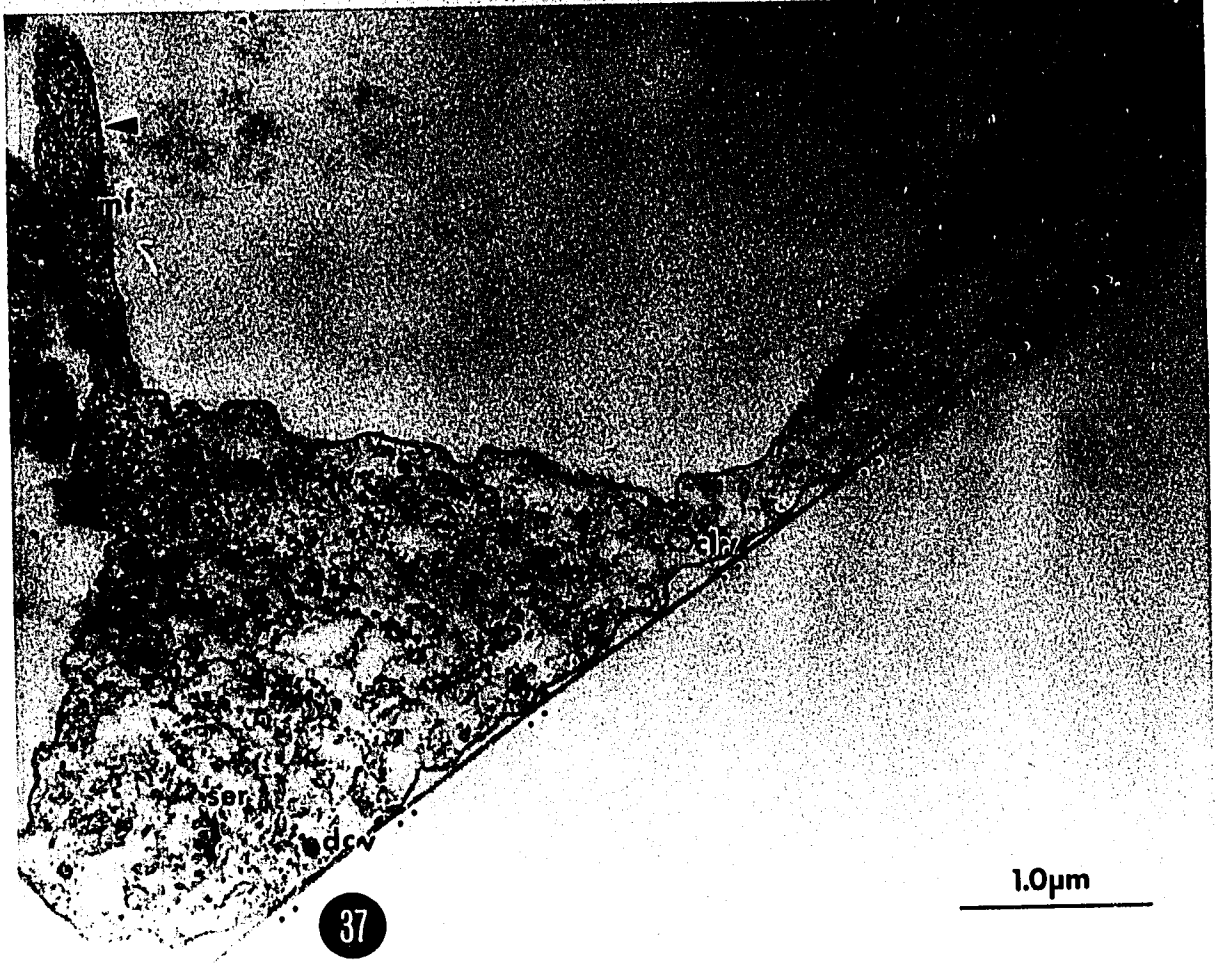
Figure 37. N2. A growth cone profile cut perpendicular to the substrate. A profile with components organized as in a growth cone is spread close to the substrate. Note that a filopodium, which probably extends from this growth cone, is projecting up from the substrate indicating that it may have been in motion, not attached to the substrate at the time of fixation. x23,500.

Key:

clv	clear vesicle(s)
dcv	dense cored vesicle(s)
mf	microfilament(s)
P	process
ser	smooth endoplasmic reticulum
..	culture substrate



36



37

1.0μm

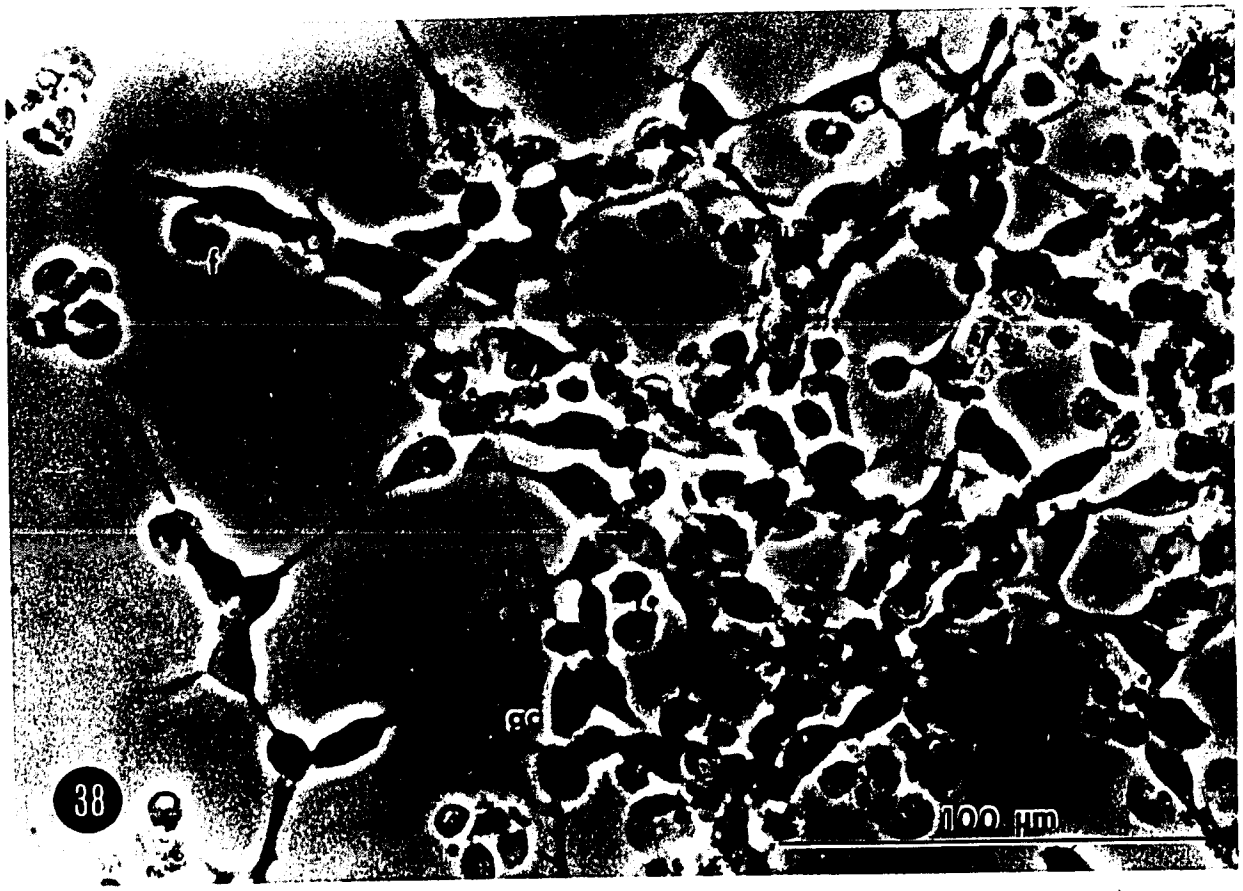
PLATE XVI.

Figure 38. F12/DME. Phase micrograph of living SY5Y cells. In F12/DME medium the cells are smaller than in N2. There are many more floating dead cells visible in the medium. Cells that remain attached however still exhibit processes and growth cones. x520.

Figure 39. F12/DME. Differential interference micrograph of glutaraldehyde fixed and air dried cells. Dead floating cells visible in living cultures are washed away, leaving only cells which were attached to the substrate. Although most of the cells which remained were `polymorphous' the highest proportions of other cell shapes were visible in this condition. Triangular, rounded, bipolar, and equilateral triangular are all visible in this micrograph. Notice the generally reduced number of processes present. x260.

Key:

b	`bipolar' cell
e	`equilateral' cell
f	floating cell
gc	growth cone
p	`polymorphous' cell
r	`round' cell
t	`triangular' cell


38
100 µm

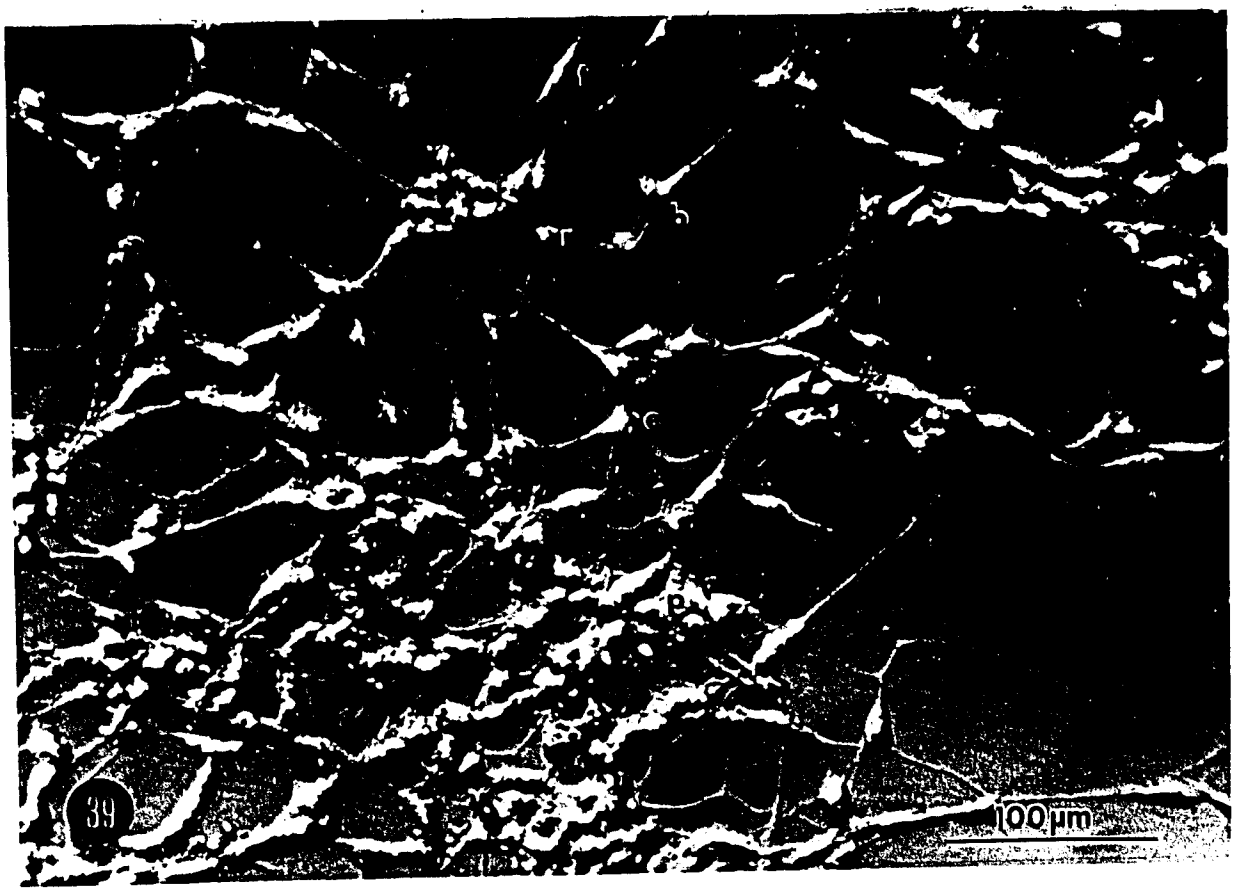

39
100 µm

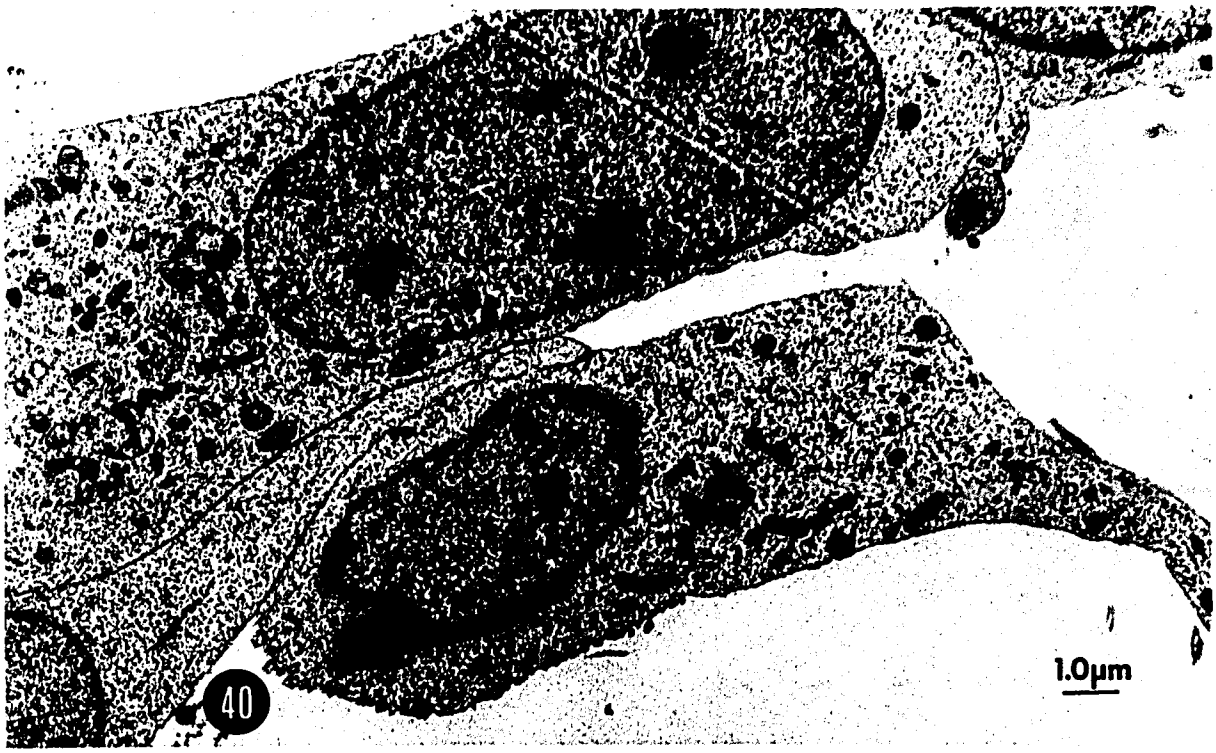
PLATE XVII.

Figure 40. F12/DME. Overview of several cells. Cells grown in F12/DME medium exibit the same overall ultrastructural characteristics seen in FCS and N2 medium. x7,000.

Figure 41. F12/DME. Microtubules and microfilaments are prominent in this section cut near the ventral surface of the cell. Several filopodia can be seen which probably extend from the cell body {arrowheads}. Note the dispersed nature of the ribosomes with few clusters, also dense cored vesicles. x23,500.

Key:

dcv	dense cored vesicle(s)
mf	microfilament(s)
mt	microtubule(s)
P	process
*	close apposition of nucleus and plasmalemma


40
1.0μm

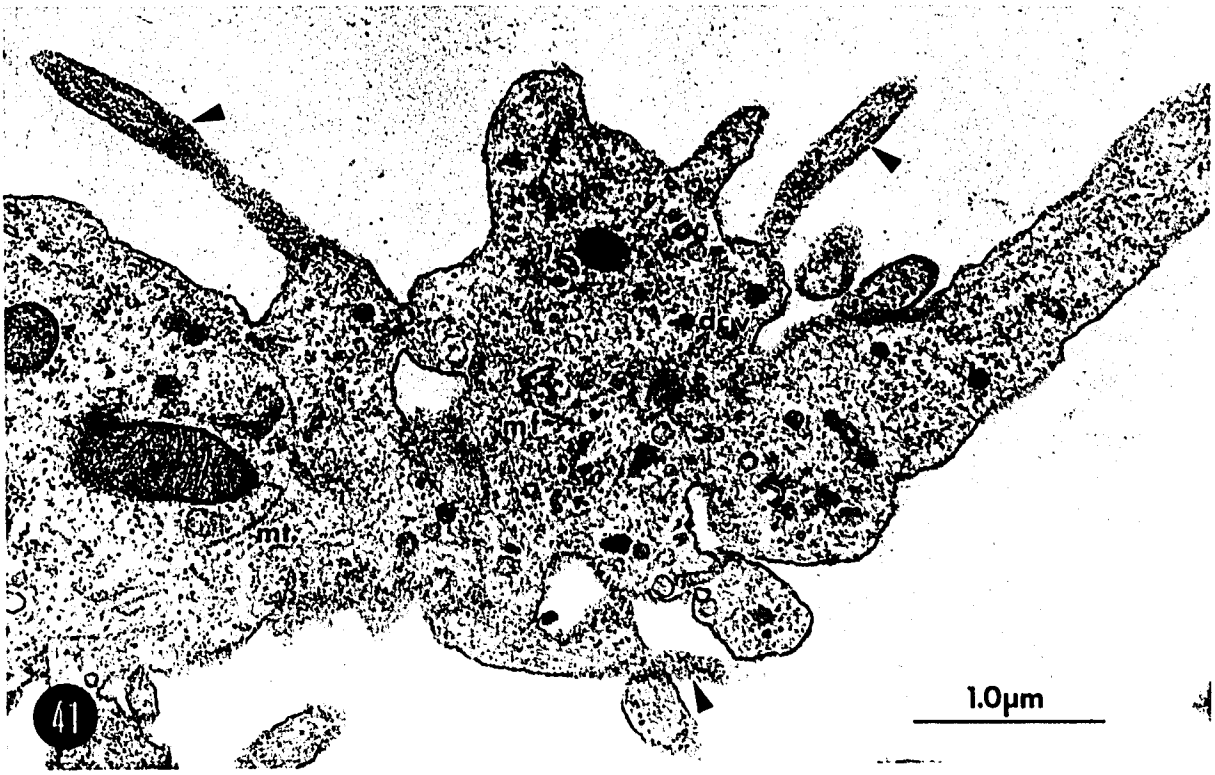

mt
mt
41
1.0μm

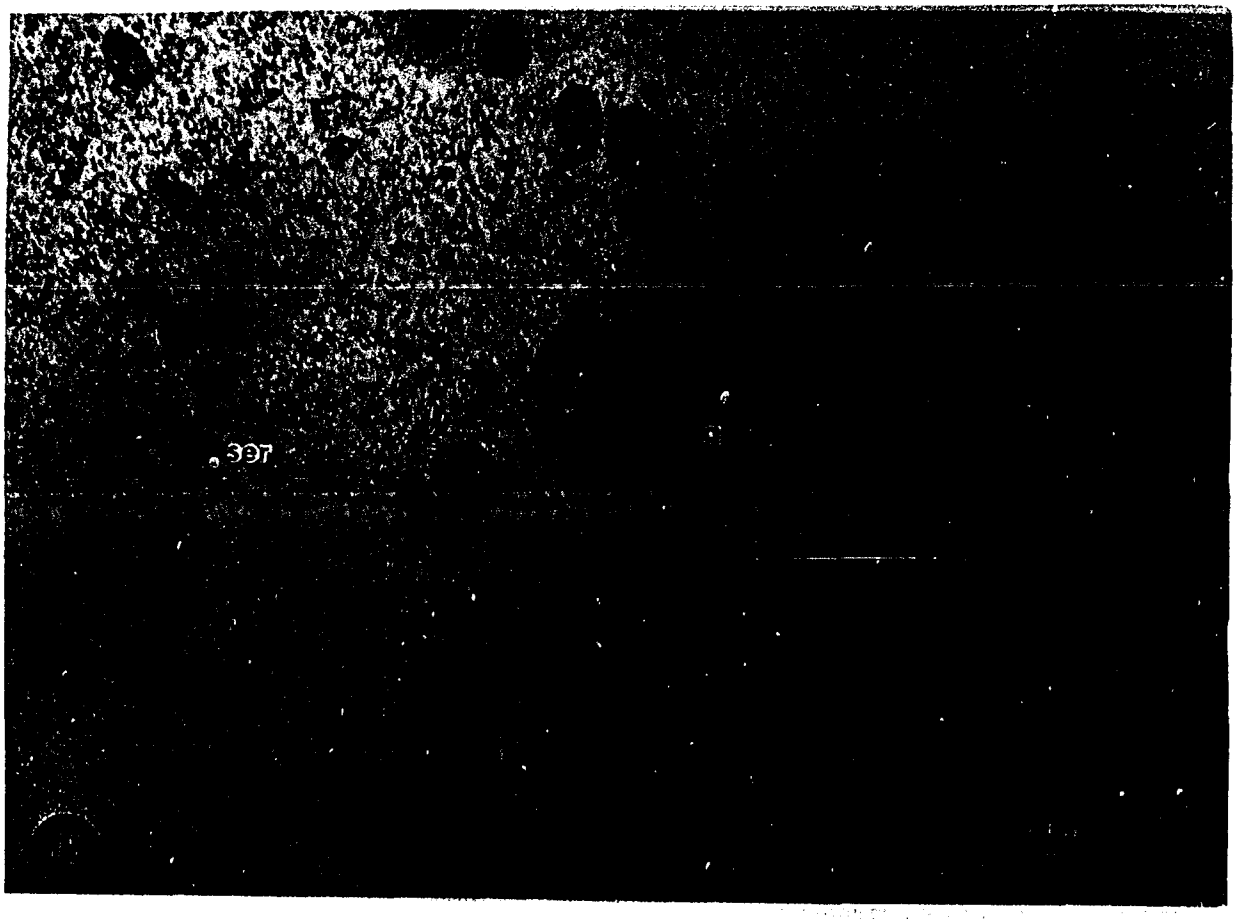
PLATE XVIII.

