Carroll, Joanne Marlene

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by

Joanne M. Carroll

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Date

Chairman of Examining Committee
Professor Katherine Lyser

4/24/82
Date

Executive Officer
Professor James A. Organ

Professor Peter Lipke — Hunter College, Institution

Professor Harvey Ozer — Hunter College, Institution

Professor Tong Joh — Cornell University Medical School, Institution

Professor Gladys Teitelman — Cornell University Medical School, Institution

_____ — _____, Institution

_____ — _____, Institution

The City University of New York

ABSTRACT

EXPRESSION OF NEURONAL CHARACTERISTICS IN HUMAN NEUROBLASTOMA CELL LINES, SK-N-BE(2) M17 AND SK-N-SH-SY5Y

by

Joanne M. Carroll

Advisor: Professor Katherine M. Lyser

Two human neuroblastoma clonal cell lines, SK-N-BE(2) M17 and SK-N-SH-SY5Y, were passaged and maintained in serum-free hormone supplemented medium, N2 (Bottenstein & Sato, 1979). Both cell lines exhibited slower growth in N2 as compared to fetal calf serum (FCS) supplementation, however, the morphology, ultrastructure and qualitative expression of neurotransmitter synthesizing enzymes, tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH), were similar.

Cultures of M17 and SY5Y in N2 medium expressed basal levels of TH lower than cultures in FCS medium. Addition of adenosine 3′,5′-cyclic monophosphate (cyclic AMP) to N2 medium elevated the specific activity of TH to the level observed in serum medium. When added to FCS medium, cyclic AMP had no effect on TH activity. Growth of these cells in N2 medium revealed the presence of a cyclic AMP-dependent process in the regulation of TH.

Cyclic AMP also increased the outgrowth of long neurites in N2 but not in FCS medium. The ultrastructure of processes studied under all conditions, however, was similar.

Nerve growth factor (NGF) treatment of SY5Y cells increases the number of long neurites. This response requires serum factors and is not observed in N2 medium (Burmeister, 1982). Despite the morphological response, NGF at concentrations up to 100 ng/ml failed to affect the specific activity of TH or DBH in either SY5Y or M17.

While both cell lines expressed enzymes specific to noradrenergic cells, as determined by activity assay and immunocytochemical staining, their ultrastructure resembled early neuroblasts of the developing sympathetic nervous system. No mature cytoplasmic features, axonal or synaptic profiles were observed. Dense core vesicles were present, however, in both cell lines under all medium conditions studied. The content of the vesicles remains to be determined.

Defined medium N2 provided an adequate medium for maintenance of human neuroblastoma lines M17 and SY5Y. In addition, it revealed effects of cyclic AMP treatment on morphology and enzyme activity not observed in the presence of serum. This medium provides an improved system in which the role of factor(s) regulating specific neuronal functions may be assayed without complications introduced by serum.

To my parents Mary and Robert Carroll

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BACKROUND

NORMAL NEURAL CREST DEVELOPMENT AND NEURONAL PLASTICITY

Neuroblastoma shares a common embryological origin with autonomic neurons being derived from the neural crest. The ontogeny of the neural crest has been the subject of extensive study since its original description by His (1868). The neural crest arises as a distinctive but transient structure at the lateral edges of the developing neural tube and subsequently undergoes extensive migration to provide cells for many diverse neuronal and nonneuronal tissues. Ablation studies, vital staining of embryos and later crest transplant experiments mapped the courses of neural crest cells to destinations including the peripheral nervous system both sympathetic (Detwiler, 1937; Yntema & Hammond, 1945; LeDouarin, 1973) and parasympathetic ganglia (vanCampenhout, 1946; LeDouarin & Teillet, 1973), neuroendocrine cells of the adrenal medulla (Fusari, 1893), skeletal and connective tissue of the head (Johnston et al., 1974; Noden, 1978), sensory ganglia (His, 1868; Fusari, 1893; Noden, 1978) and melanocytes (Weston, 1963).

Different axial levels of the neural crest migrate to particular final sites to provide cells for the various derivatives. That the cells in the crest are not determined as to their final fate has been demonstrated by heterotopic transplantation of crest cells from one axial level to another. Taking advantage of the differential staining of chick and quail nucleoli by Fuelgen-Rossenback technique, the donor cells can be distinguished from the host cells in interspecies chimeras. The migratory route as well as the final differentiative fate are determined by the environmental factors encountered after the cells leave the crest proimordium (Le Douarin & Teillet, 1974; LeDouarin et al., 1975).

Cells along the migratory route and at the final site provide cues essential for expression of the adrenergic or cholinergic phenotype. Adrenergic differentiation of crest cells requires interaction with somites, ventral neural tube and notochord (Cohen, 1972; Norr, 1973). Trunk neural crest cells migrate to paravertebral sites and aggregate to form sympathetic ganglia, whose principle neurons are adrenergic. Trunk crest cells do not normally migrate to colonize the gut, but if transplanted into the gut become cholinergic and form myenteric plexi while expression of adrenergic enzymes is suppressed (Smith et al., 1977). If the trunk crest primordium is incubated in the aneural hindgut in the presence of the notochord and neural tube, adrenergic ganglia are found in addition to myenteric plexi (Teillet et al., 1978). Cultures of early mesencephalic crest from 3 1/2 day chick embryos just beginning migration exhibit choline acetyltransferase activity. If incubated in the presence of notochord and sclerotomal somite, the cells become adrenergic (LeDouarin, 1980; Fauquet et al., 1981).

The neural crest derived neurons remain multipotential with respect to transmitter choice even after aggregation of the cells into a ganglion. Peripheral ganglia from 4.5-6 day embryos backgrafted into a 2 day host resume migration and colonize structures in the host embryo. Sensory dorsal root ganglia at this stage migrate to both autonomic and sensory structures. The sympathetic cells preferentially home to sympathetic structures indicating some early differentiation of a sympathetic line and a sensory line by 4.5 days. Cells from a 13 day embryonic ciliary ganglion migrate normally when placed in the trunk region of 2 day host embryo. (LeLievre et al., 1980). Similar transplants using the nodose ganglion, in which neural crest derivatives are exclusively nonneuronaal satellite cells, demonstrate that the nodose cells migrate to and populate sympathetic ganglia. Further, the transplanted cells exhibit

formaldehyde-induced fluorescence suggesting the presence of catecholamine. Release from the influence of the placodal environment allows for the new truncal environment to induce adrenergic expression in cells which normally express nonneuronal phenotype (LeDouarin & LeLievre, 1981).

In vitro experiments with early postnatal rat sympathetic ganglia also suggest retention of plasticity with respect to transmitter choice for some time after the phenotype appears to be fixed in vivo. Adrenergic and cholinergic differentiation can be influenced by the culture environment. Rat superior cervical ganglia, grown in medium which reduces the nonneuronal population, express the adrenergic phenotype (Mains & Patterson, 1973). The presence of nonneuronal cells or medium conditioned by nonneuronal cells enhances the expression of cholinergic phenotype in superior cervical ganglia (Patterson & Chun, 1974; Patterson & Chun, 1977). The pattern of proteins released into the extracellular medium by sympathetic neurons is dramatically altered by conditioned medium. These secreted proteins may be correlated to transmitter choice and have a role in the intercellular communication so crucial to nervous system development (Sweander, 1981). The establishment of preganglionic nerve activity may stabilize the adrenergic phenotype in vivo and antagonize effect of the cholinergic factor. Cultures of superior cervical ganglia, in the presence of conditioned medium, retain the adrenergic phenotype in the presence of high potassium (Walicke & Patterson, 1977).