Figure 42. F12/DME. Rough endoplasmic reticulum is absent from this section and the ribosomes are scattered apparently randomly. x23,500.

Figure 43. F12/DME. A well formed cilium protrudes from the cell, emerging from an area with a golgi apparatus. x68,500.

Key:

Ci	cilium
g	golgi complex(es)
ser	smooth endoplasmic reticulum



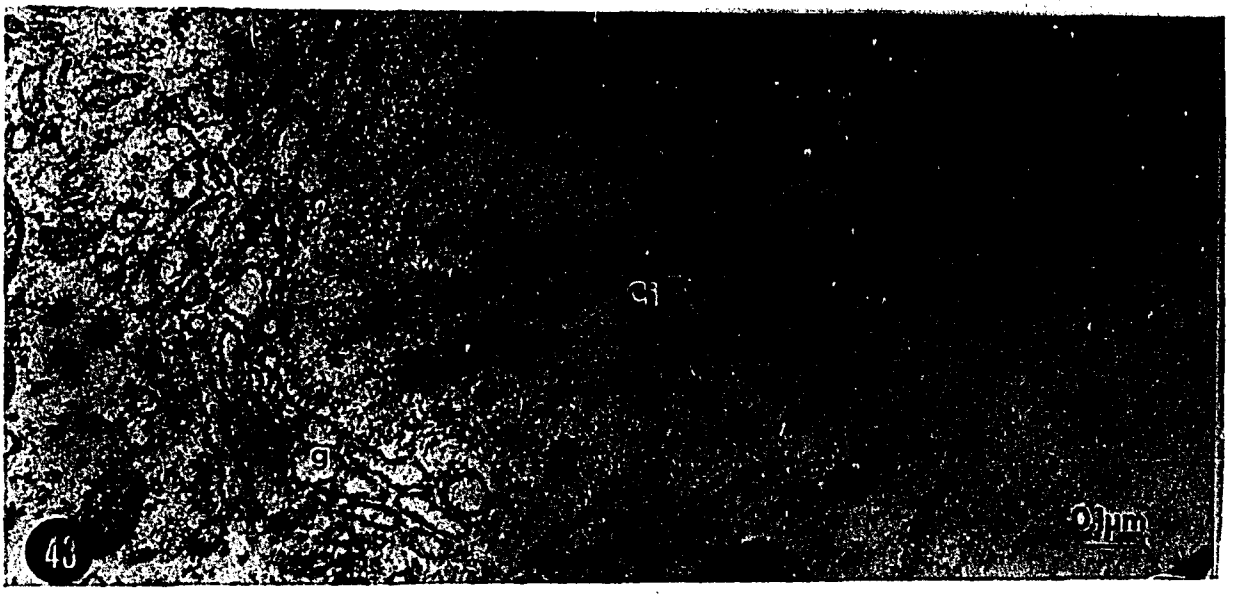

43
0.1μm

PLATE XIX.

Figure 44. F12/DME. This process exhibits many of the features of processes seen in FCS and in N2 media. x34,500.

Figure 45. F12/DME. A process with a looser appearance. Microtubules are still present but small membranous elements and free ribosomes predominate. x23,500.

Figure 46. F12/DME. A process containing a multivesiclular body. The density of microtubules varies along the length of this profile. x23,500.

Figure 47. F12/DME. This process consists predominantly of whispy material and ribosomes although some microtubules and dense cored vesicles are present. x23,500.

Figure 48. F12/DME. A relatively microtubule rich process also contains several myelin bodies (arrowheads). x23,500.

Figure 49. F12/DME. Transverse section of a process. The substrate is just beyond the lower margin of the picture x23,500.

Key:

dcv	dense cored vesicle(s)
mf	microfilament(s)
mt	microtubule(s)
mvb	multi-vesiclular body
ser	smooth endoplasmic reticulum
w	wispy material

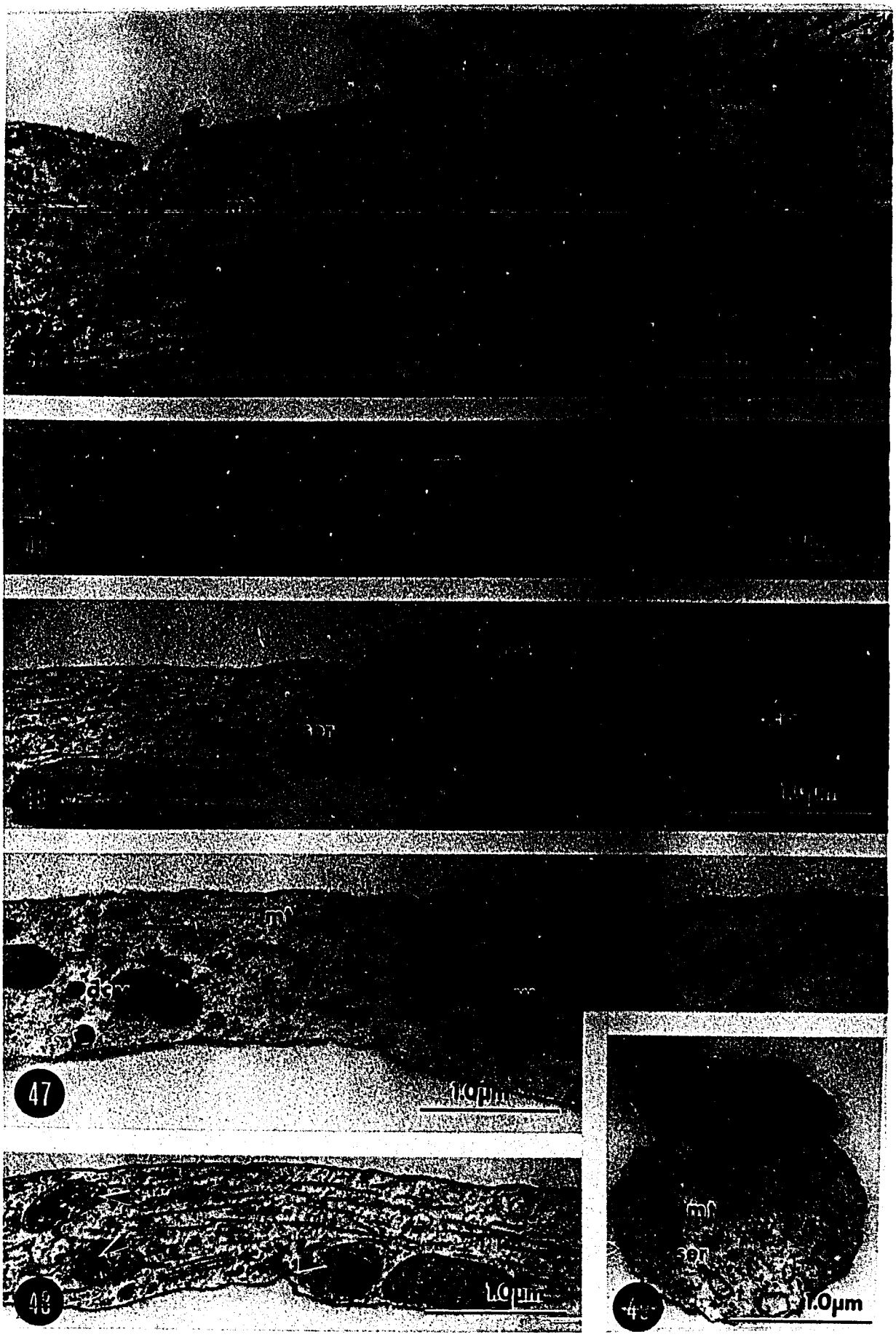

47
1.0μm
48
1.0μm
49
1.0μm

PLATE XX.

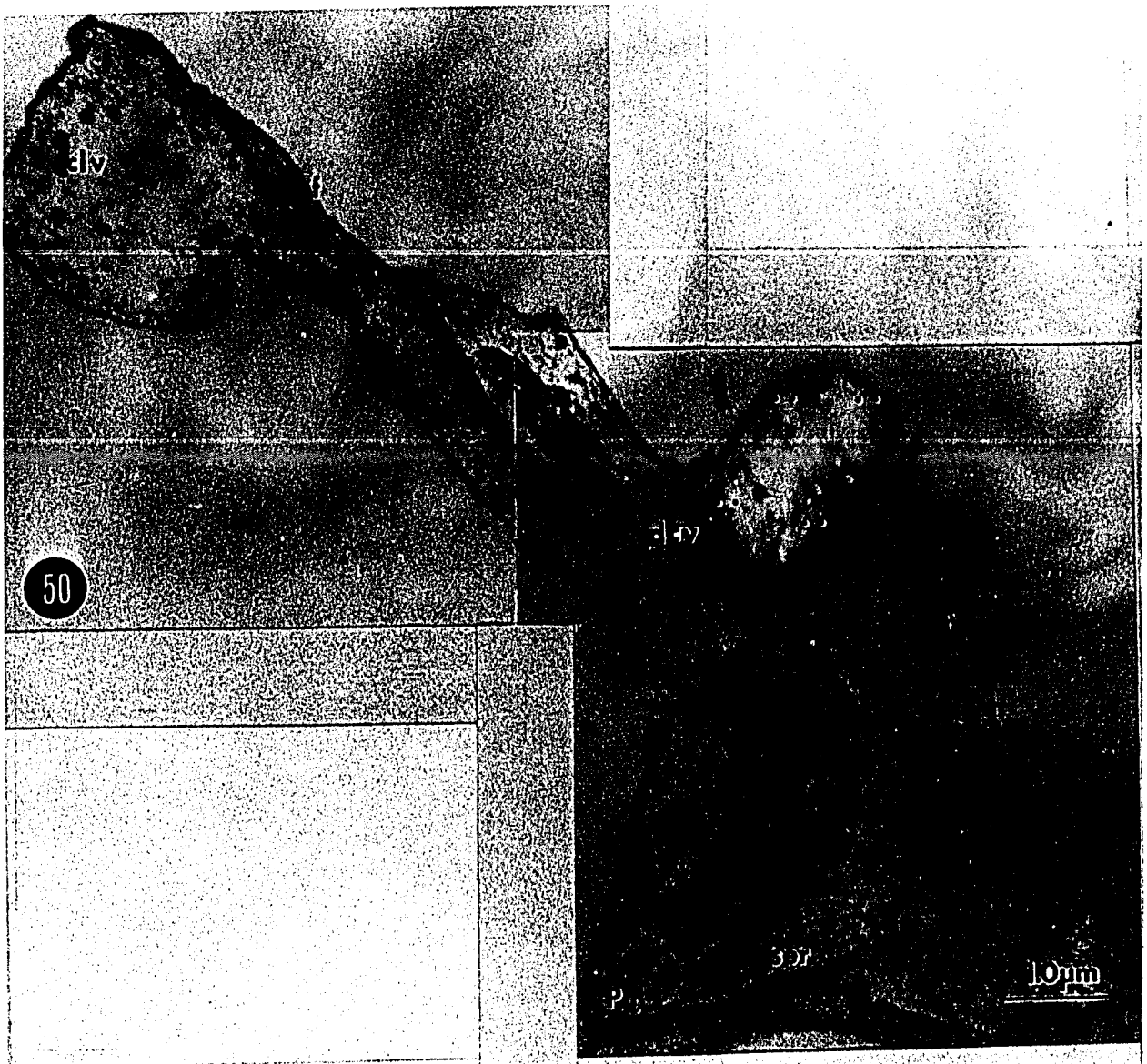
Figure 50. F12/DME. Montage of the tip of a process. This process tip exhibits all the features of a growth cone. The process expands into a region of splayed microtubules, smooth endoplasmic reticulum and mitochondria. The more distal regions consist of wispy material with some clear vesicles. A long filopodium is visible, probably extending from the expansion. Dense cored vesicles are also present. The white region {..} is a thin region of Epon caused by an irregularity in the culture substratum. It illustrates the closeness of the growth cone to the substrate. x14,300.

Figure 51. F12/DME. The tip of a process. The process leading into this expansion contains few microtubules, but the expansion contains the other organelles of a neuronal growth cone. x14,000.

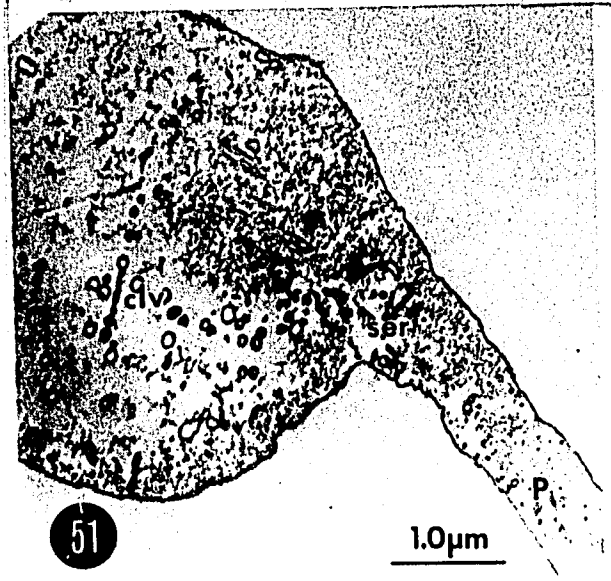
Figure 52. F12/DME. The tip of a process. The tip of this process appears depleted and small but still contains the organelles seen in growth cones. x22,000.

Key:

clv	clear vesicle(s)
dcv	dense cored vesicle(s)
f	filopodium
P	process
ser	smooth endoplasmic reticulum
..	culture substrate



50



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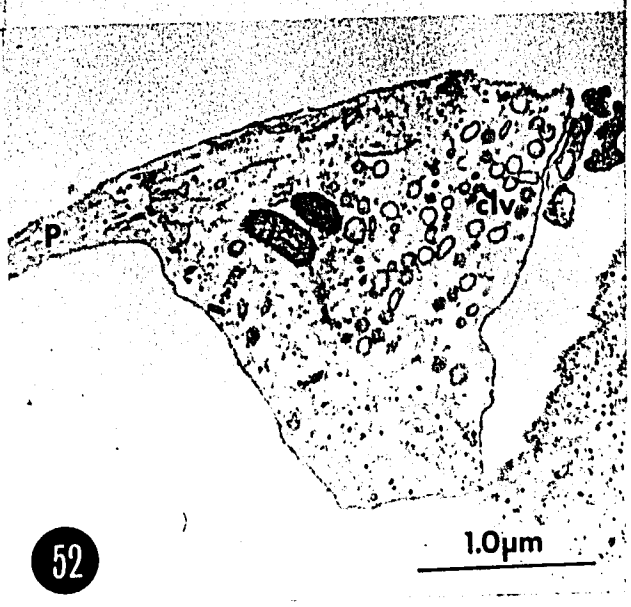


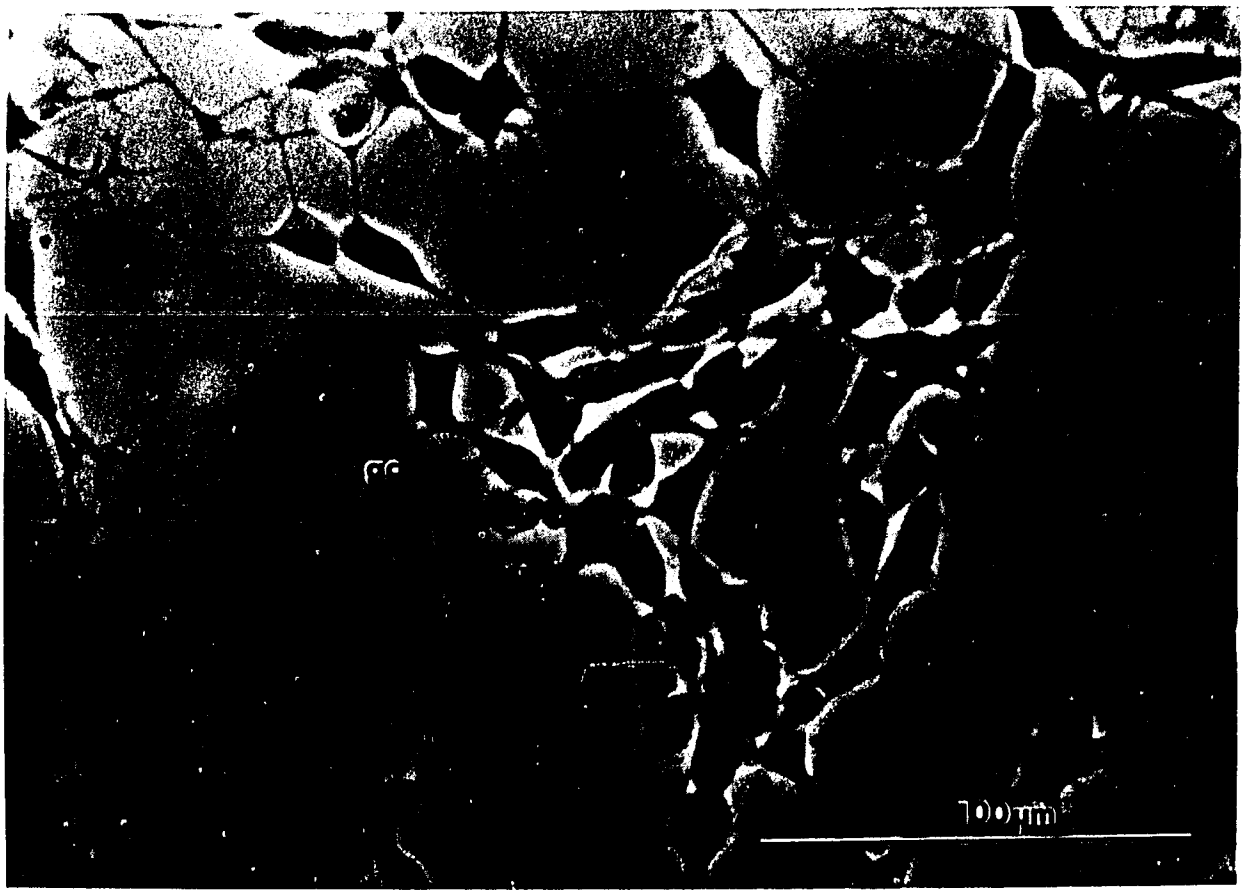
52

PLATE XXI.

Figure 53. FCS NGF. Phase contrast micrograph of living SY5Y cells. The cells appear similar in overall appearance to the cells in FCS medium. x560.

Figure 54. FCS NGF. Differential interference micrograph of glutaraldehyde fixed, air dried cells. As in FCS medium most cells have a `polymorphous shape'. Visible in this micrograph are cells classified as unipolar, bipolar, and equilateral triangular. Note that most cells are in direct contact with the cell substrate. x520.

Key:		
	b	`bipolar' cell
	e	`equilateral' cell
	gc	growth cone
	u	`unipolar' cell


100μm

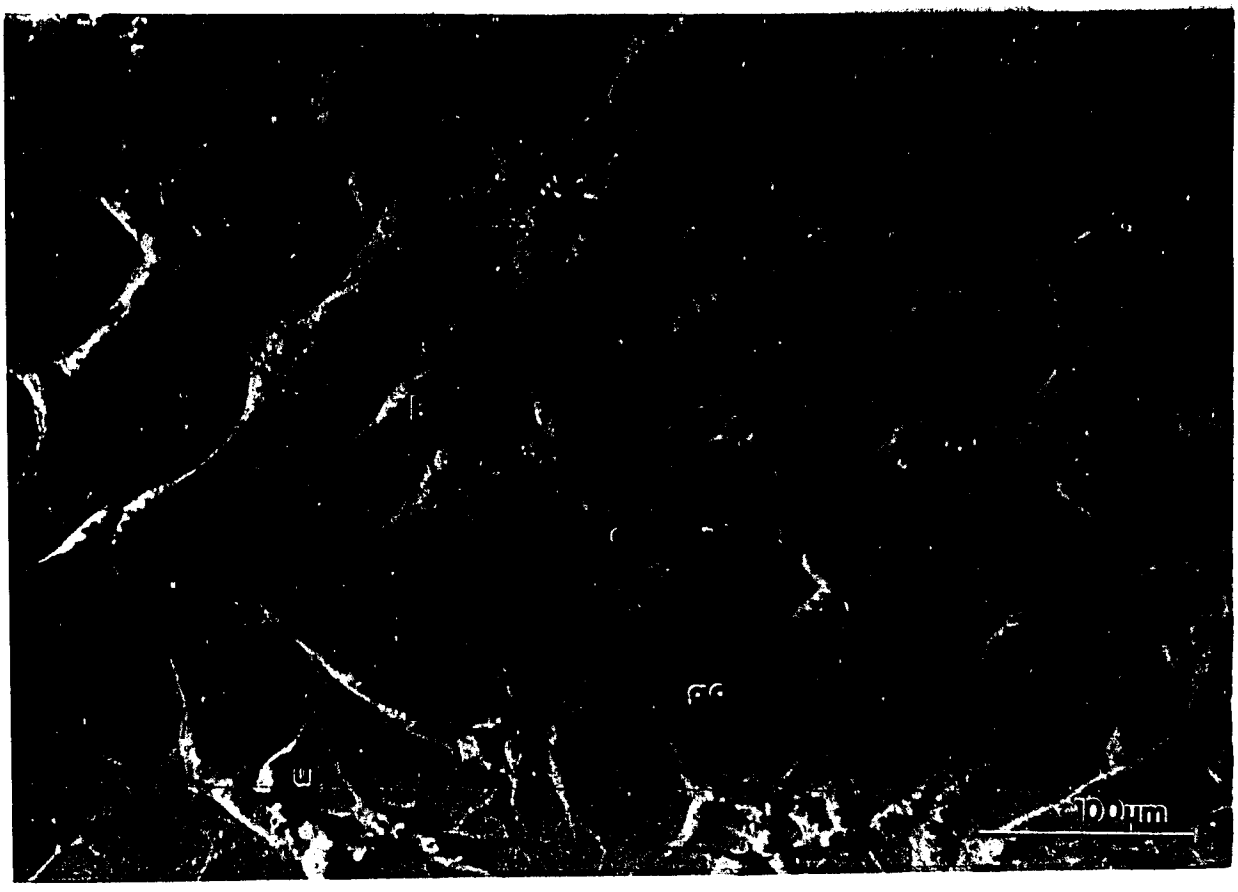

100μm

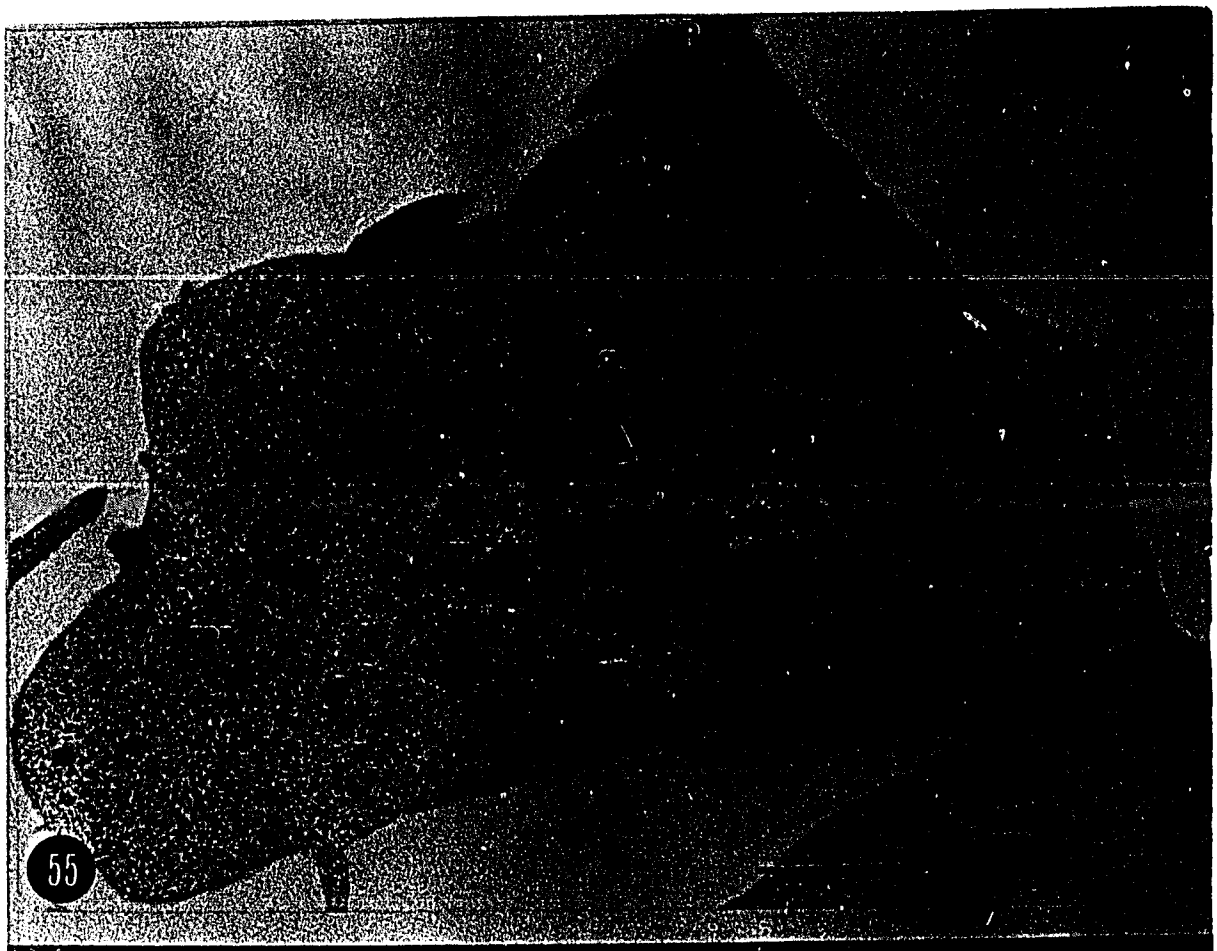
PLATE XXII.

Figure 55. FCS NGF. A typical cell. This cell exhibits an eccentric nucleus with several nucleoli and a rim of darkly stained material {arrowhead}. The cytoplasm is compartmentalized into a region rich in mitochondria and golgi and a region reletively rich in rough endoplasmic riticulum which tends to exclude mitochondria and golgi. {P} is a microtubule rich process from another cell. x5,200.

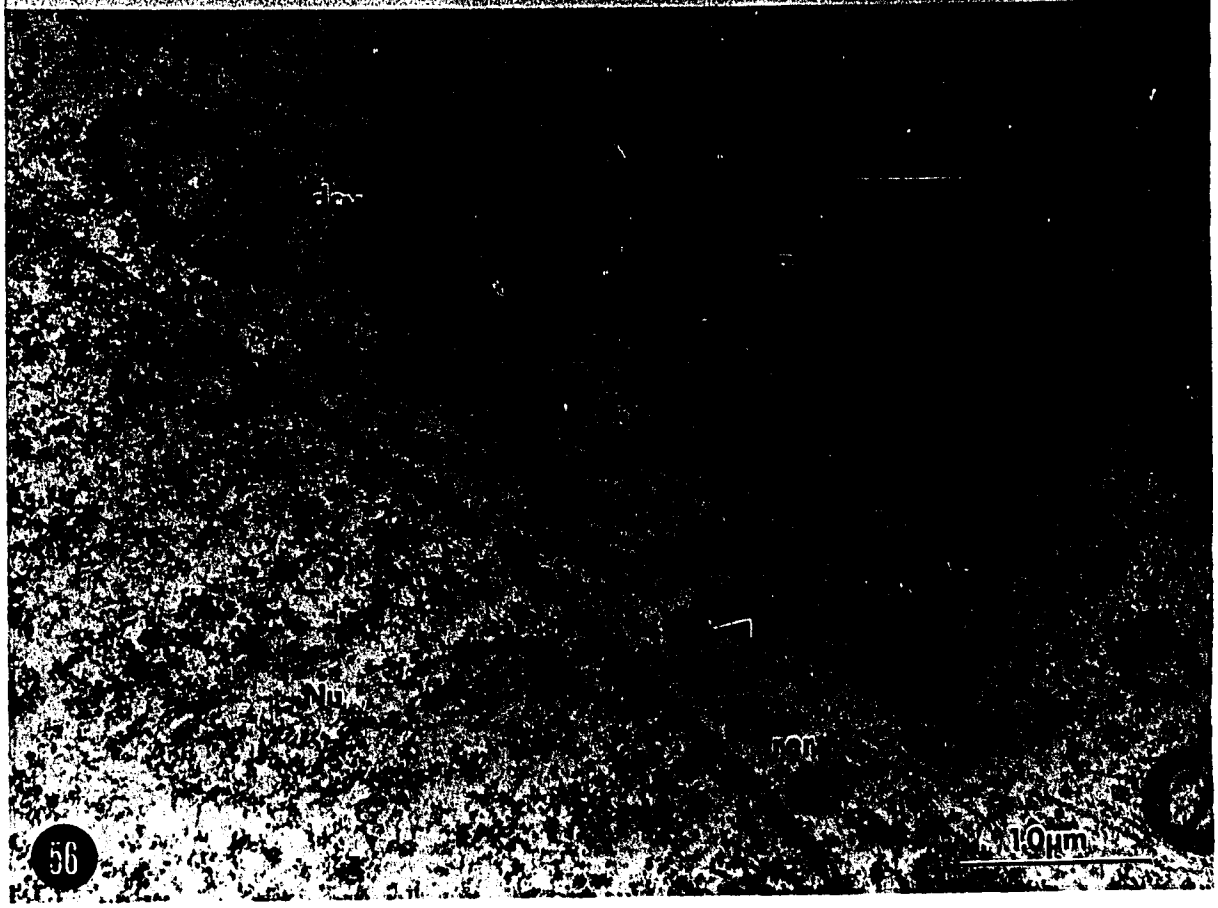
Figure 56. FCS NGF. Detail of figure 55. The arrowhead indicates a large, coated vesicle containing a dark center. x23,500

Key:

dcv	dense cored vesicle(s)
g	golgi complex(es)
Nu	nucleus
P	process
rer	rough endoplasmic reticulum
*	close juxtaposition of nucleus and plasmalemma



55



56

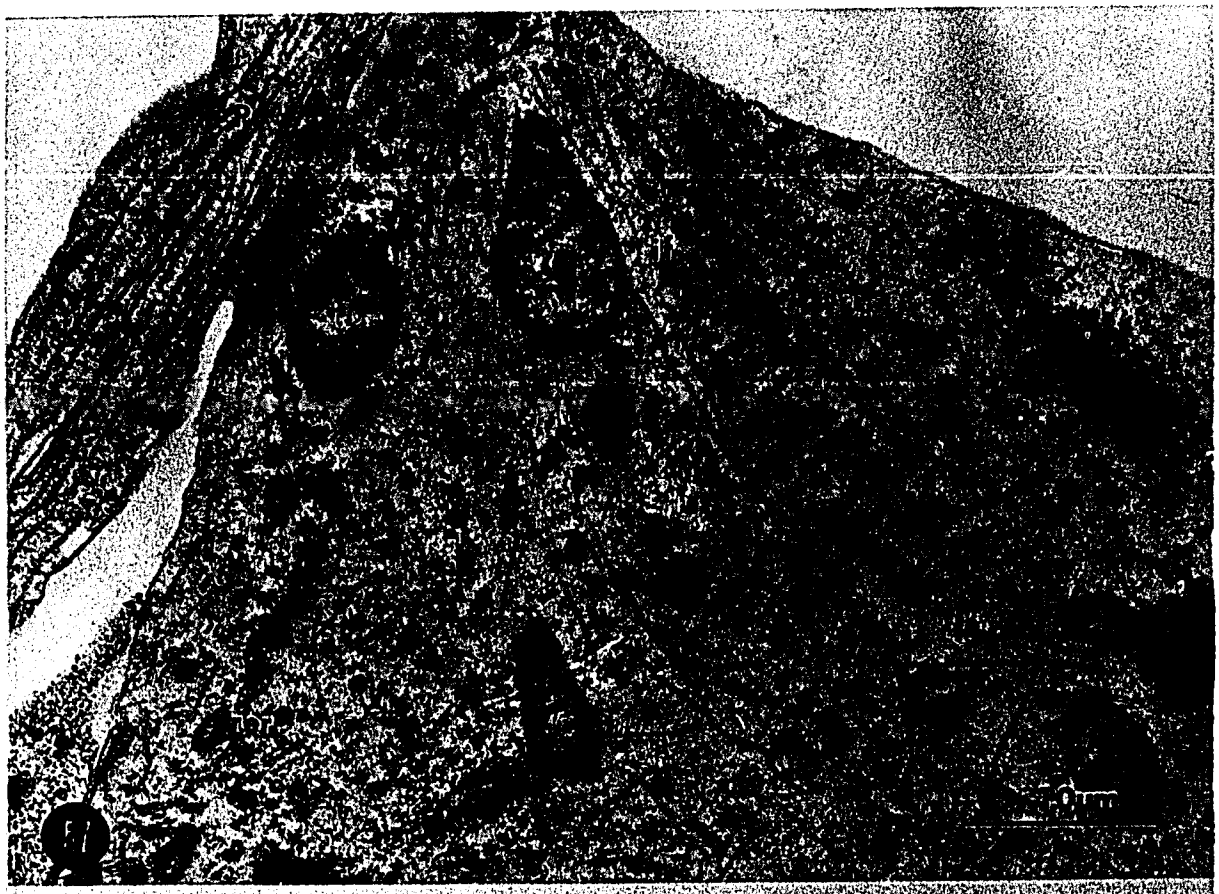
PLATE XXIII.

Figure 57. FCS NGF. This detail of figure 55. shows a funneling of microtubules and intermediate filaments like that seen at the base of cell processes. x23,500.

Figure 58. FCS NGF. Two processes. The lower process is expanded and shows a 'mound'-like structure. x23,500.

Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
P	process
Q	lines of clear vesicles
rer	rough endoplasmic reticulum



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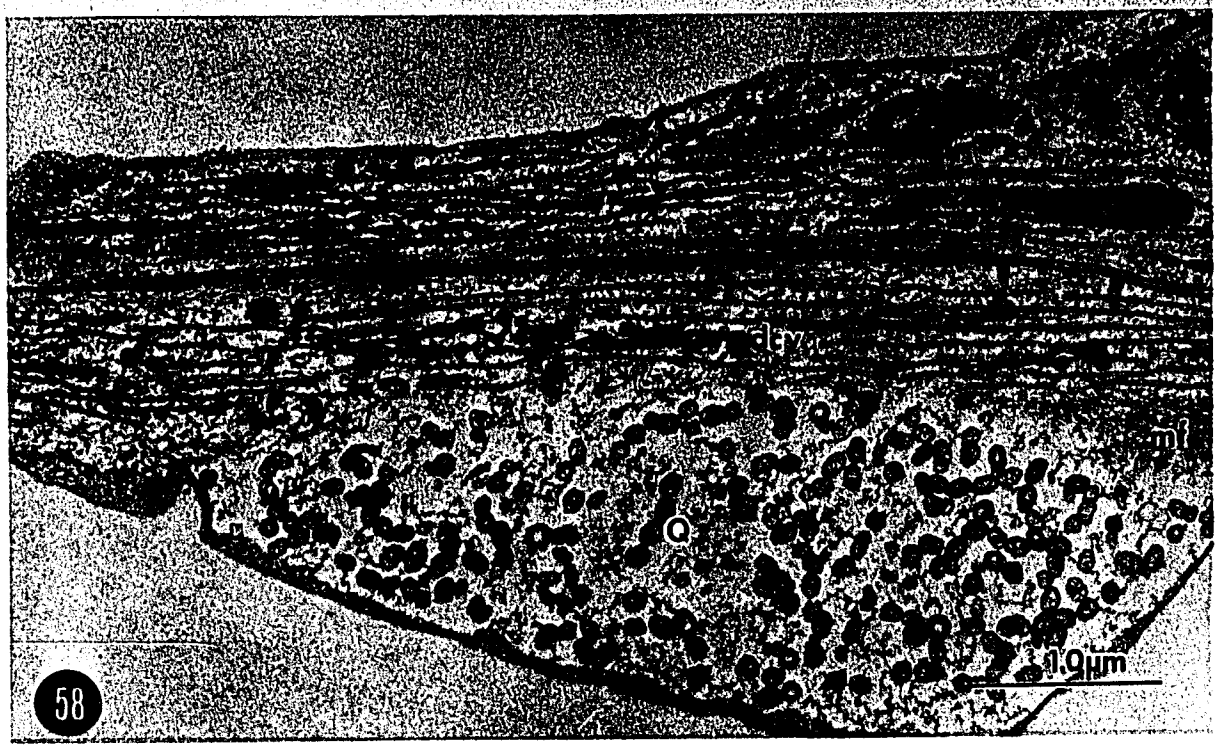


58

PLATE XXIV.

Figure 59. FCS NGF. Cell process displaying many of the same features seen in FCS medium. x23,500.

Figure 60. FCS NGF. Cell process with a rich array of microtubules interspersed with free ribosomes. 23,500x.

Figure 61. FCS NGF. This thin process with expansions containing mitochondria and smooth endoplasmic reticulum may be a long filopodium or possibly a tangential section of a typical process sectioned through the subplasmalemmal layer of microfilaments. x23,500.

Figure 62. FCS NGF. Transverse section of a process showing typical features. x44,500.