That individual cells may be multipotential with respect to neurotransmitter synthesis has been demonstrated in microcultures. Single ganglion cells can be seeded onto islands of cardiac myocytes and electrophysiological studies of these isolated cells sometimes reveals dual function with respect to transmitter release (Furshpan et al., 1976). Sequential studies of the same microculture, possible in

a few cases, suggest a transition from adrenergic to dual function to cholinergic. Further analysis of the cultures suggests the possibility that peptide neurotransmitters may also be released along with norepinephrine or acetylcholine (Potter et al., 1980).

Correlated electron microscopic and biochemical studies on explanted superior cervical ganglia at several postantal developmental stages indicate that as the phenotype becomes stabilized in vivo, the ability to modify neurotransmitter synthesizing enzyme expression in vitro is decreased. Neurons become increasingly refractory to developmental signals as the mature phenotype is stabilized in vivo (Johnson et al., 1980; Landis, 1980; Schwab & Landis, 1981).

However, even superior cervical ganglia from postnatal rats of 12.5 days grown in dissociated cell culture are capable of forming hexamethonium sensitive nicotinic cholinergic synapses between each other. If skeletal myotubes are added to the culture, curare sensitive Ca^{++}-dependent synapses develop (Wakshull et al., 1979). Autonomic neurons may retain some capacity for multiple neurotransmitter expression for a time period during development more extended than prevoiusly suggested. This capacity may be unmasked in culture by appropriate adjustment of the culture environment.

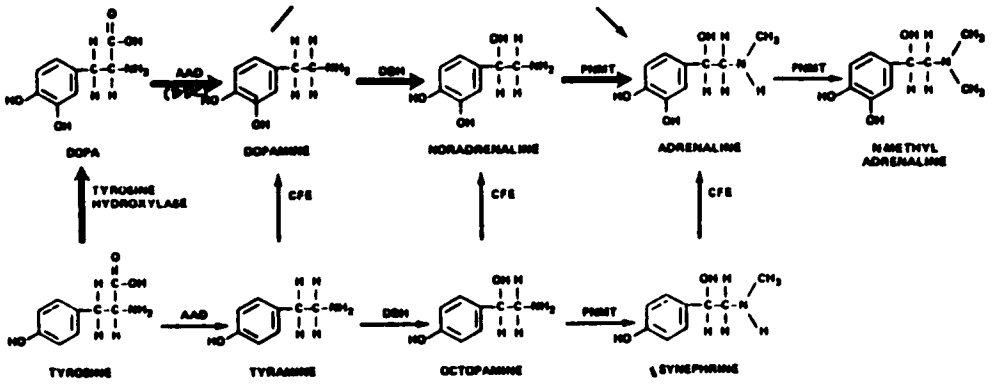
Predominantly adrenergic neurons of the perinatal rat superior cervical ganglion grown in conditioned medium are found to increase cholinergic expression at the expense of adrenergic expression (Patterson & Chun, 1974; Landis, 1976; Ross et al., 1977; Johnson et al., 1980). However, recent biochemical and immunocytochemical studies on dissociated cells from the rat superior cervical ganglia suggest that the cells are capable of dual neurotransmitter synthesis for as long as seven weeks in culture (Iacovitti et al., 1981). In addition, 90 % of the cells demonstrated to form functional cholinergic synapses by electrophysiolgical methods are

immunocytochemically stained with antibody to tyrosine hydroxylase (Higgins et al., 1981).

Neural crest cells in culture provide a convenient system in which to study the factors involved in the expression of neuronal phenotype and the molecular mechanisms regulating this gene expression. The early stages of cellular determination and differentiation remain largely unknown. Recently, cultures of early neural crest have been established (Seiber-Blum & Cohen, 1980; Fauquet et al., 1981; Loring et al., 1981). These should provide important information about early events related to these processes. However, only small amounts of material can be obtained from embryonic and postnatal animals. Continuous cell lines derived from tumors of neural crest origin could provide large amounts of homogenous cell populations. These cells may facilitate biochemical studies and be of potential use in examining the regulation of gene expression in the neural crest with respect to neurotransmitter synthesizing enzymes or other aspects of differentiation. It is necessary, however, to first establish that such cell lines express the enzymes of interest and exhibit regulatory mechanisms consistent with those found in normal cells. One aim of this thesis project was to evaluate the potential for neuroblastoma cells to express differentiated features and compare the responses of these cells in culture to those found for normal neural crest cells in vitro.

ONTOGENY OF NEUROTRANSMITTER SYNTHESIZING ENZYMES

The enzymes of the catecholamine synthetic pathway are diagrammed below.



(Axelrod, 1977)

Tyrosine hydroxylase (E.C. 1.14.16.2 tyrosine-3-monooxygenase) (TH) is a mixed function oxidase requiring tetrahydropteridine cofactor, O_2, and Fe^{++} (Nagatsu et al., 1964). It is the rate limiting step in the catecholamine pathway (Levitt et al., 1965) and subject to feedback control of the endproduct norepinephrine (Ikeda et al., 1966; Weiner, 1970).

Dopa decarboxylase (EC 4.1.1.26 L-3,4-dihydroxyphenylalanine carboxylase) (DDC) is a pyridoxal requiring enzyme and it exhibits low specifity for substrate, decarboxylating dopa as well as other aromatic-L-amino acids (Axelrod, 1977).

Dopamine-β-hydroxylase (EC 1.14.17.1 dopamine-β-monooxygenase) (DBH) is a Cu^{++}- requiring mixed function oxidase which β-hydroxylates phenylethylamines such as dopamine (Kaufmman & Friedman, 1965). The enzyme is highly localized in the storage vesicles of sympathetic nerves and chromaffin granules of the adrenal medulla (Potter & Axelrod, 1969; Viveros et al., 1969).

Phenylethanolamine-N-methyltransferase (EC 2.1.1.28 S-adenosyl-L-methionine:phenylethanolamine-N-methyltransferase) (PNMT) catalyzes the methylation of noradrenaline and other B-hydroxylated phenylethanolamine derivatives. S-adenosyl methionine serves as the

methyl donor. The enzyme is present in the cytosol of the adrenal medulla where it catalyzes the conversion of norepinephrine to epinephrine (Axelrod, 1977).

The levels of TH and DBH are modulated in the neuron to match the catecholamine synthetic rate to the demands for neurotransmission. Both short term changes in catalytic activity in response to acute neural stimulation as well as long term inductive changes in response to chronic depletion of catecholamine operate in the above capacity. Some details of these mechanisms operative in the mature sympathetic cell will be discussed below with reference to changes in enzyme activity observed in neuroblastoma cultures. During embryonic and early postnatal development of catecholaminergic systems increases in enzyme activity are also stimulated by factors in the cellular environment.

The ontogenetic appearance of the neurotransmitter synthesizing enzymes tyrosine hydroxylase (TH), dopa decarboxylase (DDC), dopamine-hydroxylase (DBH) in the sympathetic nervous system occurs at a time which appears to be coincident with the aggregation of cells into a ganglion structure (Cochard et al., 1978). Histofluorescence is detected in the 11 day rat in sympathetic primordia but not at earlier embryonic stages in cells of the dorsal neural crest or on the ventrally migrating stream of cells moving to populate the ganglia and adrenal medulla (Cochard et al., 1978). The appearance of histofluorescence is temporally coincident with the detection of the neurotransmitter synthesizing enzymes by immunocytochemical staining (Teitelman et al., 1979). The synchronous appearance of TH, DDC and DBH suggests that there is a simultaneous expression of the synthetic enzymes at about day 11 in the embryonic rat.

The regional appearance of the TH and DBH immunoreactivity follows a rostrocaudal gradient. At day 14 of gestation TH and DBH

immunoreactivity can be seen in the adrenal anlage corresponding to the arrival of migrating crest cells at this site while the appearance of PNMT is delayed until day 17. The expression of PNMT appears to be specific to the adrenal as no ectopic sites of PNMT immunoreactivity have been observed. The expression of PNMT may require additional environmental factors, perhaps glucocorticoids supplied by the surrounding cortical cells (Teitelman et al., 1979). Administration of glucocorticoid to embryonic rats fails to accelerate the appearance of the PNMT activity in the adrenal or to stimulate extraadrenal expression of the enzyme (Teitelman et al., 1979; Bohn et al., 1981). Scattered fluorescent and immunoreactive cells appear transiently in the gut at embryonic day 11.5 in the rat but disappear by day 14 and are not observed in the adult (Cochard et al., 1978; Teitelman et al., 1979). The disappearance of these transient catecholaminergic cells may represent cell death of neurons or the conversion of cells to an alternative phenotype due to environmental cues encountered in the gut.

Cells in the sympathetic primordia from 12-14 day rat embryos or chicks stages 26-27, contain radioautographically labelled cells if incubated in ^{3}H-thymidine for 2-4 hours prior to fixation (Rothman et al., 1978). At this stage cells also contain cytoplasmic TH and DBH immunoreactivity. At progressively later stages fewer and fewer dividing cells are observed indicating the withdrawl of mature sympathetic cells from the cell cycle. The withdrawl from cell cycle does not precede nor is it required for the expression of catecholamine biosynthetic activity in the ganglion as demonstrated by simultaneously FIF-positive and ^{3}H-thymidine labelled cells (Rothman et al., 1978). These cells probably represent an intermediate stage of development.

The aquisition of mature characteristics by dividing sympathicoblasts is characteristic of peripheral but not central

nervous tissue (Rothman et al., 1980). The maturation of central catacholaminergic neurons occurs during the postmitotic stage (Rothman et al., 1980). The difference between the two systems may be related to their different embryological origins. Developmental sequences and regulation may be fundamentally different in neural crest (peripheral nervous system) and neural tube (central nervous system) derivatives (Rothman et al., 1980).

Several influences are important to the subsequent development of the sympathetic ganglion cells. Though the initial expression of neurotransmitter synthesizing enzyme activity precedes the reception of the preganglionic innervation (Coughlin et al., 1977; Cochard et al., 1979; Teitelman et al., 1979; Rothman et al., 1980) these levels undergo significant increases coincident with the arrival of preganglionic fibers and the establishment of morphologically identifiable synapses (Black, 1971a). The arrival of the preganglionic fibers can be monitored biochemically by the appearance of choline acetyltransferase (CAT) in the ganglion or by morphologically distinct cholinergic synapses. Choline acetyltransferase progressively reaches adult levels in the ganglion during the first three postnatal weeks and this rise is coincident with an increase in cholinergic synapses and functional neuronal maturation (Black, 1971a).

The development of the noradrenergic ganglion cells, the postsynaptic element, includes increased TH activity. TH exhibits a biphasic increase one increase at birth to 3 days postnatal precedes the arrival of the preganglionic fibers (Black et al., 1971b). During the phase immediately following the appearance of increased synapse formation, TH rapidly increases to adult levels. This period of biochemical maturation occurs after the cessation of mitosis (Black et al., 1971b). Transection of preganglionic fibers (Black et al., 1972) or pharmacological blockade with chlorisondamine (Black, 1973)

abolishes the second phase increase in TH activity, indicating that one necessary but probably not sufficient cause for stimulation of TH to mature levels is preganglionic input (Black et al., 1972).

The ontogenetic increase in TH activity is associated with new protein synthesis not activation of preexisting molecules. This is demonstrated by the sensitivity of the induction to protein synthesis inhibitors (Black et al., 1972) and by immunotitration of increased numbers of TH molecules with the postnatal increase in activity (Black et al., 1974).

In the SCG, DDC and DBH are also regulated by transsynaptic mechanisms during critical developmental periods (Black & Geen, 1973). Basal levels of TH activity are sensitive to nicotinic receptor blockade or decentralization throughout the first month of life (Black & Geen, 1973). In later stages and in the adult the basal levels of TH and DBH are not effected by decentralization but inductive responses are dependent upon intact preganglionic innervation (Mueller et al., 1970; Molinoff et al., 1972). DDC levels are not specifically elevated by transynaptic induction (Otten & Thoenen, 1976). The specific increases in enzyme activity in the perinatal period are accompanied by increases in cell number and volume in the ganglion which may also be related to transsynaptic modulation (Black & Geen, 1974). The perinatal effects differ from the responses of the mature ganglion in that the range of response is more selective in the mature ganglion. Though mediated by acetylcholine in both cases, the mature ganglion exhibits increases in TH and DBH only, with no changes in ganglion cell number or volume. The postsynaptic process may be a modified version of the perinatal response as the postsynaptic response to the same stimulus becomes increasingly specialized (Black & Geen, 1974).

The ability of the sympathetic neuron to establish functional synapses at the peripheral target organ is influenced by preganglionic

neurotransmission. In decentralized ganglia, NGF stimulates submaximal increases in tyrosine hydroxylase at the cell body but not the terminals in the irides. The maturation of the terminals with respect to NGF responsivity appears to be prevented by decentralization of the ganglion. The response to NGF in the cell body, however, is lower than that found with intact preganglionic innervation. This could be related to the critical periods for each of these, i.e., at the time of transection the basal level of TH and DBH has been established whereas the formation of synapses may be just reaching the stage where preganglionic activity is crucial.

NERVE GROWTH FACTOR

Nerve growth factor (NGF), a protein growth hormone, is required for normal development of sympathetic and sensory neurons. It was originally recognized as an activity present in mouse sarcoma cells which had been implanted into the body wall and caused an extensive neurite outgrowth from sensory (Bueker, 1948) and sympathetic ganglia both at the level of the tumor transplant and at remote sites (Levi-Montalcini & Hamburger, 1953). Upon closer examination it was found that the hypertrophic effects include not only the ganglia directly innervating the peripheral field at the level of the tumor, but ganglia at all levels of the neural axis. The diffusible factor responsible for this effect has since been isolated from a number of sources most notably in high quantities from the male mouse salivary glands (Varon, 1967). The NGF molecule has been purified and sequenced (Angeletti & Bradshaw, 1971) and the biological activity found to be associated with the βsubunit of the dimeric molecule (Bocchini & Angeletti, 1969).