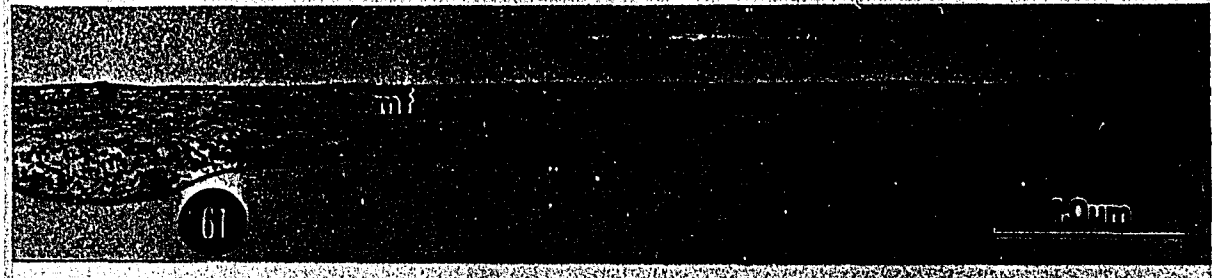
Figure 63. FCS NGF. Ribosomes are seen throughout and associate with the endoplasmic reticulum at one point. x23,500.

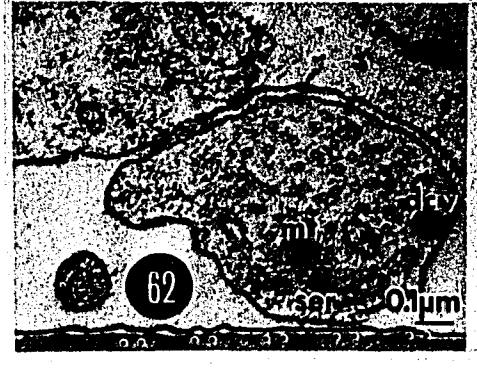
Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
rer	rough endoplasmic reticulum
ser	smooth endoplasmic reticulum
..	culture substrate


mf
dav
mf
1.0μm
60


dav
60


mf
61


dav
mf
62
0.1μm

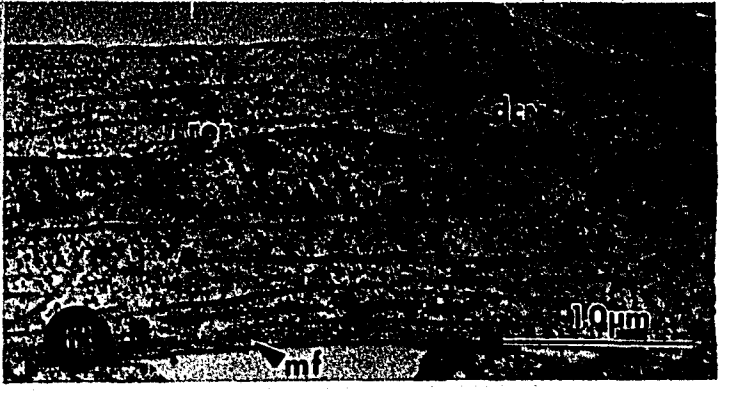

dav
mf
1.0μm

PLATE XXV.

Figure 64. FCS NGF. A transverse section through a growth cone. A profile consistent with growth cone morphology extends one filopodium {f1} to the culture substrate and one out to the side {f2}. x23,500.

Figure 65. FCS NGF. This structure at the end of aprocess displays abundant smooth endoplasmic reticulum and probably ends in one of several filopodia {arrowheads}. The dark area at the lower left is a tangential section of the plastic tissue culture dish indicating how close the growth cone structure is to the substrate. The small dots seen in the clear Epon are a staining artifact that developed after several months storage of the grid, the spots are predominantly in the area surrounding the cells not in the cell cytoplasm! x6,700.

Key:

clv	clear vesicle(s)
dcv	dense cored vesicle(s)
f	filopodium
P	process
ser	smooth endoplasmic reticulum
..	culture substrate


clv
f1
1.0µm
64

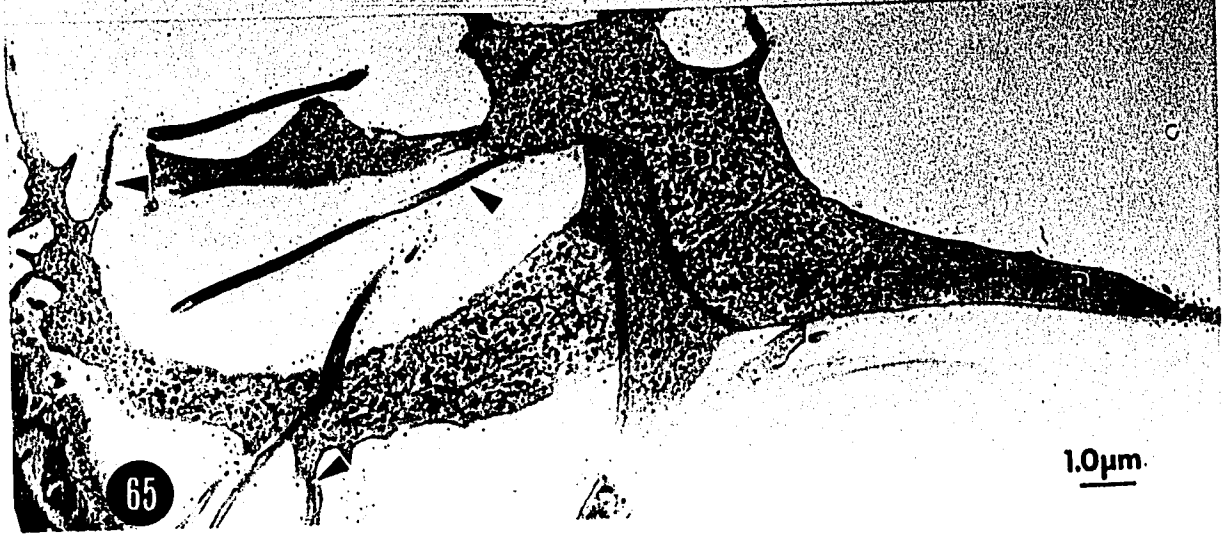

65
1.0µm

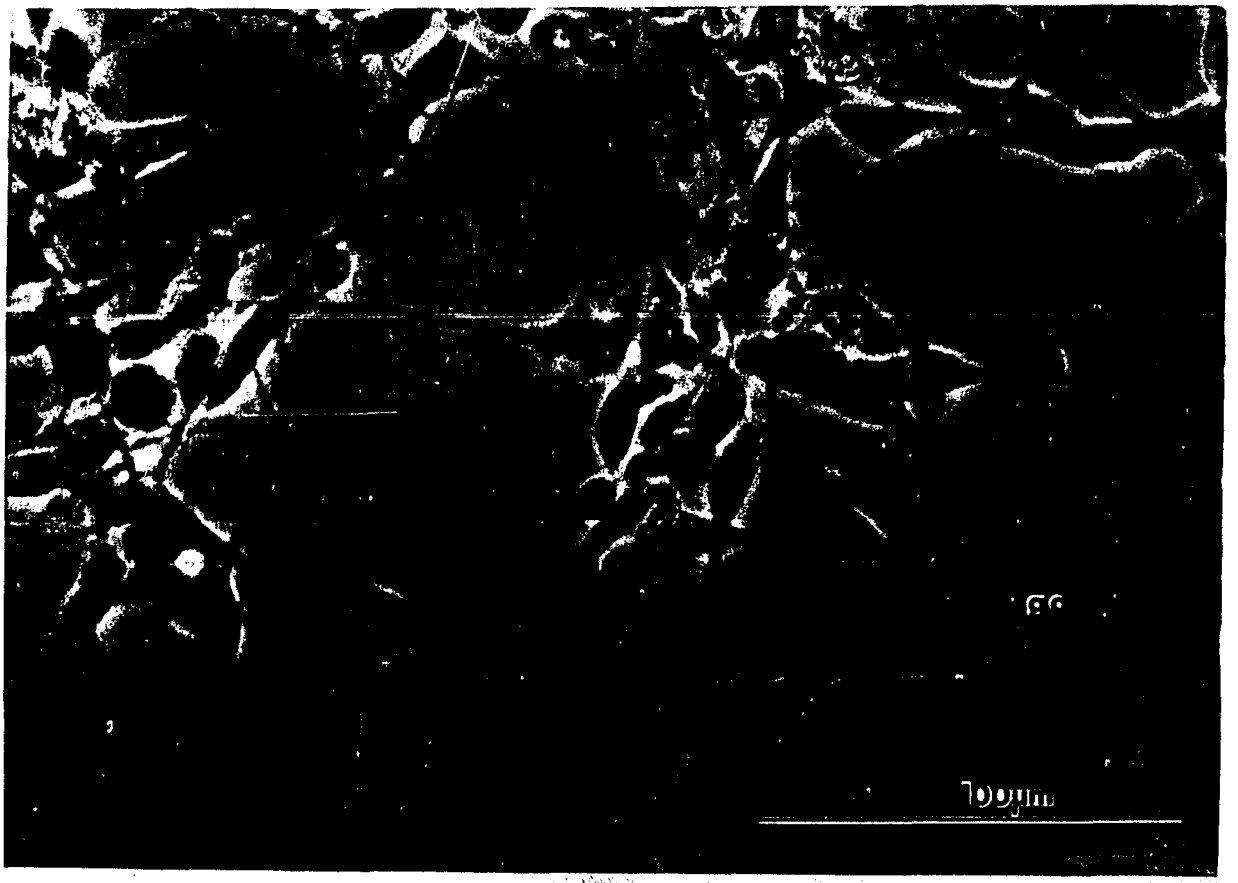
PLATE XXVI.

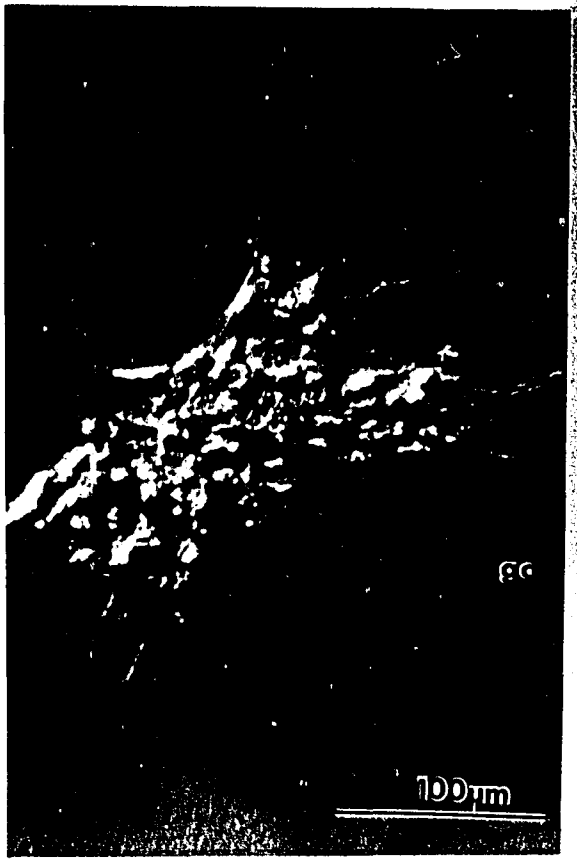
Figure 66. N2 NGF. Phase micrograph of living SY5Y cells. In N2 NGF medium there is a strong tendencey for the cells to form aggregates. The cells are more rounded in appearance than in FCS NGF with fewer processes emering per cell. x520.

Figure 67. N2 NGF. Differential interference micrograph of glutaraldehyde fixed, air dried cells. This micrograph illustrates the tendency for cells to clump together leaving large areas of the culture substrate free of cells. In the smaller clumps, such as this, many cells may be in contact with the substrate. x260.

Figure 68. N2 NGF. Differential interference micrograph of a large clump of cells. In this clump the cells are highly compacted and many are apparently adherent only to other cells. x260.

Key: gc growth cone


gc
100μm


gc
100μm


100μm

PLATE XXVII.

Figure 69. Growth of SY5Y near scratches on the culture substrate. Phase contrast pictures of SY5Y near scratches {S} on the substrate reveal that except in N2 NGF (E, F) cells and processes grow very close to the scratch (illustrated with arrowheads). The 50 um border area excludes cell bodies in N2 NGF but not cell processes. All pictures 170x, bar = 100um.

Figure 69A. FCS.

Figure 69B. N2.

Figure 69C. F12/DME.

Figure 69D. FCS NGF

Figure 69E. N2 NGF. Dense region of plate.

Figure 69F. N2 NGF. A sparser region of the same plate as 69E. A few cells do approach the scratch near the end of the mark.

Figure 69G. FCS CAMP.

Figure 69H. N2 CAMP.

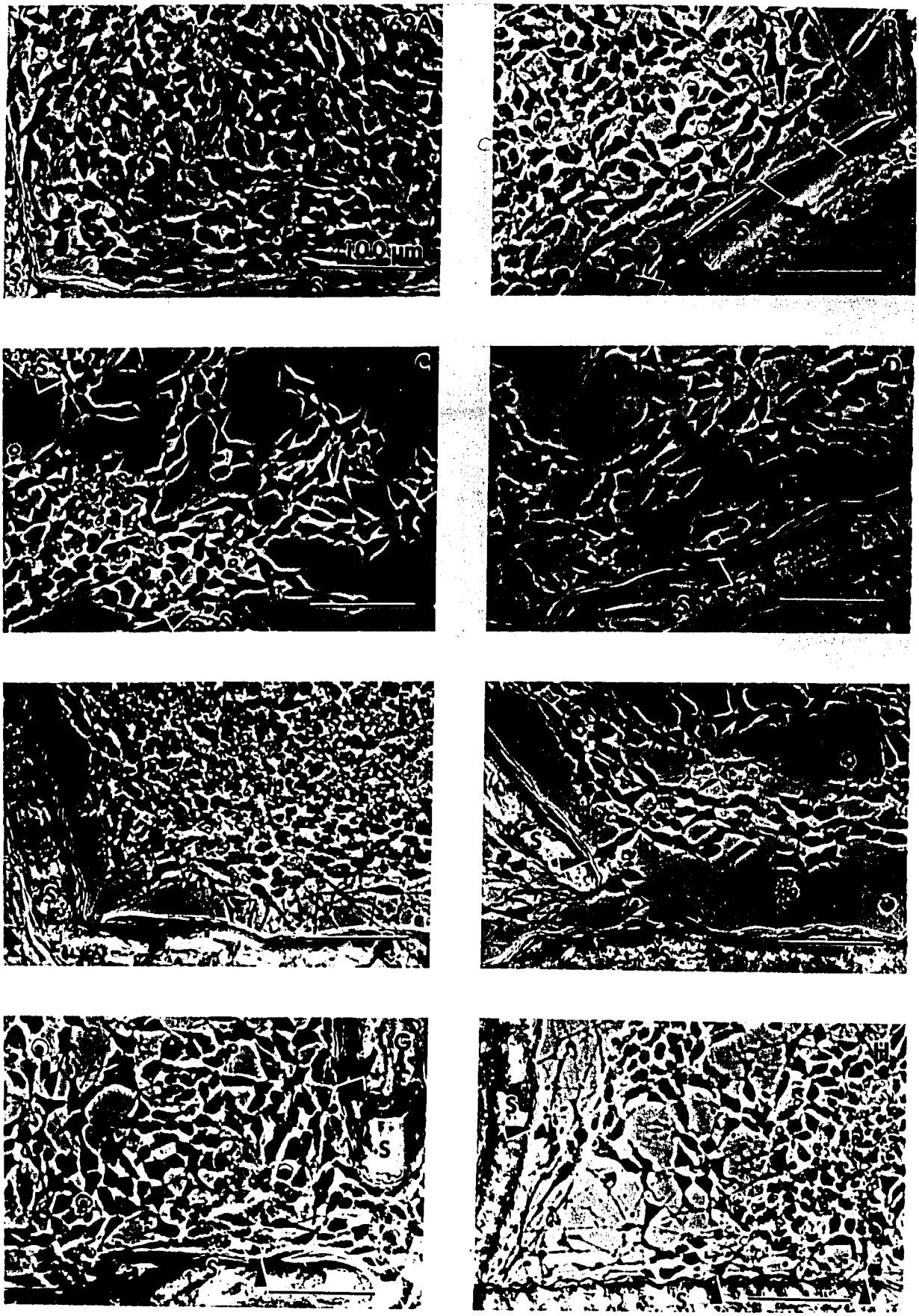

A
100μm
B
C
D
S

PLATE XXVIII.

Figure 70. N2 NGF. Cell {A} has an eccentric nucleus but little rim chromatin. The cytoplasmic components are compartmentalized as under other conditions, but the cell is unusually rich in rough endoplasmic reticulum. Cells {B} and {C} are more typical of the cell population and more like cells in other conditions. x7,000.

Figure 71. N2 NGF. The area of rough endoplasmic reticulum from cell {A} figure 70. Note the absence of organelles other than rough endoplasmic reticulum and ribosomes. 23,500x.

Key:

g	golgi complex(es)
Nu	nucleus
rer	rough endoplasmic reticulum


A
B
C
1.0μm
70

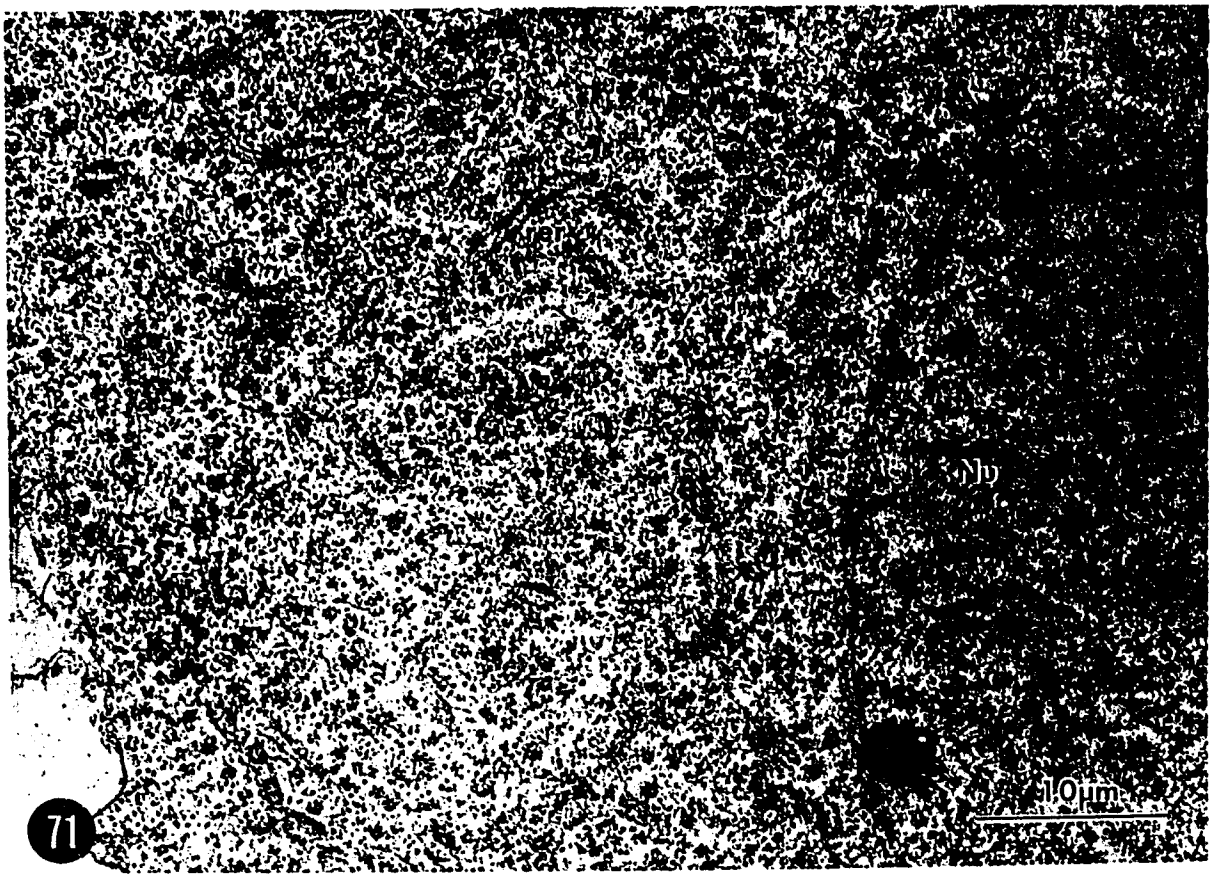

Ny
1.0μm
71

PLATE XXIX.