NGF enhances the expression of differentiated morphologic and biochemical characteristics in sympathetic neurons. Neuroblasts treated with NGF exhibit extensive cytological differentiation

characterized by the formation of extensive rough endoplasmic reticulum, Golgi cisternae, neurofilaments and neurotubules (Angeletti & Bradshaw, 1971) and an accelerated outgrowth of neuritic processes (Levi-Montalcini & Angeletti, 1968). Specific induction of the neurotransmitter synthesizing enzymes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH), results from the treatment of both adult and neonatal sympathetic ganglia with NGF (Thoenen et al., 1971; Stoeckel et al., 1974).

NGF is also required for the ontogenetic development of sensory ganglia though, in contrast to the sympathetic cells, this dependence does not continue into adult stages (Levi-Montalcini, 1966). The adrenal medulla, an embryologically related tissue derived from the neural crest and complementary to the sympathetic nervous in some of its physiological functions, was not initially thought to be influenced by NGF during its development as it did not exhibit morphometric decreases in the presence of NGF antibodies nor was NGF responsible for its survival and maintenance in the adult (Angeletti et al., 1972). However, recent studies in vivo (Otten et al., 1977; Aloe & Levi-Montalcini, 1979) and in vitro (Unsicker et al., 1978) suggest that chromaffin cells may be capable of a response to NGF administration in a manner which resembles their neuronal counterparts.

Administration of NGF to newborn or adult rats elicits a selective increase in TH and DBH in the adrenal medulla with a time course that corresponds with the arrival of the NGF molecule in medulla (Otten et al., 1977). The maximal response, while not of the magnitude of the sympathetic response, was significant and a direct effect of NGF, independent of the splanchnic innervation to the gland (Otten et al., 1977). Tissue culture of adrenal chromaffin tissue has also revealed affects of NGF on the morphology of the adrenal cells stimulating the outgrowth of neurites which closely resemble neuronal processes (Unsicker et al., 1979). Dexamethasone (Unsicker et al., 1980) or

cyclic AMP (Zeigler & Unsicker, 1981) antagonize the NGF effect and the authors suggest that this is related to the processes in vivo which function to maintain an endocrine phenotype in preference to a neuronal one in the adrenal medulla.

That the action of NGF may not be restricted to neuronal development is demonstrated by studies where treatment during the embryonic period with NGF or anti-NGF antibodies significantly influences the chromaffin cells. Administration of antibodies to NGF at embryonic day 17 and again during the first 8 postnatal days causes extensive cellular degeneration and disorganization in the adrenal medulla. Administration of NGF during this same embryonic and early postnatal period transforms the medulla into a structure resembling a ganglion with neuron-like cells and extensive neuritic outgrowth which invades the surrounding cortical tissue. The transformation of the chromaffin cells is only partially stimulated if NGF treatment commences at birth and is virtually absent with administration after the first two postnatal weeks. The ectopic ganglion formed by NGF treatment is dependent upon exogenous NGF presumably due to the lack of normal target tissue to supply the trophic factor (Aloe & Levi-Montalcini, 1979).

The regeneration of neurites observed in explanted ganglia may represent a process distinct from neurite initiation with first exposure to NGF. The PC12 pheochromocytoma cell line, which possesses a rounded cellular morphology without NGF, elaborates an extensive network of processes when incubated with NGF for several days to one week (Greene & Tischler, 1976). Accompanying the morphologic change is a reduction in cell division and DNA synthesis (Gunning et al., 1981b), increased levels of choline acetyltransferase (Greene & Rein, 1977), acetylcholinesterase (Greene & Rickenstein, 1981), and a specific large external glycoprotein, NILE (McGuire et al., 1978).

The outgrowth of neurites in response to NGF proceeds via transcription-dependent and independent pathways. In PC12 cells not previously exposed to NGF, the initiation of neurite outgrowth requires protein synthesis. However, cells pretreated with NGF when divested of their neurites and then replated, regenerate neurites with a significantly shorter time course by a transcription-independent process. Even cells treated with NGF in suspension culture, where no neurites are expressed due to lack of surface attachment, elaborate neurites within 24 hours of plating onto an appropriate substrate. This event has been termed "priming" and appears to involve the accumulation of cellular material which can support rapid expression of neurites upon subsequent presentation of NGF. Cells are capable of a transcription-independent response to NGF providing these molecules are present in sufficient quantity (Burstein & Greene, 1978). Neurite outgrowth observed in explanted ganglia is rapid and transcription-independent probably due to <u>in vivo</u> "priming".

Another function of NGF is the maintenance and survival of sympathetic neurons. In serum-free medium PC12 cells require NGF and this survival effect is separable from NGF effects on neurite outgrowth. The presence of RNA synthesis inhibitors prevents neurite outgrowth but does not influence cell viability suggesting that the survival effects are mediated by transcription independent processes (Greene, 1978). The slowing of the cell division rate occurs by a temporally distinct process. The initial outgrowth of processes occurs during log phase growth and the inhibition of cell division occurs at a time when neurite outgrowth is maximal (Gunning et al., 1981a). ^{3}H-thymidine incorporation into morphologically differentiated cells suggests that NGF acts via multiple pathways on its target cells (Gunning et al., 1981b).

NGF specifically induces levels of the adrenergic neurotransmitter synthesizing enzymes in sympathetic ganglia. The effects on tyrosine hydroxylase and dopamine-β-hydroxylase are mediated by mechanisms separable from those influencing survival and morphology (Stoeckel et al., 1974).

The PC12 line of rat pheochromocytoma has been extensively characterized for its morphological response to NGF and, as discussed above, a priming model (Burstein & Greene, 1978) involving a multistep process to account for NGF action on neurite outgrowth has emerged from this work. However, PC12 cells do not exhibit NGF inducible TH activity despite the presence of specific receptors as evidenced by the effects on CAT (Edgar & Thoenen, 1978) and morphology (Greene & Tischler, 1976). This could be the result of a defect in the cellular machinery which acts subsequent to receptor binding to influence TH (Edgar & Thoenen, 1978). Alternatively, it could represent a response which is appropriate to the determination as an endocrine cell or to neoplastic origin. PC12 cells demonstrate that biochemical and morphological effects of NGF need not be expressed coordinately and the mechanisms subserving each effect may be separable.

A few neuroblastoma lines have been reported to exhibit NGF induced changes in morphology (Perez-Polo et al, 1979; Kobler et al, 1974). One human neuroblastoma line, NJB, exhibits a morphological response to NGF treatment which is accompanied by increased colchicine binding activity in measured in cell extracts (Kobler et al., 1974). These findings, in contrast to work done on neurite outgrowth from isolated ganglia, suggest an increase in cellular content of tubulin subunits in response to NGF (Kobler et al., 1974). Also increased was the level of neuron specific protein 14-3-2 (Kobler et al., 1974). The "priming " events associated with neurite outgrowth in PC12 required RNA and protein synthesis, but the nature of the proteins

synthesized is not known (Burstein & Greene, 1978; Gunning et al., 1981a). In the SK-N-SY5Y (SY5Y) alterations in morphology, population growth and electrical excitability are found associated with NGF treatment (Kuramoto et al., 1981; Perez-Polo et al., 1979). NGF also prevents the cytotoxic effects of 6-hydroxydopamine in SY5Y cells (Tiffany-Castiglioni et al., 1979). These morphological effects of NGF have been confirmed and further characterized (Burmeister, 1982).

This thesis examined the NGF effect on the levels of neurotransmitter synthesizing enzymes TH and DBH in SY5Y and the previously uncharacterized clonal line SK-N-BE(2)-M17 (M17). The SY5Y cells possess high affinity NGF receptors on the cell surface but in low quantity (Sonnefeld & Ishii, 1979). Of several human neuroblastoma cell lines studied, SY5Y had the lowest receptor content but was the only one to exhibit the morphological response of neurite extension (Sonnefeld, personal communication). The M17 clone has not been examined as yet for receptor content.

CYCLIC AMP: ACTION AS A SECOND MESSENGER FOR NGF?

The action of NGF may involve second messengers. NGF introduced directly into target cell cytoplasm by cell fusion with NGF-loaded erythrocyte ghosts fails to elicit any any effects in the PC12 target (Heumann et al.,1981). This suggests that surface binding is prerequisite to actions of NGF. Anti-NGF antibodies introduced into the PC12 in this manner fail to prevent the stimulation of neurite outgrowth by NGF in the medium suggesting that release of free NGF into the cytoplasm and direct effects on cytoplasmic organelles is unlikely (Huemann et al., 1981). The nature of the intervening steps mediating NGF action may involve multiple pathways and no definitive data is yet available on any of these though several possibilities are under investigation.

Transitory elevations of intracellular cyclic AMP following NGF treatment have been reported in chick dorsal root ganglia (Skaper et al., 1979), embryonic sensory neurons (Narumi & Fujita, 1978), superior cervical ganglion organ cultures (Nikodijevic et al, 1975), PC12 (Schubert & Whitlock, 1977) and the N18 mouse neuroblastoma clone (Narumi & Fujita, 1978). However, others have failed to measure increases in cyclic AMP following NGF treatment in culture (Hatanaka et al., 1978) or in sympathetic ganglia after in vivo NGF injection. In addition, treatment of cells with agents which are known to elevate cyclic AMP, as isoproteronol, fails to induce tyrosine hydroxylase, a well known inductive effect of NGF on sympathetic neurons (Otten et al., 1978). Cyclic AMP cannot substitute fo NGF in mediating the survival of embryonic chick sympathetic ganglia (Greene & Shooter, 1980).

Exposure of PC12 cells to cyclic AMP analogues or agents which stimulate intracellular cyclic AMP, prostaglandin E1 or cholera toxin, can mimic some of the effects of NGF treatment (Schubert et al., 1978). Distinctive differences have been found in the neurite elongation found to be stimulated by cyclic AMP and NGF as well as in the "priming" process (Burstein & Greene, 1978). Neurites induced by cyclic AMP appear with a different time course than those produced by NGF, appearing rapidly and being unstable, regressing at times when NGF induced neurites continue their elongation (Gunning et al., 1981). The NGF priming of PC12 cells could not be reproduced by cyclic AMP nor could cyclic AMP accelerate the process if added simultaneously with NGF (Gunning et al., 1981). Cyclic AMP initiates neurite outgrowth by a process independent of priming.

NGF causes rapid and characteristic changes in the cell surface morphology of PC12 prior to the observed effects on neurite outgrowth which occur over a time course of 70 hours (Connolly et al., 1980). Cyclic AMP fails to stimulate the protrusive activity on the surface

and rather elicited outgrowth of neurites without the characteristic surface alterations over a time course of 4-6 hours (Gunning et al., 1981). Finally, cyclic AMP stimulated neurite extension appears to be transcription-independent and the neurites thus stimulated cannot be maintained by replacement of cyclic AMP by NGF (Gunning et al., 1981a).

Though evidence from these cellular studies with sympathetic ganglia and PC12 indicates that cyclic AMP action is not sufficient to account for all the observed actions of NGF, cyclic AMP dependent events may play a role in mediating some actions of NGF. The limitations of the methods employed to detect changes in intracellular molecules and their subsequent effects may be the crucial aspect in elucidating the mechanism of action for NGF.

Notably, on the molecular level, protein phosphorylation in the cell is altered by NGF and cyclic AMP. The pattern of protein phosphorylation observed following NGF, cyclic AMP or cholera toxin treatment of PC12 cells is virtually identical while EGF and insulin are not. Both cytoplasmic and nuclear proteins are affected though the phosphorylation of the nuclear proteins occurs more slowly than the cytoplasmic events (Halegoua & Patrick, 1980).

Whether the lag between the phosphorylation in the two compartments is related to translocation of protein kinase subunits (Jungmann et al., 1975) or to translocation of NGF and interaction with nuclear receptors (Yanker & Shooter, 1979) remains to be determined.

Increased phosphorylation of a cytoplasmic protein of molecular weight of 60,000 which is immunoprecipitated by antibody specific to TH is found following cyclic AMP or NGF treatment (Halegoua & Patrick, 1980). Though the phosphorylation of TH is stimulated by NGF

treatment enzyme activity is not induced in PC12 by NGF. This indicates that phosphorylation alone may not be sufficient to account for the inductive effects of NGF.

The phosphorylation of nuclear proteins may be involved with transcriptional events mediating NGF actions. The specific phosphorylation of high mobility group protein, HMG-17, which has been associated with active chromatin, by NGF but not insulin or EGF suggests that this plays a unique role in NGF action (Halegoua & Patrick, 1980). The phosphorylation of nuclear proteins has been observed with both sympathetic cells and PC12. A single chromatin associated nuclear protein of molecular weight of 30,000 exhibits specific increase in phosphorylation in response to NGF, EGF or cyclic AMP treatment of the cells (Yu et al.,1980). Whether all phosphorylations of the protein are at the same site is not known, but it was postulated that phosphorylation at different sites could influence the effect on transcription and be involved in the differential responses to NGF and EGF (Yu et al., 1980).

CYCLIC AMP AND NEOPLASIA

Cyclic AMP-dependent mechanisms are implicated in growth control and cell cycle traverse in normal tissues (Pastan et al., 1975). Defects in the metabolism of cyclic AMP or the utilization of cyclic AMP could contribute to neoplastic growth. Interest in this area was stimulated by reports of reduced adenyl cyclase activity in a polyoma transformed hamster cell line (Burk, 1968). A number of tumor cell lines, including neuroblastoma, exhibit cellular resposes to cyclic AMP which include slowing of growth rate, morphological differentiation and increased activity of specific enzymes associated with differentiated function (Prasad & Vernadakis, 1972).

Neoplastic cells often exhibit abnormalities in cyclic AMP

metabolism (Ryan & Heidrick, 1974) possibly due to altered adenyl cyclase activity (Pastan et al., 1975), elevated phosphodiesterase activity (Pradad & Sinha, 1976), or defective cyclic AMP binding proteins (Prashad et al., 1980; Prashad et al., 1977; Cho-Chung et al., 1980; Imashuku, 1980). As treatments which elevate intracellular cyclic AMP in tumor cell cultures often stimulate the expression of more differentiated features, several investigations have focused on the cyclic AMP levels and content of binding proteins found in neoplastic cells during rapid growth and regression. However, correlations between tissue content of cyclic AMP and neoplastic growth is not always observed (Hunt & Martin, 1981).