Figure 72. N2 NGF. This process has a typical distribution of organelles. Note the variation in organelle content from area to area. x23,500.

Figure 73. N2 NGF. A typical process. x23,500.

Figure 74. N2 NGF. A typical process enriched in membranous components. x23,500.

Figure 75. N2 NGF. A typical process. x23,500.

Figure 76. N2 NGF. This process, next to a cell body, is enriched in 10nm intermediate filaments. x23,500

Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
mvb	multi-vesiclular body
ser	smooth endoplasmic reticulum

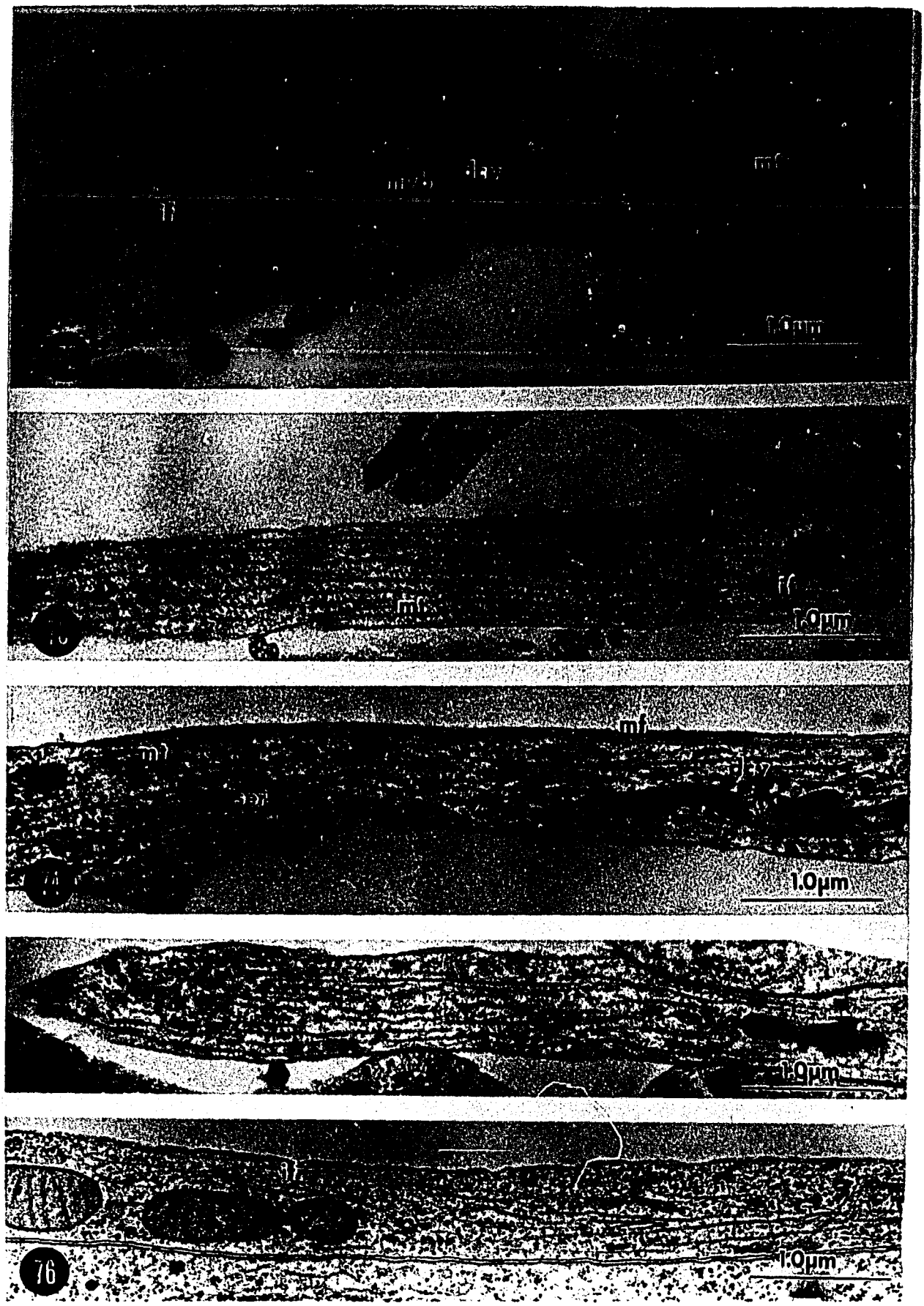

1.0μm
1.0μm
1.0μm
1.0μm
76

PLATE XXX.

Figure 77. N2 NGF. The tip of a process exhibiting all the features of a neuronal growth cone. Process {P} expands into a region of smooth endoplasmic reticulum and dense cored vesicles. Further out is a mound structure filled with clear vesicles. The expansion ends in several microfilamentous filopodia {arrowheads}. The dark smudge in the center of the field {..} is the probably the interface of a cell or growth cone and the culture dish x23,500.

Key:

clv	clear vesicle(s)
dcv	dense cored vesicle(s)
M	mound of clear vesicles
P	process
ser	smooth endoplasmic reticulum
..	culture substrate

77

PLATE XXXI

Figure 79. FCS CAMP. Phase contrast micrograph of living SY5Y cells. The cells appear much as in FCS (compare fig. 8), except the processes appear to be straighter and thinner. x520.

Figure 80. FCS CAMP. Differential interference micrograph of glutaraldehyde fixed, air dried cells. As in FCS most of the cell are classified as `polymorphous' in shape. Cells displaying round, unipolar and equilateral triagular morphologies are also present in this field. x260.

Key:
- e `equilateral' cell
- r `round' cell
- u `unipolar' cell


100μm


100μm

PLATE XXXII.

Figure 81. FCS CAMP. Typical cells. The cells in FCS CAMP exhibit eccentric nuclei with one or several nucleoli and a dark rim of chromatin at the nuclear envelope. The cytoplasm is compartmentalized into an area rich in mitochondria and golgi while other areas exclude these organelles. A centriole is located some distance from the nucleus. 7,100x.

Figure 82. FCS CAMP. This portion of the figure 81. (sorry, it's mirror image reversed) displays the richness of organelles near the nucleus. x23,500.

Key:

arrow	rim chromatin
c	centriole
dcv	dense cored vesicle(s)
g	golgi complex(es)
Nu	nucleus
ps	polysome
rer	rough endoplasmic reticulum
ser	smooth endoplasmic reticulum
*	close juxtaposition of nucleus and plasmalemma

81

82

PLATE XXXIII.

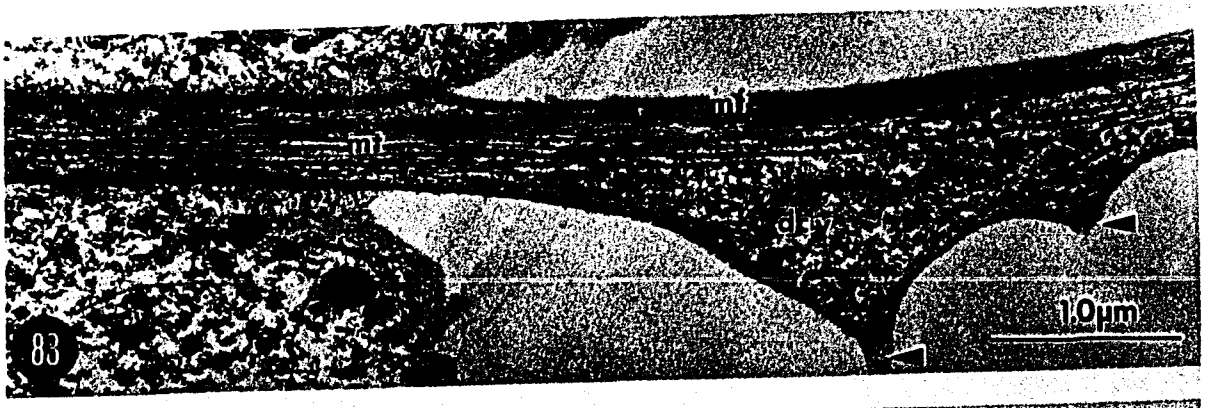
Figure 83. FCS CAMP. This process emerges from under another cell and displays an expanded area which probably extends into filopodial like regions {arrowheads}; The expansion is enriched in ribosomes and membranous organelles while microtubules and microfilaments continue uninterrupted on the other side. x23,500.

Figure 84. FCS CAMP. This process is enriched in intermediate filaments in its center and microfilaments under the process plasmalemma. x23,500.

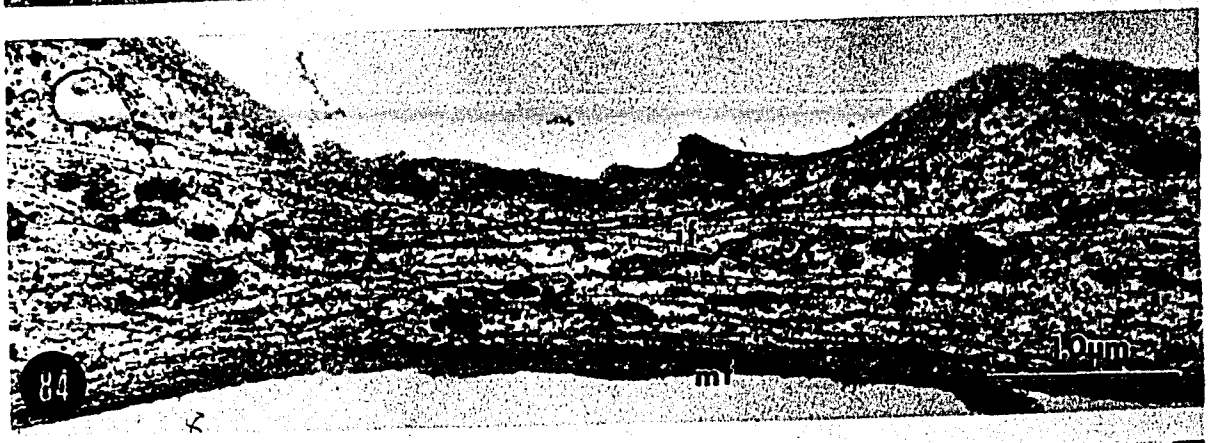
Figure 85. FCS CAMP. This typical process expands to form the structure seen in figure 90. x23,500.

Figure 86. FCS CAMP. On the left the process expands to exhibit an area enriched in ribosomes but free of microtubules and microfilaments. This may be a tangential section through an area like the lower area of figure 83. x23,500.

Figure 87. FCS CAMP. This process is filled mainly with membranous organelles and microfilaments and is notable for a string of clear vesicles located just subjacent to the plasma membrane. x23,500.



83



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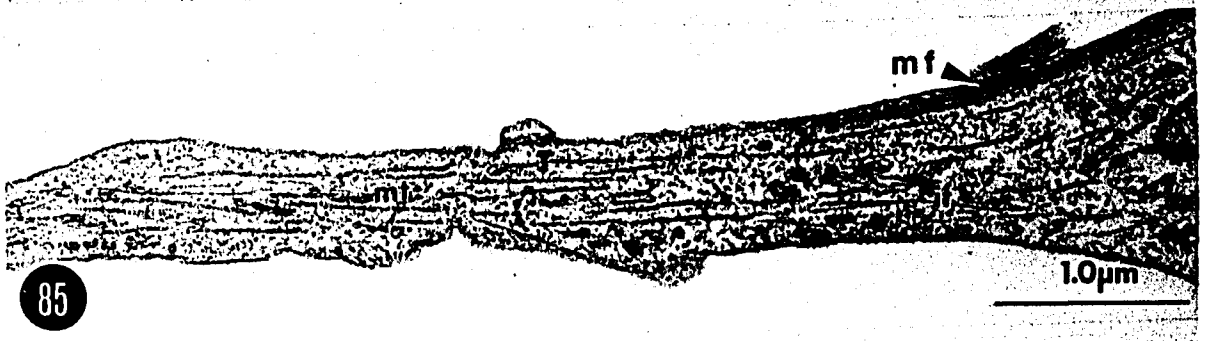
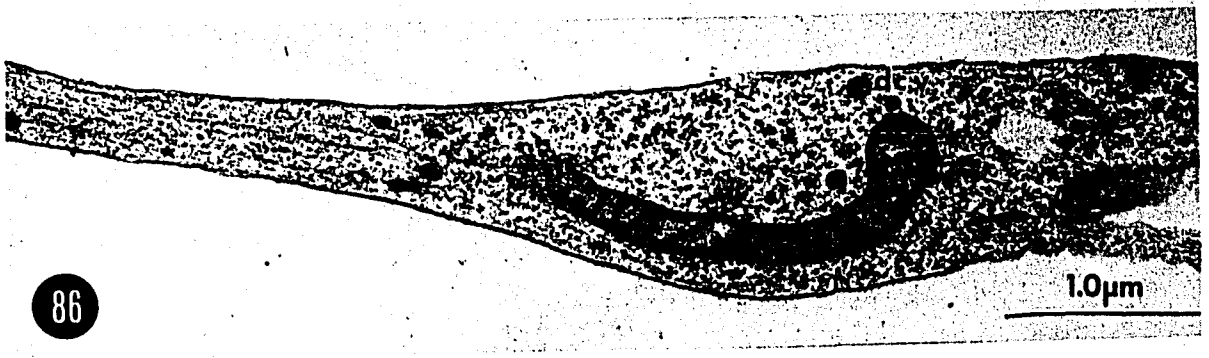
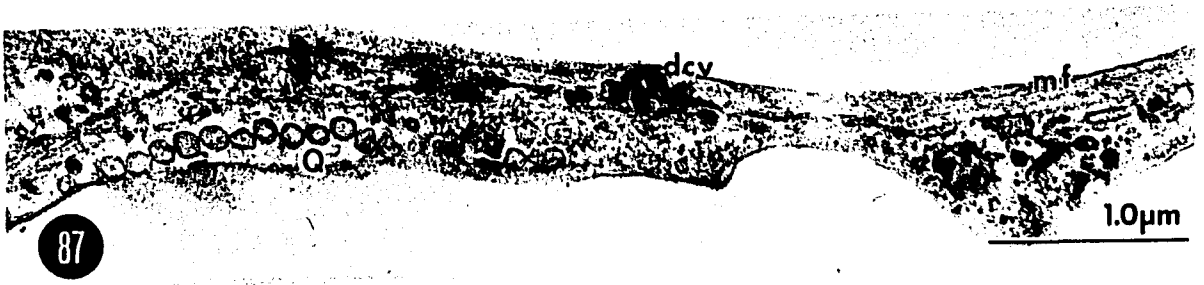


85



86



87

PLATE XXXIV.

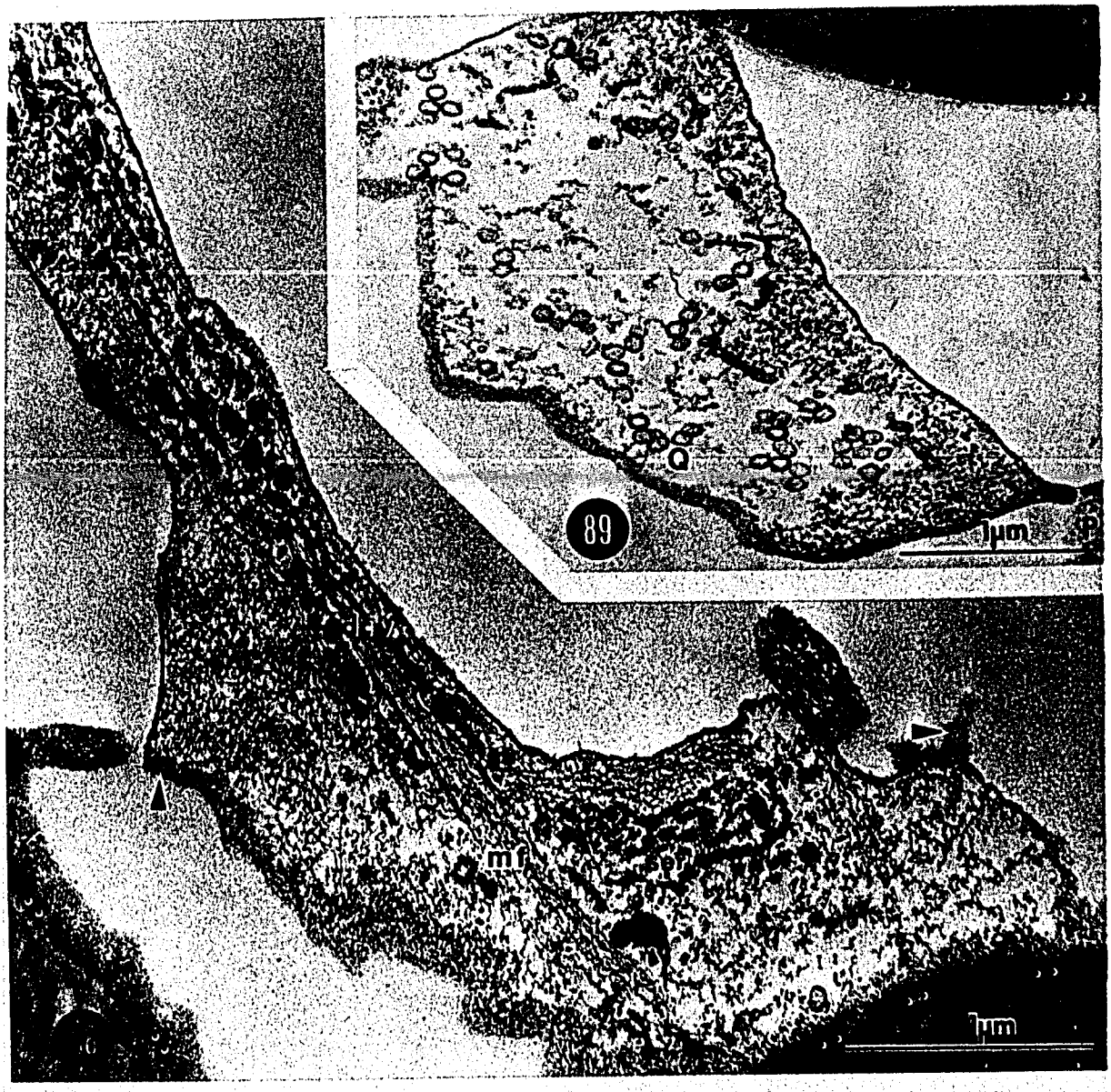
Figure 88. FCS CAMP. The tip of a process. the aligned microfilaments running the length of the growth cone are unusual. This section is extremely close to the substrate as seen by the proximity of culture dish plastic {..}. The arrowheads indicate areas which may lead into filopodia, x34,100.

Figure 89. FCS CAMP. The tip of a process. A process {P} expands into several directions not shown here. In this plane an area free of most cytoplasmic staining contains lines of clear vesicles and wispy material associated with microfilaments. 24,300x.

Figure 90. FCS CAMP. Probably not the tip of a process. This area is continuous with the process in figure 85 and has some of the features of a growth cone; splaying microtubules and intermediate filaments and smooth endoplasmic reticulum, but the microtubules seem to reorganize and continue on at the distal end of the structure {mt'}. x16,000.

Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
P	process
Q	lines of clear vesicles


Q
89
1µm
mf
1µm

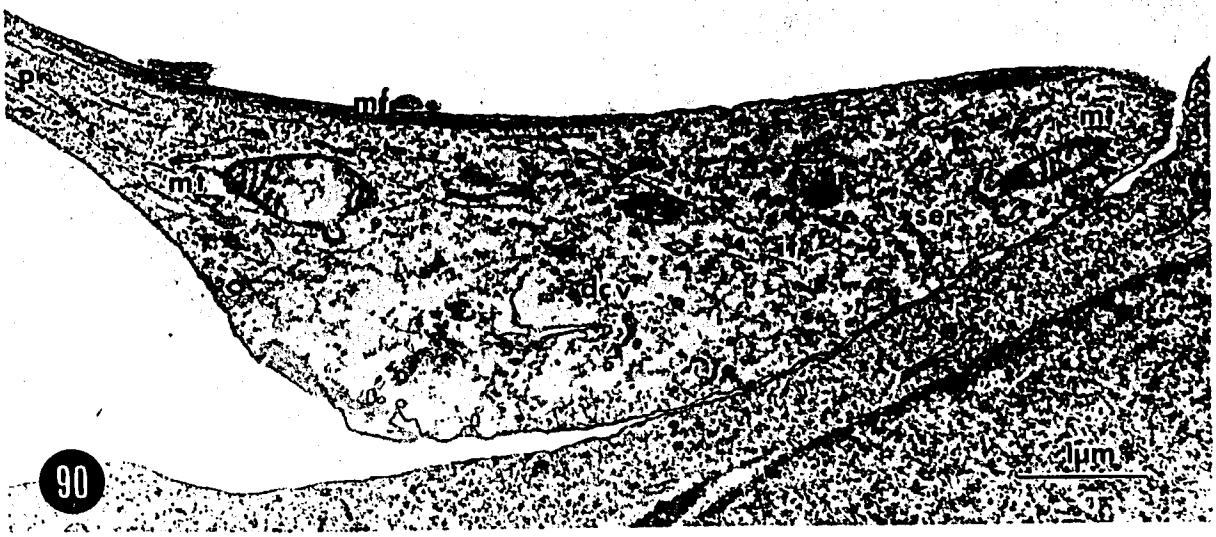
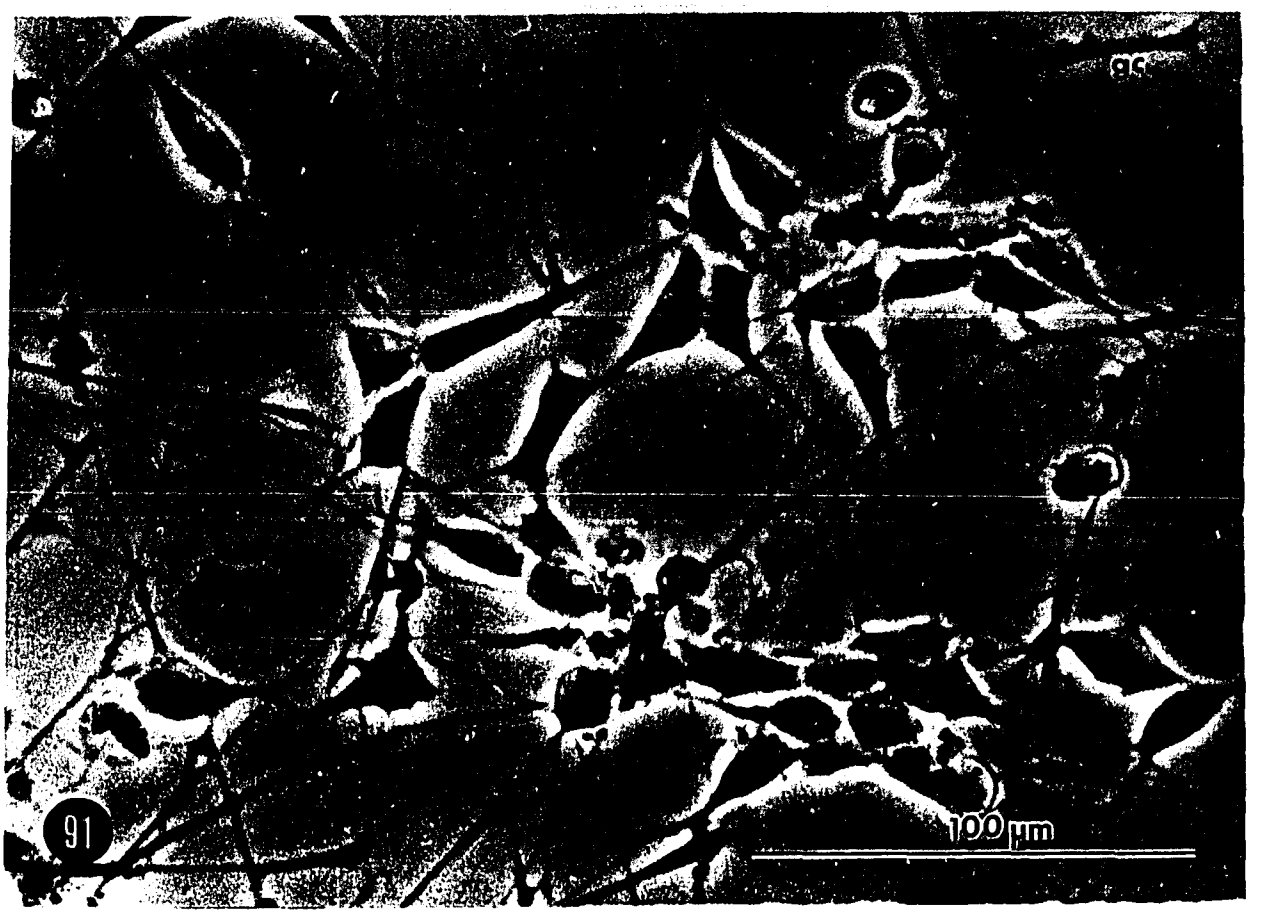

mf
mf
mf
ser
dcv
90
1µm

PLATE XXXV.

Figure 91. N2 CAMP. Phase contrast micrograph of living SY5Y cells illustrating the extensive network of processes in N2 CAMP medium. Some cells appear rounded and small clumps are seen, but neither is as accentuated as in N2 NGF (compare fig 66, 67, 68). x520.

Figure 92. N2 CAMP. Differential interference micrograph of glutaraldehyde fixed, air dried cells. All the cells in this field exhibit a 'polymorphous' morphology. x260.

Key: gc growth cone


91
100 µm


100 µm

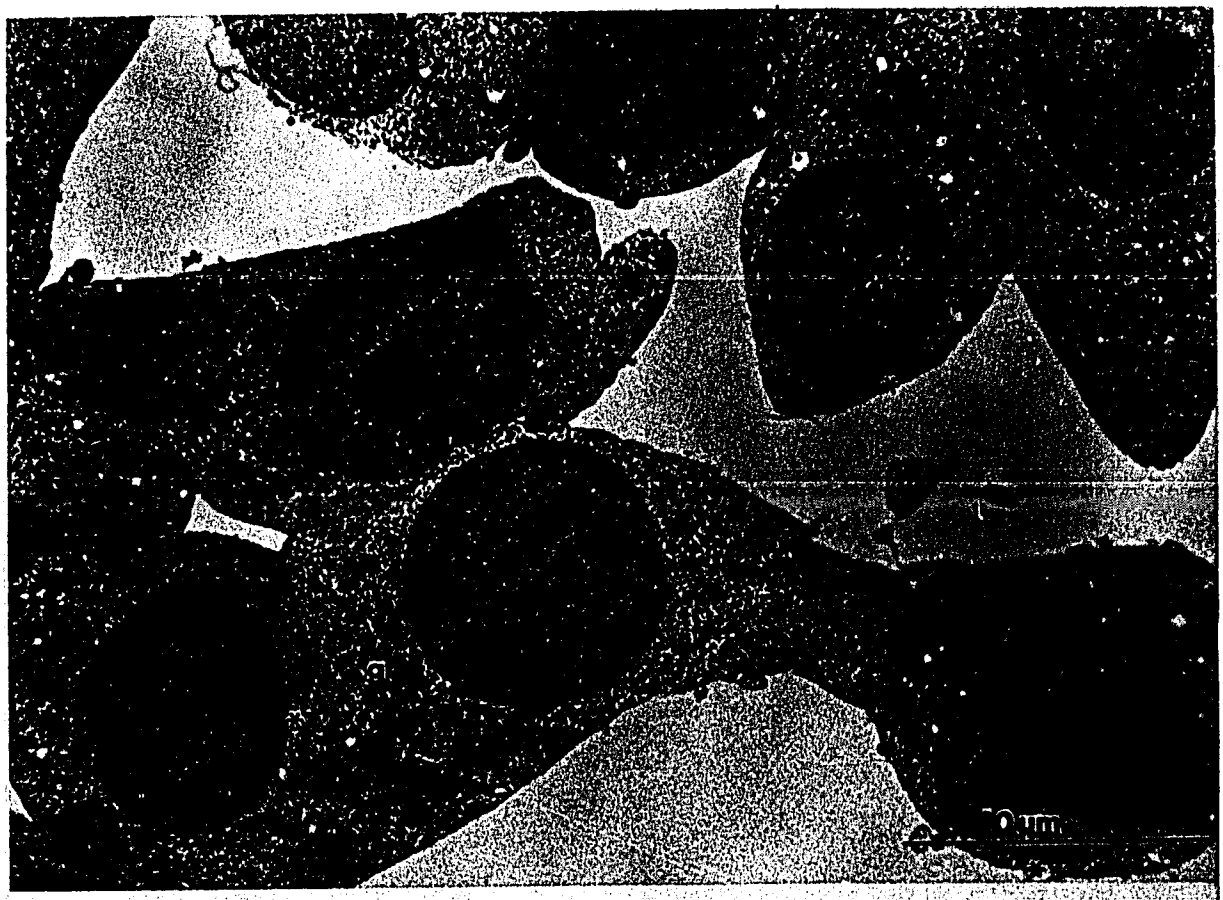
PLATE XXXVI.

Figure 93. N2 CAMP. Overveiw of SY5Y ultrastructure. In N2 CAMP medium the cells have all the characteristics displayed under other conditions. x3,800.

Figure 94. N2 CAMP. The areas rich in golgi and mitochondria adjacent to the nucleus also contain many other organelles. x23,500.

Key:

dcv	dense cored vesicle(s)
g	golgi complex(es)
Nu	nucleus
r	ribosomes
rer	rough endoplasmic reticulum
*	close juxtaposition of nucleus and plasmalemma



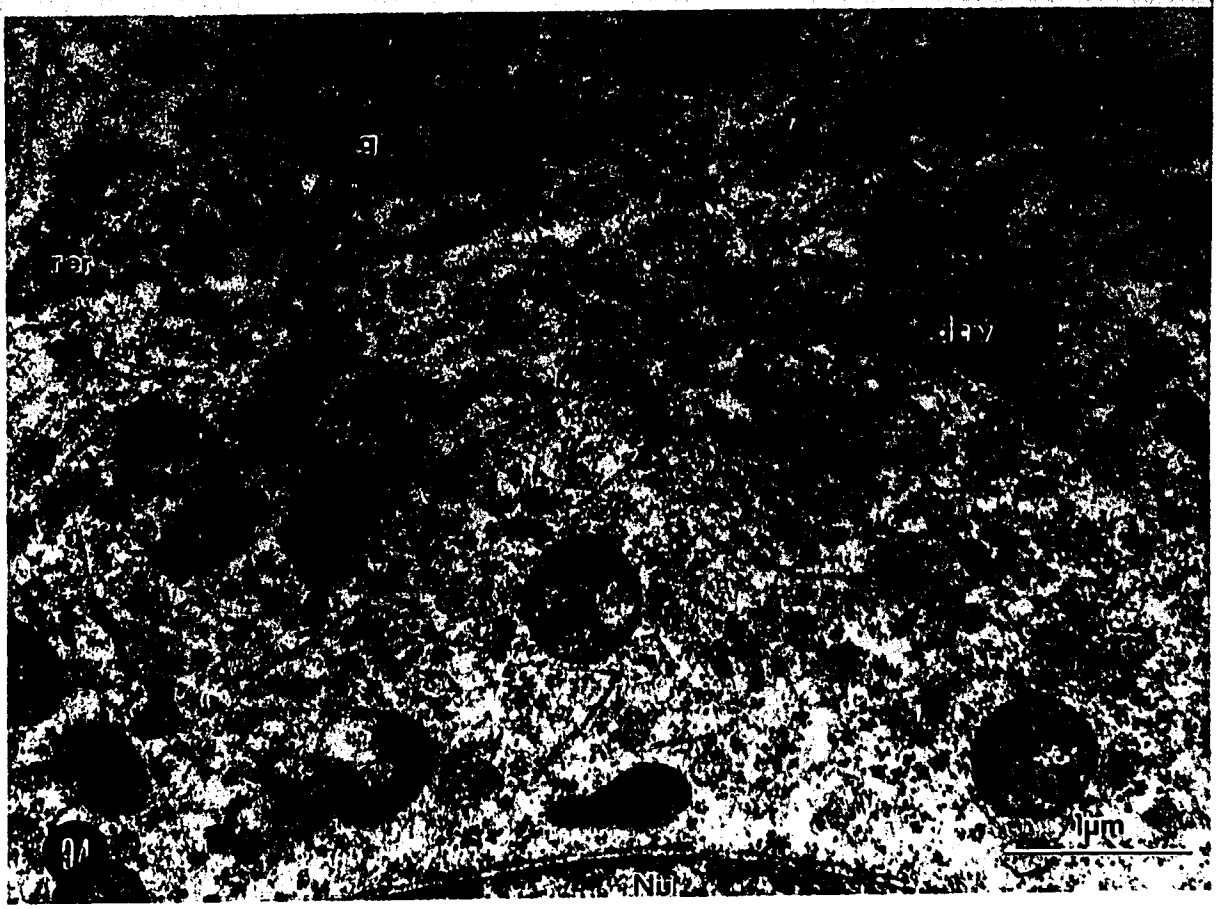


PLATE XXXVII.

Figure 95. N2 CAMP. This typical process is composed mostly of microtubules with a few intermediate filaments in the center. x23,500.

Figure 96. N2 CAMP. This process is enriched in 10nm intermediate filaments with only a few scattered microtubules. x23,500.

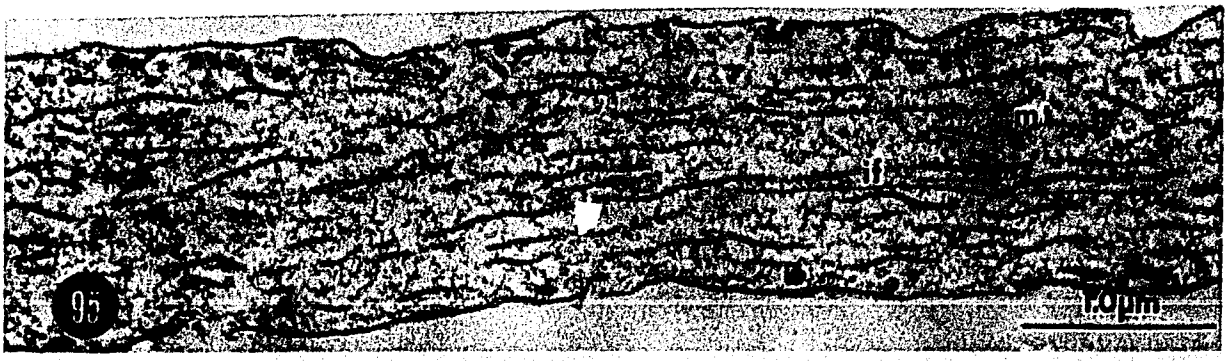
Figure 97. N2 CAMP. Microtubules are very closely packed in this process. Note the numerous ribosomes. 23,500x.

Figure 98. N2 CAMP. This typical process displays both microtubules, intermediate filaments and microfilaments. x23,500.

Figure 99. N2 CAMP. Dense cored vesicles and ribosomes are enriched in this process profile, especially in the side projections. x23,500.

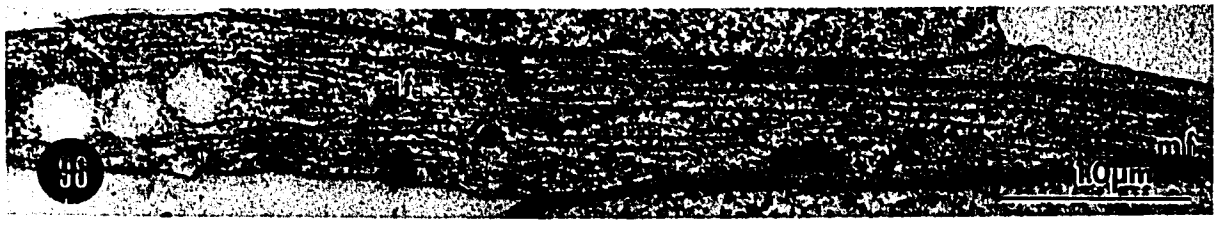
Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
mf	microfilament(s)
mt	microtubule(s)
mvb	multi-vesiclular body


95
mf
ij
1.0μm


96
dcv
mvb
ij
mf
1.0μm


97
mf
ij
dcv
1.0μm


98
mf
1.0μm

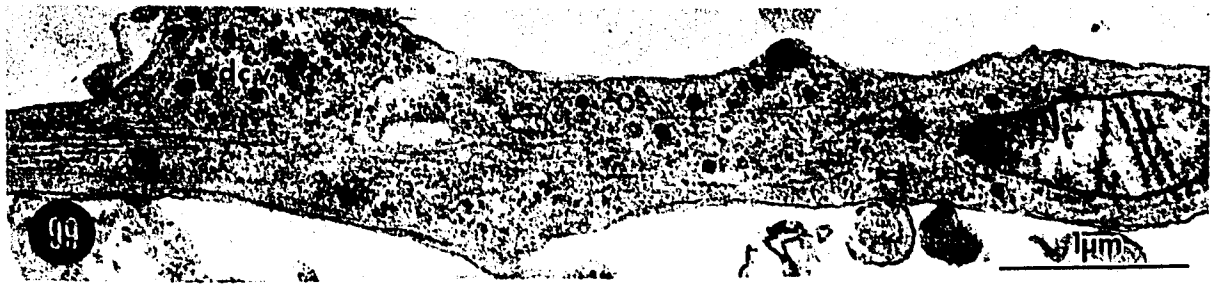

99
dcv
1μm

PLATE XXXVIII.

Figure 100. N2 CAMP. This extensive structure at the end of a process {P} displays all the characteristics of a neuronal growth cone. There is a tangle of intermediate filaments at the beginning of the expansion which is magnified in figure 102. x7,000.