Several human and mouse neuroblastomas have significantly lower mean cyclic AMP content than ganglioneuromas or human sympathetic ganglia (Imashuku et al., 1977). Addition of cyclic AMP analogues to cultures of neuroblastoma causes increased phophodiesterase activity but nonetheless an elevated level of intracellular cyclic AMP is maintained (Prasad & Kumar, 1975; Prasad & Kumar, 1974).

The action of cyclic AMP in the cell depends upon the functional integrity of binding proteins, particularly the cyclic AMP dependent protein kinases (Kuo & Greengard, 1969). The protein kinase consists of catalytic (C) and regulatory (R) subunits. Upon binding cyclic AMP the subunits dissociate liberating the C subunit to mediate the phosphorylation of cellular substrates (Gill & Garren, 1970). Two forms of cyclic AMP dependent protein kinase are found in mammalian cells, Type I and II, which differ only in thier regulatory subunit (Corbin et al., 1975). The relative amounts of type I and II isozymes differ amongst tissues and species (Corbin et al., 1977). The relative amounts of type I and II are not static in the cell and may vary during development and cell cycle. In mouse heart, the relative amounts of type I/II change from neonatal ratio of 2/1 to an adult ratio of 1/1. The shift in the ratios occurs simultaneously with the

maturation of the heart muscle and a switch from proliferative to hypertrophic growth (Malkinson & Butley, 1981).

In the IMR-32 human neuroblastoma cell line and the NPB_2 clone of C1300, a two-fold increase in cyclic AMP binding activity accompanies the differentiation of cells by the cyclic nucleotide. The increased binding activity in these cells (Prasad et al., 1976), in a mouse neuroblastoma-rat glioma hybrid line (Walter et al., 1978) and in the S20 and N18 lines of C1300 (Prashad & Rosenberg, 1978) are not accompanied by increases in protein kinase activity. The levels of intracellular cyclic AMP increase by 12-fold despite a 40% increase in phosphodiesterase activity indicating that binding may afford protection for the ligand (Prashad & Rosenberg, 1978). Alternatively, the binding to these non-kinase sites may reflect noncoordinated expression of R and C subunits or defective R subunits unable to interact with the C subunit. Such binding could compete for cyclic AMP and reduce functional binding to active protein kinase in the tumor cells (Walter et al., 1978).

In human mammary tumors the levels of type I and II were correlated with growth and differentiation. A positive correlation between the I/II ratio and the rate of cell proliferation is primarily due to elevated levels of type I. In normal cells the type II kinase is the predominant type while neoplastic cells exhibit a two-fold increase in type I kinase (Handschin & Eppenberger, 1979).

Regression of dimethylbenzanthracene (DMBA)-induced mammary tumors in rats can be achieved by removal of estrogens by ovariectomy or treatment with cyclic AMP. Increases in cyclic AMP binding activity, specifically increased type II protein kinase, antagonizes the effects of hormone in stimulating tumor growth (Bodwin et al., 1978; Cho-Chung et al., 1978). The pattern of nuclear protein kinases is altered by ovariectomy or cyclic AMP treatment.

In responsive tumors, treatment with cyclic AMP analogues leads to a three-fold increase in nuclear cyclic AMP while cytosol binding decreases by a corresponding amount. The increase in the nuclear protein kinase activity is temperature dependent and requires initial interaction of cyclic AMP with protein in the cytosol (Cho-Chung et al., 1977a). If cyclic AMP-protein kinase complex is incubated with nuclei from unresponsive tumors, nuclear binding is observed. The converse experiment with cytosol from unresponsive tumors and nuclei from responsive tumor does not lead to nuclear changes. Binding proteins from unresponsive tumors fail to bind cyclic AMP, to translocate to the nucleus or catalyze phosphorylation reactions at either site. The unresponsive tumors appear to lack appropriate binding proteins, probably protein kinase, capable of translocation to the nucleus and able to catayize phosphorylation of proteins related to tumor regression (Cho-Chung et al., 1977b).

Specific uptake of a 56 kd protein from the cytosol of cyclic AMP treated cells precedes the phosphorylation of a 76 kd nuclear protein. The type II protein kinase purified from bovine heart phosphorylated this protein as well. The 56 kd cytosol protein from hormone unresponsive tumors fails to be translocated to the nucleus and no phosphorylation of the 76 kd protein is observed in nuclei from responsive or unresponsive tumors (Cho-Chung et al., 1979).

These experiments suggest that the cytosol protein in responsive cells associated with nuclear events that may be correlated with regression is protein kinase type II. Cells unresponsive to hormone withdrawl or cyclic AMP treatment fail to regress and do not appear to possess protein kinase able to interact with the catalytic subunit or to be translocated to the nucleus (Cho-Chung et al., 1980).

In view of the possible actions of cyclic AMP as a second

messenger for hormones or factors and in growth regulation, its effects on the growth and development of the human neuroblastoma cell lines SY5Y and M17 were investigated.

NEUROBLASTOMA: CLINICAL CHARACTERISTICS

Neuroblastoma is a tumor which most commonly occurs in early childhood. Primary tumors most frequently appear in the adrenal medulla or in retroperitoneal tissue adjacent to the gland. Tumors appearing in the thorax or pelvis are usually at paravertebral sites in the posterior or lateral body wall in the vicinity of the autonomic ganglia. The location of the primary tumors suggests their derivation from the neural crest (Jones & Campbell, 1976). Variable degrees of differentiation are associated with the cells of neuroblastoma tumors. Some tumors consist mainly of early neuroblast-like cells with rather undifferentiated appearance while others exhibit features resembling more mature stages of sympathetic ganglion cells (Russell & Rubenstein, 1971). Benign tumors consisting of predominantly mature neuron-like elements are ganglioneuromas (Staley et al., 1967)

The ultrastructure of undifferentiated neuroblastoma is characterized by sparse rough endoplasmic reticulum, scant Golgi, neuritic profiles with variable resemblance to mature axons, and nuclei with highly invaginated borders, prominent nucleoli and masses of irregularly condensed chromatin within the nucleoplasm underlying the nuclear envelope (Misugi et al., 1968; Greenberg et al., 1969; Goldstein et al., 1971; Lyser, 1974). The more mature cells exhibit a sympathetic morphology possessing abundant rough endoplasmic reticulum, extensive Golgi, neurite processes containing arrays of microtubules and neurofilaments and nuclei of more rounded or oval shape with reduced amounts of condensed chromatin (Staley et al., 1967).

Organ cultures of primary neuroblastoma tumors or lines derived from the tumor retain similar differentiated features. Plasma clot cultures of neuroblastoma exhibit a halo of neurite outgrowth and resemble cultured sympathetic ganglia. Cultures from tumor biopsies can be useful in the diagnosis of neuroblastoma (Murray & Stout, 1947). The ultrastructure of organ cultures of neuroblastoma resembles that of the original biopsy (Lyser, 1974). In long-term culture some more differentiated morphology may develop (Kadin & Bensch, 1971; Goldstein & Bradshaw, 1972; Goldstein et al., 1964). Continuous cell lines with immature ultrastructure have also been reported (Barnes et al., 1981).

Catecholamine synthesis in tumors is variable and some tumors express cholinergic characteristics in place of or in addition to adrenergic enzymes. Most neuroblastomas exhibit tyrosine hydroxylase activity and the large amounts of dihydroxyphenylalanine (DOPA) and dopamine released from the tumor mass lead to urinary excretion of catecholamines and their metabolites, vanillactic acid or 3-methyl tyrosine and homovanillic acid. More highly differentiated neuroblastoma tumors, ganglioneuromas or pheochromocytomas also produce amounts of norepinephrine by B-hydroxylation of dopamine catalyzed by DBH. Metabolism of norepinephrine or epinephrine releases vanillmandelic acid. The pattern of metabolite excretion is diagnostically valuable (Jones & Campbell, 1976).

A poor survival prognosis is correlated with higher levels of DOPA metabolites, 3-methyltyrosine or vanillactic acid, and dopamine metabolite, homovanillic acid, relative to the norepinephrine metabolite homovanillic acid. This suggests that the biochemically more immature tumor may be associated with a more aggressive clinical course (Romansky et al., 1978; Laug et al., 1978). With maturation of the tumor to a benign ganglioneuroma, increased norepinephrine content of the tumor is measured (Bohoun & Comay, 1970).

The ultrastructural detection of increased numbers of neurosecretory granules is positively correlated with differentiation of neurotransmitter synthesizing ability of the tumor. Tumors judged by both criteria to be more advanced correlate with a more favorable prognosis (Greenberg et al., 1969; Romansky et al., 1978). The expression and nature of neurite processes is not correlated with the biochemical maturity or prognosis (Romansky et al., 1978). This may be related to the independent developmental appearance and regulation of these neuronal characteristics.

One intriguing clinical aspect of neuroblastoma is the phenomenon of spontaneous regression in which malignant tumor cells, by an as yet undetermined process, adopt a more benign course and become ganglioneuromas (Cushing & Wollbach, 1927; Beckwith & Perrin, 1963). During this regression cells assume a morphologically more differentiated phenotype (Beckwith & Martin, 1968) and biochemically decrease catecholamine production as evidenced by reduced excretion of metabolites in the urine (Evans et al., 1971). The frequency of this regression is higher than that of other malignancies (Evans et al., 1971). The incidence of neuroblastoma in fact is larger than estimated from the clinically manifesting cases. A high incidence of regressed tumors was discovered upon autopsy of children who had died of unrelated causes (Beckwith & Perrin, 1963). This spontaneous regression to a more benign form may be related to the early developmental stage and further suggests that the immature tumor cells have the capacity for progressive differentiation under the influence of as yet unidentified factors in vivo. This has stimulated interest in culturing neuroblastoma cells as a means of investigating the cellular and molecular processes involved in regression as well as possibly using neuroblastomas as a source of developmentally early stage sympathoblasts.

INTRODUCTION

Neuroblastomas could arise from neoplastic events in cells at a variety of early developmental stages and as such may be a potential source of neuronal material with which to study early neuronal differentiation. As the tumors apparently arise from the neural crest, an embryonic structure which undergoes extensive migration within the embryo to supply cells for many diverse tissues, transformation may result in cells following aberrant migratory routes and settling in ectopic sites where they do not receive or fail to respond to normal developmental signals. Such cells might continue to proliferate and may metastisize to other sites perhaps related to the continuation of the normal embryonic migratory process. However, if neuroblastoma develops by the transformation of the cells to a malignant phenotype due to extensive chromosomal damage, loss, mutation or rearrangement, the ability to influence the expression of differentiated functions in the cells by addition of factors in culture would be severely limited. As the lines used in this study contain near diploid chromosomal complements (Beidler et al., 1980), and have been shown to variably express neuron specific enzymes (Ross et al., 1981), they present a more promising model than the extensively used mouse neuroblastoma which has modal chromosomal content in the tetraploid range.

NEUROBLASTOMA CELL LINES

The most widely studied neuroblastoma cell line studied to date is the C1300 mouse neuroblastoma derived from a spontaneous tumor which arose as an abdominal mass in a Jackson mouse in 1940 (Dunham et al., 1953). It was passaged as a subcutaneous tumor in mice and later was established in culture (Augusti-Tocco & Sato, 1969). Several lines and clones have subsequently been developed and studied (Schubert et al., 1969; Klebe & Ruddle, 1969; Seeds et al., 1970). These sublines and clones vary in their morphology, most notably in

the ability to extend neurite-like processes and the levels of neurotransmitter synthesizing enzymes. The major classes with respect to neurotransmitter synthesizing enzyme activity are adrenergic, cholinergic, inactive and mixed function (Amano et al., 1972).

The C1300 neuroblastoma and the lines derived from it express some differentiated features which can be manipulated in culture. The elaboration of of neuritic processes or elevation of levels of neurotransmitter synthesizing enzyme activity have been observed by treatment of the cells with a variety of agents. Increased numbers of neurites are stimulated by treatment of the cells with a variety of agents including cyclic AMP (Prasad & Hsie, 1971; Furmanski et al., 1971); bromodeoxyuridine (Schubert & Jacob, 1970), serum withdrawl (Seeds et al., 1970), hypertonic medium (Ross et al., 1975), serum type (Prasad, 1978). A large number of agents have been reported to influence neurite outgrowth in C1300 (see Burmeister, 1982). No common mechanism for the actions of the various agents is apparent and it is likely that neurite outgrowth can be stimulated via effects at several cell levels. Cessation of cell division while it enhances neurite outgrowth (Schubert et al., 1971; Prasad, 1972; Klebe & Ruddle, 1969), is not prerquisite for the extension of processes (Prasad & Hsie, 1971; Schubert & Jacob, 1970).

The stimulation of neurite outgrowth in C1300 by cyclic AMP (Prasad & Hsie, 1971), bromodeoxyuridine and serum-free medium (Schubert, 1971) involves protein but not RNA synthesis. Low serum (Schubert, 1971) or serum withdrawl (Seeds et al., 1970) causes neurite extension without protein synthesis. The different metabolic requirements reported may reflect different cell types present in the original tumor and selection for different populations over time. Alternatively, the cells may be capable of neurite extension and stimulation at one of several levels may be sufficient to enhance this capability.

The processes elaborated by C1300 cells contain numerous microtubules neurofilaments and variable content of organelles including ribosomes,vesicles mitochondria, endoplasmic reticulum. The vesicle population includes dense core and clear varieties of 150-200 nm annd 40-60 nm, respectively. Primitive junctions between cells have been observed but no synaptic morphology found (Ross et al., 1975).

Neurotransmitter synthesizing enzymes, tyrosine hydroxylase (TH), dopamine-β-hydroxylase (DBH) and choline acetyltransferase (CAT) are present in the various C1300 lines. Elevation of TH by cyclic AMP analogues involves actinonycin D and cycloheximide sensitive processes (Waymire et al., 1972; Richelson, 1973). DBH is elevated concomitantly with TH by cyclic AMP treatment (Waymire et al., 1978a). This induction occurs independently of analogue effects on cell division (Waymire et al., 1978b).

The increased TH and DBH activity involves increased Vmax and immuntitration indicates that at least part of the increase is due to de novo synthesis of TH molecules. TH also exhibits a reduced Km for pteridine cofactor while Km for substrate is unaltered (Waymire etal., 1978a). Lysine deprivation prevents expression of enzyme induction by cyclic AMP. When lysine is added back to cyclic AMP treated cultures along with actinomycin D, the elevation of TH is observed. This indicates that cells accumulate specific transcriptional products associated with the cyclic AMP-induced increase in TH (Waymire et al., 1979).