Key:

arrow	filopodium
clv	clear vesicles
dcv	dense cored vesicle(s)
if	intermediate filament(s)
P	process
ser	smooth endoplasmic reticulum


P
100
1µm

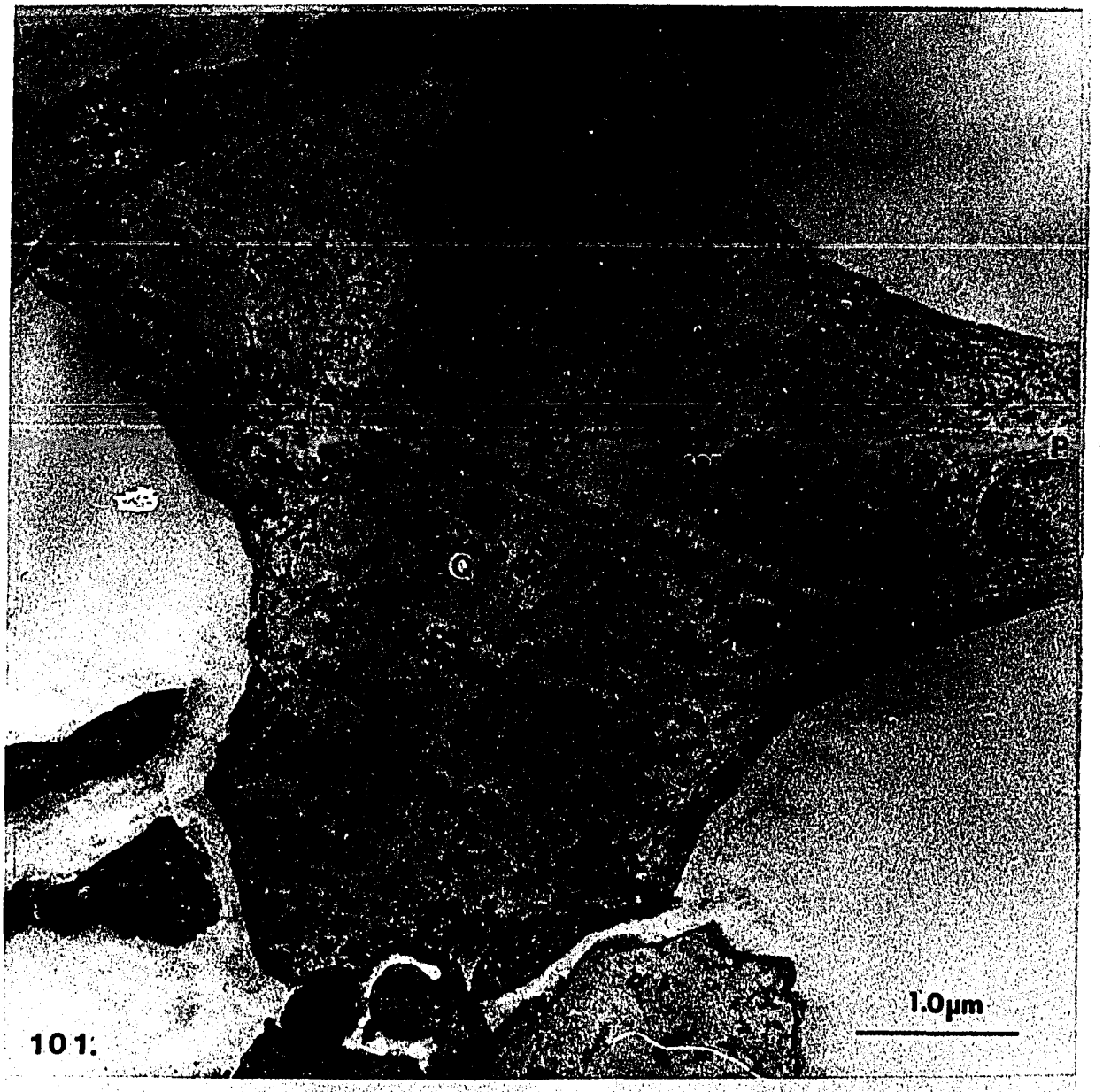
PLATE XXXIX.

Figure 101. N2 CAMP. This growth cone-like region displays a number of clear vesicles arranged in queues and a tangle of intermediate filaments. x22,600.

Figure 102. N2 CAMP. The proximal region of the growth cone in figure 100 where the process enters the growth cone is dominated by a tangle of intermediate filaments x23,500.

Key:

dcv	dense cored vesicle(s)
if	intermediate filament(s)
P	process
Q	lines of clear vesicles
ser	smooth endoplasmic reticulum



101.

102.

DISCUSSION

The studies on SY5Y are centered on three general topics: 1. An evaluation of the suitability of SY5Y as a model system for the study of neuronal morphogenesis. 2. The effects of nerve growth factor on the growth and morphology of SY5Y. 3. The relationship between the NGF effects and those of increased levels of cAMP.

SY5Y AS A MODEL OF SYMPATHETIC DEVELOPMENT

The SY5Y clone has a number of features in common with other neuroblastomas and with sympathetic ganglion cells [Beidler et al., 1978; Perez-Polo et al., 1979; Ross et al., 1980; Kuramoto et al., 1981].

As shown in this study, SY5Y cells have much the same ultrastructure as developing sympathetic neurons. The bodies of SY5Y cells contain all the organelles of sympathetic neurons, but they are more like embryonic than mature neurons. Eranko [1972] has classified developing rat sympathetic cells into three continuous categories: early sympathicoblasts, late sympathicoblasts and young sympathetic nerve cells. The SY5Y cells, under most conditions studied, resemble most closely the early and late sympathicoblasts. The nuclei of human sympathetic cells, corresponding to early sympathicoblast stage, exhibit the same close juxtaposition to the plasma membrane as SY5Y, and the distribution of chromatin into unevenly dispersed clumps with an irregular rim of chromatin at the nuclear membrane is characteristic of early stages of development in human ganglion cells [Hervonen et al., 1978] as well as SY5Y.

The quantity of individual cytoplasmic components of SY5Y varies among the seven conditions examined. Under most conditions the rough endoplasmic reticulum and the golgi apparatus are present but sparse in comparison to mature neurons. In N2 NGF medium some cells were seen with a more developed rER (figure 70), corresponding to the late sympathicoblast stage or early sympathetic neuron stage of rat [Eranko, 1972]. This response by SY5Y is similar to the response of neonatal sympathetic neurons in vivo after NGF injection, where there is an increase in the amount of rER present [Angelletti et al., 1971]. However, the amount of rER in SY5Y was not nearly equal to the amount seen in the NGF stimulated neurons, or indeed in normal mature neurons. The increased golgi and its presence throughout the somal cytoplasm seen in NGF stimulated neonatal neurons [Angelletti et al., 1971] was not evident in SY5Y.

In F12/DME rER and polysomes were extremely sparse. In addition, there was an apparent increase in lysosomal and myelin vesicles. Combined with the population data, which showed either no growth or declining cell numbers, these observations testify to the unhealthy state of the cells in this medium. The SY5Y cells differ markedly from many C 1300 mouse neuroblastoma clones which respond to serum withdrawal with increased process formation, often refered to as 'differentiation' [Seeds et al. 1970].

Under no condition tested was the cytoplasm filled with the overwhelming quantity of compact rER and golgi seen in normal mature sympathetic neurons [Palay and Palade, 1955; Pick, 1964; Eranko, 1972].

Under all the conditions tested dense cored vesicles ranging in size from 80 to 110 nm in diameter were observed in the somal cytoplasm, processes, and growth cones. The dense cored vesicles correspond to the large dense cored vesicles seen in embryonic stages of development [Eranko, 1972; Hervonen et al., 1978], not the

50 nm in diameter vesicles seen in mature adrenergic nerve terminals [Bondareff, 1965], nor do they conform to the slightly larger and more irregularly shaped dense cored vesicles seen in adrenal medullary cells.

In mature nerve cells dense cored vesicles are well correlated with the presence of catecholamines [Grillo, 1966; Hokfelt, 1969]. SY5Y contains the catecholamine synthesizing enzymes dopamine-β-hydroxylase and tyrosine hydroxylase [Beidler et al., 1978, Carroll, 1982]. However there is some controversy over whether the dense cored vesicles seen in early embryonic sympathetic ganglion cells actually contain catecholamines.

In embryonic human sympathetic ganglia, Hervonen et al., [1978] found numerous dense cored vesicles in the cell body and processes using conventional glutaraldehyde-osmium fixation, but were unable to demonstrate catecholamines in vesicles using potassium permangenate fixation, which preferentially stains catecholamines. Recently, potassium permangenate staining (as well as chromate-dichromate staining) of dense cored vesicles in early human sympathetic ganglia has been demonstrated, although the number of positive vesicles is small [G. D. Zeevalk, personal communication].

Barnes et al. [1981] found little correlation between the reported levels of catecholaminergic neurotransmitter enzyme activity and the occurence of dense cored vesicles in human neuroblastomas. The present study is in agreement with this. In the SY5Y parental line SK-N-SH there are few dense cored vesicles. In SY5Y there are frequent, although not overly abundant, dense cored vesicles, despite levels of dopamine-β-hydroxylase much lower than in SK-N-SH [Barnes et al., 1981; Ross et al., 1980; Carroll, 1982].

The question of the identity of the dense cored vesicles is of particular interest because dense cored vesicles have been reported in

several neuronal types not known to express catecholamines, including dorsal root ganglion cells in culture [Yamada et al., 1971] and regenerating goldfish optic ganglion cells [Lanners and Grafstein, 1980]. The status of the dense cored vesicles in SY5Y is currently being investigated [Carroll and Lyser, personal communication].

The SY5Y cells have neurite-like processes whether NGF or cAMP is added or not. Under all the seven conditions studied the processes are rich in microtubules, with a subplasmalemmal distribution of microfilaments and a dispersed irregular distribution of intermediate filaments. Mitochondria, ribosomes, smooth endoplasmic reticulum, multivesicular bodies, coated vesicles and dense cored vesicles were also observed under all conditions. The components were dispersed much as in both mature sympathetic ganglion cells in vivo (see especially Elvin 1963, figure 2., page 409) and in developing rat sympathetic ganglion cells in vitro [Bunge, 1973] (compare figures 11 and 13 pages 723 and 724). Embryonic human sympathetic ganglion cells in vitro also produce neurites with the same subcellular components [Zeevalk and Lyser, 1982].

Recently, Barnes et al. [1981] in a study of several human neuroblastoma lines including SK-N-SH, the parental line of SY5Y, have argued that the presence of ribosomes in neuroblastoma cell processes suggests that the term `neurite-like' may not be appropriate. Ribosomes are not generally found in the distal portions of axons [Peters et al., 1976] although ribosomal proteins are known to be moved down the axons of mature neurons [Bondy and Purdy, 1975]. However, ribosomes are often seen in the proximal portions of axons [Peters et al., 1976], in developing neurites both in vitro [Bunge, 1973] and in vivo [Tennyson 1970; Lyser, 1964] and in dendrites [Peters et al., 1976]. The neuroblastoma processes could be described as developing neurites, dendrites, or the proximal sections of axons. The presence of ribosomes throughout the processes of SY5Y cells should not be considered abnormal, but rather another characteristic

held in commmon with normal neurons. They are only abnormal if the processes of SY5Y are thought to be mature motor axons.

Processes in SY5Y are tipped with structures which are isomorphic with growth cones seen in vivo and in vitro [Tennyson, 1970; Bunge, 1973]. The 'cône de croissance' was first recognized as a specific structure by Ramón y Cajal [see, 1937]. It was seen, in action, by Ross Harrison in the first neural tube explants.

> "The most remarkable feature of the fiber is its enlarged end from which extend numerous fine, simple or branched filaments. The end swelling bears a resemblance to certain rhizopods and close observation reveals a continual change in form, especially as regards the origin and branching of the filaments. In fact, the changes are so rapid that it is difficult to draw details accurately. It is clear we have before us a mass of protoplasm undergoing amoeboid movements."
>
> [Harrison, 1907].

The most elegant demonstrations of the growth cone structure were provided by studies where living growth cones were observed and photographed in vitro and the same structures then observed in the electron microscope [Yamada et al., 1971; Bunge, 1973; see also Peters et al., 1976, for finer micrograph reproduction]. In these studies the proximal regions of the growth cones show a splaying of the neurite microtubules and neurofilaments into the growth cone, with both elements extending only a relatively short distance into the growth cone expansion. The areas closest to the neurite are rich in all types of membranous organelles, especially tubular smooth ER, but also in small clear, and large, dense cored vesicles. The dense cored vesicles are large, 65-160nm in diameter, but similar in appearance to the vesicles seen in catecholamine releasing cells. They were seen in both sympathetic [Bunge, 1973], and dorsal root ganglion cell growth cones [Yamada et al., 1971]. Coated vesicles, large vacuoles (presumably pinocytotic vesicles), various lysosomal vacuoles and mitochondria are also seen. Ribosomes are present but sparse.

Occasionally present in growth cones are areas near the plasmalemma which contain clusters of clear, irregular vesicles, about 100

μm in diameter. Although structures of this type, 'mounds', are often associated with growth cones [Bunge, 1973; Pfenninger and Bunge, 1974] and have been used as a criterion for the identification of growth cones in vivo [del Cerro and Snider, 1968] they are caused by glutar- aldehyde fixation and are found in other parts of neuronal and non-neuronal cells [Hasty and Hay, 1978; Nuttall and Wessells 1979] and are not necessarily diagnostic for growth cones.

In more distal portions of the growth cone the density of organelles is reduced and the filopodia consist nearly exclusively of elongated meshworks of microfilaments.

This basic structure has been seen in numerous studies with only minor variations [Grainger and James, 1970; Molliver and van der Loos, 1970; Kawana et al., 1971; Hinds, 1972; Hinds and Hinds, 1972; del Cerro, 1974; Skoff and Hamburger, 1974; Rees et al., 1976; Epstein et al., 1980].

The physical compartmentalization of the growth cone may reflect functional differences.

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The physical compartmentalization of the growth cone may reflect functional differences.

The filopodia at the tip of the growth cone are the most active

portion of the organelle and are analogous in many ways to the periphery of other cell types. The microfilaments are rich in actin and myosin [Ishikawa et al., 1969; Burton and Kirkland, 1972; LeBeux and Willemot, 1975; Sanger, 1975; Marchisio et al., 1978; Kuczmarski and Rosenbaum, 1979; Letourneau, 1981], and the knowledge that actin and myosin are present in the growth cone has strengthened the long standing idea that the filopodia are force generating structures, pulling the rest of the growth cone forward.

The filopodia are one of the areas of the growth cone that are closest to the substrate [Letourneau, 1979] and similar areas of close contact on other cultured cells are areas of tight adhesion to the substrate [Harris, 1973]. Extended filopodia which do not make strong contacts with the substrate can be seen to retract, while filopodia making contact with small objects on the substrate can be seen tugging and deforming them towards the growth cone [Nakai and Kawasaki, 1959]. If the formation of microspikes is cyclic, composed of an extension phase followed by a contraction, weak adhesion of the microspike would lead to retraction whereas a strong adhesion would result in the forward movement of whatever cytoplasmic anchor the filopodium has. Direct experimental evidence consistent with this was obtained by Wessells and Nuttall [1978a]. They repeatedly passed a small glass needle under one side of a chick ciliary ganglion cell growth cone, disrupting filopodial contact on that side. The growth cone then turned towards the opposite side. Of course the action of the needle could extend beyond the simple dislodgement of the filopodia to damage of some sort to other growth cone structures.

The region of the growth cone proximal to the filopodia is quite distinct in both structure and function. This region is characterized by accumulations of membranous organelles and is probably involved in the addition of new membrane to the growing tip.

Pfenninger [1980] has followed the incorporation of ^{3}H-glycerol

into cells of the rat superior cervical ganglion, in vitro, with electron microscopic autoradiography. After a 15 minute pulse followed by a short chase, most label is found in the cell body, but after a 60 minute chase label is concentrated at the growth cones. The highest concentration of grains is located over large clear vesicles just below the plasma membrane; vis., the glutaraldehyde induced 'mounds' [Nuttall and Wessells, 1979; Rees and Reese, 1981]. The physical changes brought about by glutaraldehyde fixation may be unmasking a structural inhomogeneity in the plasmalemma, the site where new plasma membrane is being added.

The function of the most proximal region of the growth cone undoubtedly involves the regulation of microtubules and neurofilaments. However, whether tubulin or neurofilament polymerization occurs here, or in the cell soma is unknown [Bray and Gilbert, 1981].

The neuronal growth cone is a highly organized organelle system that plays the major role in the development of axons and dendrites. The functions of testing the environment, mechanically advancing and elaborating the neurite structure are all performed within the growth cone and are reflected in its structure. The presence of structures isomorphic with the neuronal growth cone in the human neuroblastoma line SY5Y is one of the major pieces of evidence leading to the belief that studies of this line can shed light on neuronal development.

There is variety among the SY5Y growth cone profiles observed, but the profiles are all consistent with a structure of splayed cytoskeletal elements at the proximal portions of the growth cone, areas rich in smooth endoplasmic reticulum and other membranous organelles in medial sections and microfilamentous filopodia at the ends.

Overall, based on ultrastructural criteria, the SY5Y cells are directly comparable with early neurons of the sympathetic ganglion. There are no major discernable differences between the two at the

level of the cell body, the neurite and the growth cone.

Another feature of the SY5Y clone which is comparable with normal sympathetic neurons is the survival of SY5Y in the serum-free medium N2 of Bottenstein and Sato [1979]. This medium selectively allows the survival of neurons but is not favorable for the growth of non-neuronal cells [Bottenstein et al., 1979]. SY5Y does not thrive in F12/DME medium alone, but with the five additives of N2 medium the cells survive and multiply (table 2; figure 1). Of course SY5Y cells differ from normal sympathetic cells in continuing to divide while neuronal ganglion cells do not.

The SY5Y clone has several drawbacks as a model system, many of them more technical than theoretical in nature. 1. SY5Y is a slow grower. The doubling time of the cells in serum is about 2 days, under experimental conditions such as N2 it is 3 days. Not only does this make experimentation a lengthly matter, but it also makes the use of potentially toxic drugs difficult. 2. SY5Y cells do not stick well to the culture substrate. The cells become conspicuously less adherent over time in the culture dish, especially in serum-less medium. This forces compromises in experimental design such as restricted feeding schedules and limited growth periods. Treatment of the cells for electron microscopy or histochemistry becomes difficult in later time periods because of loss of cells. 3. The levels of the neurotransmitter enzymes tyrosine hydroxylase and dopamine-β-hydroxylase are low, making sampling and quantitation difficult [Carroll, 1982]. 4. Although the karyotype of SY5Y [Perez-Polo et al., 1979] and neurotransmitter enzyme levels are stable over time [Ross et al., 1980] there is phenotypic variation. In our hands, the ability of one line of SY5Y to respond to NGF was absent after a year of continuous non-selective subculturing. The reason for the lack of NGF response is unclear. 5. Although the cells respond to NGF, the response is stochastic; not all the cells in the population respond to treatments. This makes accurate and extensive sampling of the

population necessary. It is impossible simply to look at one area of the culture dish and determine if there has been a morphological response.

On a broader scale, the use of malignant cells as models of normal behaviour is fraught with difficulties. The SY5Y clone has an abnormal karyotype [Perez-Polo et al., 1979, Biedler et al., 1973], and, although it displays a number of neuronal characteristics, it is descendent from cells which, in fact, did not respond appropriately in the body. Although neuroblastomas may sometimes spontaneously mature into non-malignant cells with many of the characteristics of mature neurons [Cushing and Wollbach, 1927], the causes of the initial malignancy are unknown. Consequently, it is not known how the changes of malignancy influence the responses of the cells to experimental conditions and how these differ from the responses of normal neurons.

Taking this into consideration, however, tumor systems and the SY5Y clone in particular, can be a valuable tool in studing some aspects of development. Any cell has only a limited repertoir of responses at its disposal, and the disturbances of malignancy probably do not create entirely new structures and patterns of behaviour. Although the functional aspects of neurite growth may not necessarily be reflected in cell's ultrastructure, the high correlation between structures seen in normal developing neurons and SY5Y cells, combined with a functional response which mimicks normal development, makes it reasonable to assume that the forces acting to produce processes in SY5Y cells play a role in normal neuronal development.

THE EFFECTS OF NGF ON SY5Y

The SY5Y clone is not unique; other human and murine neuroblastomas have a neuronal fine structure [Barnes et al., 1981; Kataoka et al., 1980; Ross et al., 1975] and the ability to form neurite-like processes [Prasad, 1975]. What makes SY5Y distinctive is its response to nerve growth factor. Although a morphological response by primary

cultures of neuroblastoma to NGF has been reported [Waris, 1973], SY5Y is one of only two established neuronal cell lines to have this ability.

NGF WITH SERUM

The response of SY5Y to NGF is greatly influenced by the presence or absence of fetal calf serum in the medium. In the presence of serum the response is simple. After four days exposure to NGF there are twice as many long (>100 μm) processes as there are in the non-NGF treated controls. There is no difference in the ultrastructure of the cells, the processes, or the growth cones (figures 53 to 65). There is no increase in the number of processes emerging from the cells (table 5) or the number of 25 μm or greater processes (table 4), there is no change in the size of the cells (table 6) and little change in the distribution of cell shapes in the population (table 7) In experiments run concurrently with the experiments done for this thesis, Carroll [1982] has found no increase in tyrosine hydoroxylase or dopamine-β-hydroxylase activities with added NGF. Finally there is no change in the growth rate with added NGF (table 2). The only effect observed is the presence of long processes.

The response of SY5Y to nerve growth factor in serum-containing medium differs in several respects from the responses observed by Perez-Polo et al. [1979]. The morphological response was not quantitated by these workers so that direct comparisons of process length and number are not possible. My observations do confirm however that there is a significant increase in long processes with the addition of NGF.

Perez-Polo et al. [1979] report that the cell bodies of SY5Y in NGF are more rounded and larger than controls. As evidence they present their figure 2E (p. 347, Perez-Polo et al., 1979), which shows a number of highly refractile cells, along with some flatter

cells. In this figure the refractile cells are in fact noticably smaller in diameter than the flattened cells, and no attempt is made to measure the volume of the cells. In a lower magnification picture (figure 2F, p. 347) a large majority of the cells are seen to be flattened cells corresponding to the polymorphous category of cell shape presented in this thesis. Highly refractile cells are seen in FCS NGF (figure 55) as well as in FCS medium (not illustrated). In the present study when the number of cells in six different shape categories is compared in FCS NGF and FCS media, there is no significant difference between the conditions (table 7). When the length, width, length to width ratio, and area of the substrate covered by the cell are compared, again, no differences are discernible. Given the variability of morphology inherent in these cells from area to area in the culture dish, and the quantitative analysis presented here, the assertion that NGF in fetal calf serum medium significantly alters the somal morphology of SY5Y cells cannot be substantiated.

Perez-Polo et al. [1979] report the formation of large aggregates of cells formed by the migration of cells into clumps. These `psuedo-ganglia' were observed by me in this study, however they were infrequent, and the clumping did not include "most cells". This difference in response may well be caused by the differences in culture conditions as discussed below.

The most serious difference from the work of Perez-Polo et al. [1979] is in regard to cellular proliferation. They report that after 24 hours in NGF the SY5Y population is significantly smaller than in non-NGF controls. The incorporation of radioactive thymidine is reduced after only 6 hours exposure to NGF. Under my FCS NGF conditions the growth of SY5Y was identical with FCS controls (table 2).

There are several differences between the conditions of Perez-

Polo et al. [1979] and the conditions presented here. Dr. Perez-Polo's cells were grown in a richer medium, a medium containing 15% fetal calf serum and 85% Ham's F12. The FCS NGF medium used in my experiments is 45% Ham's F12, 45% Dulbecco's modified Eagle's medium (a less rich formulation), and only 10% fetal calf serum. FCS NGF does include, however, the 5 additives of Bottenstein and Sato's [1979] N2 medium. Dr. Perez- Polo's cells received 1 μg/ml 2.5S NGF, as opposed to 500 ng/ml 7S NGF or 100 ng/ml 2.5S NGF in my experiments. Finally Dr. Perez-Polo's cells were fed every 2 days as opposed to only once at time 0 in my own case.

Using the direct line of SY5Y from which my own cells derive Sonnefelt and Ishii, [1982] found that concentrations of 2.5S NGF up to 2 ug/ml do not inhibit cell proliferation, and that process extension reachs a maximum with 50 ng/ml of 2.5S NGF. This indicates that the differences observed between Dr. Perez-Polo's cells and my own are probably not due to the levels of NGF used. The effects of NGF on population growth in Dr. Perez-Polo's hands can be seen after 24 hours, the short time course of this effect ruling out the possible degradation of NGF over time as a possible cause of the lack of growth control. My growth curves do not show any difference from controls at 48 hours, the time of the first feeding in Dr. Perez-Polo's experiments, although the morphological effect is seen at day 4.

Although definitive comparisons have not been made in regard to the NGF effect in different labs, it is possible that three phenotypic varients of SY5Y exist, 1, cells which do not respond to NGF with morphologic or growth changes [Lyser et al., 1980], 2, cells which have only a morphologic response [this thesis, Sonnenfelt and Ishii, 1982], and 3, cells which respond with both morphologic and population growth responses [Perez-Polo et al., 1979].

The responses of SY5Y to NGF differ from the responses of the rat pheochromocytoma cell line PC12. In serum containing medium PC12

cells have a rounded morphology with only short cell extensions emerging; and making contact with the substrate [Greene and Tischler, 1976; Luckenbill-Edds et al., 1979]. When NGF is added to the medium the cells extend long branching processes. These processes have been well characterized on an ultrastructural level and are in general similar to neuronal processes in embryos. This neuronal morphology is not seen in cells which do not receive NGF, and it appears only after several days exposure to NGF.

This is not what happens with SY5Y cells. Whether NGF is present or not, most cells are well spread on the substrate and extend neurite-like processes. The increased number of long processes observed in FCS NGF medium is not the result of a new type of process emerging, the ultrastructure of processes in FCS and FCS NGF is identical. It is not due to an increased sprouting of processes by the cell, the average number of processes emerging from the cell is the same in both conditions as is the percentage of cells with processes exceeding 25 μm. The increase is due exclusively to the lengthening of processes already present on the cells.

The production of processes in PC12 is dependent on RNA synthesis. When PC12 cells are exposed to NGF and RNA synthesis inhibitors at the same time, no processes emerge. If PC12 cells are grown in medium with NGF for a week or two, are allowed to extend processes, and are then dissociated from the culture dish so that the processes retract, when they are replated they will immediatly extend processes in NGF medium whether RNA synthesis inhibitors are present or not. Replated in medium without NGF these cells do not extend neurites. The previous exposure to NGF allows the accumulation of an RNA synthesis dependent product, which will then permit the cells to respond to NGF without additional RNA synthesis. This 'priming' of the cells gradually disappears over time. If cells are primed, replated, then left without NGF for increasing lengths of time the cells become less and less responsive to added NGF in the presence of

RNA synthesis inhibitors. The effects of NGF on PC12 is thus twofold: there is an initial step which is transcription dependent, and a second expressive step which utilizes the RNA synthesis product in the formation of neuronal processes [Burstein and Greene, 1978; Greene and Shooter, 1980].

Process extension by SY5Y in the presence of RNA synthesis inhibitors was not tested, however, it is clear that the 'priming' action of NGF is not required for the expression of processes by SY5Y; neurite-like processes form with and without added NGF. In this respect SY5Y and other neuroblastomas [Prasad, 1972; Schubert et al., 1971; Seeds et al., 1970] are similar to normal neurons. In vitro, the extension of neurites by sympathetic, and sensory neurons can occur in the presence of actinomycin D [Partlow and Larrabee, 1971; Mizel and Bamberg, 1976; .

The ability to produce processes is one of the primary diagnostic characteristics of the neuroblastomas [Wright, 1910], and the ability to form processes spontaneously, in culture, without added NGF is a characteristic of many, if not all, primary cultures of human neuroblastomas [Murray and Stout, 1947; Reynolds et al., 1981]. Similarly, there is an apparent NGF-independent production of processes by early embryonic sympathetic explants. Mouse sympathetic ganglion cells from embryonic day 14 can survive for several days in culture without added NGF. During this time the cells extend processes much the same as do later explants in the presence of NGF [Coughlin et al., 1977]. Although the question of regeneration of processes versus the initiation of processes is raised in this experiment, cells isolated from the neural crest at the time of neural crest migration also produce processes with a neuronal ultrastructure, in vitro, without added NGF [Sieber-Blum and Cohen, 1980].

The environment of the ganglion in the embryo is complex and it is not known to what extent the cells are exposed to NGF before

explantation [Thoenen and Barde, 1980]. So it is not known if the cells are 'primed' by endogenous NGF and then express processes in the permissive environment of the tissue culture dish. Neither is it known whether the requirement of NGF for neurite extension by PC12 is representitive of neurite elongation by all NGF sensitive cells or is an aberration of this particular pheochromocytoma. Curiously, the cells of the normal counterpart of PC12 , the adrenal medulla, which normally do not send out processes, will elaborate long neurite-like processes in culture in response to NGF [Ziegler and Unsicker, 1981].

SY5Y cells are similar to PC12 in the dissociation of process elongation and population growth. Although Greene and Tischler [1976] reported a withdrawal from the cell cycle concomitant with process extension, Gunning et al. [1981a] find that process extension occurs without population slowing, with the cells able to incorporate radioactive thymidine and extend processes at the same time.

NGF WITHOUT SERUM

Without serum in the medium, but with the five additives of N2 medium needed for survival, a new spectrum of responses to NGF is revealed. Carroll [1982] has found no increase in SY5Y of the neurotransmitter enzymes tyrosine hydroxylase and dopamine-B-hydroxylase in N2 NGF medium. The ultrastructure of the processes and growth cones in N2 NGF is the same as in N2 alone (figures 66 to 77), and the cell size is the same (table 6). However, in every other parameter measured, the cells respond differently to NGF in N2 than they do in FCS.

Unlike cells in FCS NGF cells in N2 NGF exhibit a decline in the rate of population growth (fig. 2) , but no increase in long process production (table 3) compared to controls. There is also a dramatic change in the distribution of cell shapes (table 7) which results from a large degree of cell clumping (fig. 68).

In N2 NGF medium some cells exhibited an increase in the amount of rough endoplasmic reticulum and a diminution of the amount of rim chromatin (figure 70 cell [A]). These changes are consistent with a more mature sympathetic cell type [Eranko, 1972; Hervonen et al., 1978]. Whether such changes would continue over time to produce mature looking cells has not been determined. The vast majority of cells in N2 NGF do not exhibit this change.

In N2 NGF the cells display the ability to differentiate between areas of the substrate (fig. 69); the cells will not adhere to an area about 50 μm wide adjacent to scratches in the substrate. Cells in N2 medium display some tendency to avoid this area (fig. 69B, but there is a pronounced avoidance in N2 NGF. The effect of NGF on cell bodies is different from its effect on growth cones. This is evidenced by the differential adhesion of the two near the scratches; the growth cones in N2 NGF do not avoid this area.

Differences between cell body and growth cone are not surprising given the differences in function of the two, but many experimenters have failed to recognize this possibility (for example see Marchase, 1977; and Gottlieb and Glaser, 1980). Recently Krystosek and Seeds [1981] , have observed differences between the growth cones and cell bodies in the expression of fibrinolytic activity in many but not all of the cells of a C1300 neuroblastoma clone. Concanavalin A will bind to the cell bodies but not the neurites of other C1300 cells [Denis-Donini and Augusti-Tocco, 1980]. The work with heart conditioned medium by Collins [1978] also illustrates differences in adhesion; ciliary ganglion cell bodies can adhere to the substrate without the neurite adhesion factor present. The behavior of SY5Y cells in N2 NGF is, I believe, the first observation of a difference in the adhesion of the cell and growth cone under conditions in which both can adhere to the substrate.

The exact nature of the substrate and the changes produced by the

scratch were not pursued. Although the exact mechanism in the production of 'tissue culture' plastic is proprietary and known only to the manufacturers, the plastic is believed to carry more of an electrostatic (negative) charge than 'bacteriological' plastic Petri plates [Martin and Rubin, 1974]. The scratch marks disrupt the substrate directly, but may also alter the electrostatic nature of the plastic in a 50 μm swath on both sides.

THE RESPONSE OF SY5Y TO NGF

Fetal calf serum acts to obscure some of the actions of NGF while it allows the expression of others.

If one assumes that the immediate actions of NGF on SY5Y are the same in both FCS NGF, and N2 NGF medium, then NGF can be seen to act at three distinct loci:

1. NGF acts on the growth cone. In serum containing medium only process elongation is affected. There are no observable changes in the cell somal morphology, including the number of processes per cell, and there is no change in process or growth cone ultrastructure. Therefore, the effect on process elongation most probably reflects actions at the level of the growth cone which lead to increased growth of already present processes.

2. NGF acts on the cell body. Without serum, NGF acts to alter the adhesion of the cell body, increasing cell-cell adhesion leading to the formation of large cell clumps. The adhesion of the cell bodies to the substrate is different from the adhesion of growth cones to the substrate. In N2 NGF the growth cones are capable of growing very close to scratch marks drawn on the culture substrate while the cell bodies preferentially grow 50 μm or more from the scratch (figure 69,E, F).

3. NGF acts to limit cell proliferation, although the presence of serum in the medium overcomes this inhibition. The molecular mechanisms for this are not known and the effect will not be pursued here, except to say that there is no correlation between population growth and the ability to produce long neurite-like processes.

Although serum has many components and actions, the effect of serum proteins on adhesion is widely recognized [Grinnell, 1978]. NGF in turn can act to increase the adhesion of growth cones [Gunderson and Barrett, 1980] as well as cell bodies [Schubert and Whitlock, 1977] to the substrate. However, in many cases, adhesion requires specific molecules on both the cell and the substrate. Collins [1980] has shown that ciliary ganglion cells are unable to extend neurites without a specific conditioned medium factor present on the substrate.

One hypothesis to explain the effects of NGF under serum and serum-free conditions is to assume that NGF acts to increase the adhesive potential of the growth cones to the substrate and in a different manner the adhesiveness of the cell bodies. NGF may lead to the increase in the adhesive potential in both serum and serum-free medium, but the expression of this potential may depend on the presence of an appropriate substrate molecule which is provided by serum. In serum containing medium NGF may increase the adhesion of the growth cones to the substrate, leading to speedier process extension, much as increased adhesion of dorsal root ganglion growth cones increases the rate of neurite extension [Ludueña, 1973]. The same changes in the growth cone induced by NGF in serum free medium would not be expressed as enhanced neurite growth if the appropriate substrate components are sparse. The number of processes remain about the same as in the control. The ability of the SY5Y cells to survive in serum free, hormone supplemented medium, and the refractoriness to NGF induced process elongation under this condition makes the testing of the serum factor hypothesis relatively straight forward. Fractionation of serum would be expected to produce specific

factors mediating NGF enhanced neurite outgrowth. This procedure would be similar to the isolation of specific fibroblast spreading factors in serum by Grinell (1978).

At the level of the cell body NGF can act to increase cell-cell adhesion. In serum-free medium clumping may occur because an NGF induced increase in cell-cell adhesion is stronger than the adhesion of the cell to the substrate. In serum containing medium clumping would not occur if the relative cell to substratum adhesion is stronger than the NGF induced interaction.

Overall, the effects of NGF on SY5Y have been to modulate the morphological characteristics already present in the cell line rather than to induce entirely new characteristics. The SY5Y cells are fully capable of producing processes and growth cones isomorphic with the processes and growth cones of developing sympathetic ganglion cells without the addition of NGF. NGF simply enhances the growth of these processes under appropriate culture conditions. The mechanism of this may be to change the adhesional qualities of the cells and growth cones, increasing the adhesiveness of both. NGF is also capable of suppressing SY5Y cell division. This action is separable from its action on cell morphology and is again highly dependent on culture conditions for its expression.

THE RELATIONSHIP OF NGF ACTIONS TO THE ACTIONS OF dbcAMP

Numerous studies have focused on the effects of increased cAMP levels on neuroblastoma cells [Prasad, 1975]. The possibility that cAMP modulates the effects of NGF in the cell has been postulated [Schubert et al., 1978]. In the SY5Y clone however the actions of dbcAMP and NGF are markedly different. In fetal calf serum containing medium dbcAMP does not cause an increase in the production of long processes, while NGF does. In serumless medium on the other hand NGF has no effect on long process production but dbcAMP causes 23% of the

cells to extend 100 μm or longer neurites (table 3).

In both serum and serum free medium dbcAMP stops population growth, although with slightly different kinetics. The only other effect in serum is to make the cells spread out more on the substrate (table 6). In serumless medium, however, dbcAMP did not cause an increase in cell spreading, but it did lead to a significant increase in the number of processes per cell, the number of 25 μm or longer processes and the number of 100 μm processes.

On an ultrastructural level dbcAMP was not seen to cause any change in the cell bodies, the processes, or the growth cones.

Although many people have used agents which raise cAMP levels to 'differentiate' neuroblastoma cells, and numerous physiological changes have been noted, no definitive, mechanistic explanations for the cAMP effects on process formation have been proposed. Based on the morphological analysis presented in this thesis, I cannot contribute to this area. What is clear from this study is that the effects of dbcAMP are quite distinct from the effects of NGF.

The complimentarity of the effects on SY5Y of NGF and dbcAMP with and without serum, prompts this proposal: Process elongation requires at least two steps at the growth cone, an adhesive step and a detachment step as the growth cone advances. Both steps are necessary for process growth. The effects of NGF and dbcAMP may be due to the enhancement of one step by NGF and the other by dbcAMP. Serum is crucial in the expression of the NGF and dbcAMP effects because the actions of serum mimic the actions of dbcAMP. The addition of dbcAMP to serum containing medium does not result in increased process elongation because the rate limiting steps are the actions enhanced by NGF. Conversely, in serum-free medium, the addition of NGF does not lead to increased process elongation, because the dbcAMP and serum enhanced steps are now rate limiting.

This proposal is in concurrance with two recent reports. Gunderson and Barrett [1980] have found that both NGF and dbcAMP can act as positive chemotrophic agents for growth cones (discussed on page 13, this thesis), however, NGF increases growth cone adhesion to the substrate while dbcAMP reduces it. The effects of NGF and dbcAMP on PC12 process formation were recently shown to be additive in nature, dbcAMP increasing the maximum rate of appearance of NGF induced processes. This indicates that, as in SY5Y, the actions are complimentary and stimulate growth at different loci within the neurite building machinery [Gunning et al., 1981b].

Overall, the effects of dbcAMP on SY5Y are modulatory in nature, as are the effects of NGF. No new morphological features are visible, there is only an enhancement of structures already present. Also, as with NGF, there is no correlation between population doubling time and process extension. Both NGF and dbcAMP can cause neurite extension but the conditions for, and probably the mechanisms behind, this expression differ.

CONCLUSIONS

Conclusions drawn from this study are:

1. Although the SY5Y clone of human neuroblastoma presents a number of technical difficulties it can be a valuable model for the study of neuronal morphogenesis. There are no major differences between the ultrastructure of these cells and the ultrastructure of developing sympathetic neurons. In particular the cells display features closely tied to the functions of neuronal growth cones, including structures involved in the exploration of the environment and the addition of new plasma membrane. These cells have an advantage over other neuroblastomas in being responsive to nerve growth factor, and over PC12 cells in having a response directly comparable to developing sympathetic ganglion cells, rather than to the responses of adrenal medullary cell in culture.

2. The response of the SY5Y cells to nerve growth factor is not representative of a classical cellular differentiation; rather NGF modulates structures within the cell which are already present. Although this modulation is in the direction of a more mature cell, the degree of response is different in different cells of the clone indicating that the modulation may be probabilistic. The effects observed were not universal; rather they were expressed as increases in the proportion of the population responding. Therefore it is essential when using this cell line to survey the entire dish to compensate for local variations in responsiveness.

3. Whether or not a cell produces a long process in response to NGF depends on factors other than NGF. In the culture conditions used for these experiments one highly important factor was the presence of serum in the medium. The serum probably exerted its effect by altering the adhesive properties of the substrate.

4. With SY5Y, the actions of NGF on cell survival, population growth and process elongation have been dissociated. SY5Y does not require NGF for survival, population growth can be affected but only under some conditions, and process elongation can occur when population growth is not affected. The NGF molecule is more than a mere survival factor for sympathetic cells. Other functions are served and the dilineation of these functions can help explain findings such as the effects of NGF on otherwise non-NGF dependent cells [Turner 1980; Yip and Grafstein, 1981].

5. The effects of NGF are different at the growth cone and the cell body. Although the mechanism is not known this study demonstrates that the pattern of somal adhesion is different from growth cone adhesion under the proper circumstances.

6. The effects of dbcAMP are clearly different from the effects of NGF. Both agents act to increase the number of long neurite-like processes under some conditions. The reciprocity seen between the NGF and dbcAMP effects with and without serum suggests that the two may act on different but complimentary phases in the mechanism of process elongation.

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