Though the term differentiation was initially used to describe the changes in neurite outgrowth or increased enzyme levels, this may not be accurate. Many effects are reversible and do not necessarily represent a progression toward a more mature and stable phenotype. As

cells usually express some neurite extension or enzyme activity even in control conditions the changes are mainly quantitative in nature. The alterations may more closely resemble regulatory processes functioning in cells at a variety of stages rather than "differentiation".

In comparison to the vast literature available on experimental studies with the mouse neuroblastoma, C1300, relatively little has been done with human neuroblastoma in culture. Human lines have been derived from a number of separate tumors (Tumilowicz et al., 1970; Biedler et al., 1973; Biedler & Helson, 1974; Kobler & Goldstein, 1974; Ruffner, 1976; Schlessinger et al., 1976; Gerson et al., 1977; Seeger et al., 1977; West et al., 1977; Sekeguchi et al., 1979). These lines exhibit several similarities, most striking among these is the presence of a near diploid karyotype and the presence of distinctive marker chromosomes. The markers are stable and unique to each line, which facilitates the identification of neuroblastoma cells from potential contaminating cell lines (Herschman et al., 1978).

These lines have been derived from primary tumors, bone marrow biopsies or peripheral blood and have been characterized according to karyotype, expression of neurotransmitter synthesizing enzymes and morphology. However, no comprehensive study including cytogenetic, biochemical and morphologic parameters has emerged. Also, in contrast to C1300, relatively little has been done with experimental manipulation of the culture environment in attempts to enhance the expression of differentiated characteristics and use the human cell lines as a probe for the study of developmental or regulatory processes. Much emphasis in the literature to date relates to the use of culture as a clinical tool with which to facilitate, explain and improve diagnostic parameters and suggest or evaluate new therapies (Murray & Stout, 1947; Prasad, 1975; Helson, 1981; Reynolds & Perez-Polo., 1981).

A few studies have used the human lines to probe developmental processes. For example, the surface glycoproteins in normal and tumor cells differ, tumors containing more complex oligosaccarides than normal cells (Glick et al., 1973; Glick et al., 1976). In neuroblastoma cells induced to form neurites, the glycoprotein pattern consists predominantly of glycoproteins with less complex oligosaccharides resembling that of normal cells. The ability of tumor cells to differentiate is correlated to the glycoprotein pattern observed (Glick, 1978). Using the CHP-134 line, a membrane glycoprotein has been isolated which is related to the development of electrically excitable membranes in this line (Littauer et al., 1979; Glick et al., 1980).

Human line, SY5Y has been reported to have NGF responsivity with respect to neurite outgrowth (Perez-Polo et al., 1979) and protection from toxic effects of 6-hydroxydopamine (Tiffany-Castiglioni et al., 1981). The NJB line also exhibits responsivity to NGF expressed in increases levels of tubulin and neuron specific protein 14-3-2 (Kobler et al., 1974). The response to NGF in NJB cells is enhanced by mitomycin C and phorbol esters though the mechanisms of such interactions remain unclear (Goldstein et al., 1980). These examples, while few, demonstrate the potential usefulness of neuroblastomas for study of the molecular and cellular processes associated with aspects of neuronal differentiation.

The distinctive chromosomal markers associated with each human line appear to be retained with passaging and cloning (Herschman et al., 1978). Regions of the chromosome which lack longitudinal differentiaton with cytogenetic banding methods, homogenously staining regions or HSRs, have been observed in several human neuroblastomas. No preferential location for the HSRs is observed but the marker is consistent in all cells of the line. (Biedler et al., 1976; Spengler & Biedler, 1979; Balanban-Malenbaum & Gilbert, 1977; Seeger et al.,

1977; Herschman et al., 1978). HSRs have been exclusively found in cell lines and not in primary tumors (Brodeur et al., 1981). Double minute chromosomes are present in both continuous cell lines and primary tumors (Brodeur et al., 1981). Some work following the appearance of double minutes and HSRs with cell passage and cloning suggest that these two chromosomal anomalies mmay represent alternate expression of the same phenomenon (Balanban-Malenbaum & Gilbert, 1977; Biedler et al., 1980). Double minutes chromosomes in mutants neuroblatoma lines are associated with multiple drug resistance (Baskin et al., 1981).

HSRs have also been observed in a drug resistant line of Chinese hamster ovary (CHO) cells. Cells selected for resistance to the toxic action of methotrexate or methasquin, antifolate drugs, exhibit cross resistance to other antifolates. All resistant cells exhibited HSRs (Biedler & Spengler, 1976). Resistance to the drug was shown to be associated with high levels of dihydrofolate reductase (Biedler & Spengler, 1976), and the HSRs apparently represent regions of gene amplification for this enzyme (Biedler et al., 1980).

No correlation between the the neuroblastoma HSRs and a biochemical abnormality related to its phenotype has been deciphered as yet (Biedler et al., 1980; Ross et al., 1980). Analysis of cell hybrids formed by the fusion of a tyrosine hydroxylase-deficient C1300 line with the human SK-N-BE(2) line is currently in progress to map the locus of the TH gene and assess the relation of HSR to its expression in the human line (Biedler et al., 1980).

The most consistent cytogenetic abnormality observed in a study of 24 neuroblastoma tumors and lines preferentially involves the short arm of chromosome 1. In the 20 near diploid tumors, 14 exhibited some abnormality associated with this site. In 11 of the 14 cases a specific deletion of the 1p32-1pter region is found, producing

monosomy for this genetic material. Of the remaining three cases, one had a translocation of 1p and 12p, a second had an insertion at band 1p13 and a third an extra band at 1p36. This is the first report in neuroblastoma of a chromosomal abnormality that may be common to a class of neuroblastoma tumors (Brodeur et al., 1981).

The significance of chromosomal abnormalities to specific biochemical defects or neoplastic progression are not known. However, the human cell lines present a means of evaluating biochemical expression in cells which appear to maintain stable karyotype. In addition, the near diploid karyotype suggests an advantage over the C1300 lines for the further investigation of developmental and regulatory phenomena in neuronal cells.

The SK-N-BE(2) cells exhibit a modal chromosome number of 44 and contain HSR regions (Biedler & Helson, 1974). Cells also exhibit a metaphase chromosome anomaly in short arm of chromosome 1 (Brodeur et al., 1981). The SK-N-SH cells have a modal chromosomal content of 47 and exhibit no HSRs or double minute chromosomes (Biedler et al., 1973). No abnormality is associated with the short arm of chromosome 1 (Brodeur et al., 1981), but 50-70% of the cells exhibited a gain in the long arm of chromosome 1 (Biedler et al., 1980). Both the BE(2) and SH lines are trisomic for the distal portion of the q arm of chromosome 17. Whether these same abnormalities are present in the clonal lines SK-N-SH-SY5Y and SK-N-BE(2)-M17 used in this study has not been determined directly.

The biochemical characterization of human neuroblastoma lines reveals four main types with respect to neurotransmitter synthesizing enzyme activity. Adrenergic, cholinergic, inactive and mixed function lines have been described (Herschman et al., 1978).

The SH line possesses variable levels of the adrenergic enzymes

tyrosine hydroxylase (TH), dopa decarboxylase (DDC), and dopamine-β-hydroxylase (DBH)(Ross et al., 1980). However, if ^{3}H-choline or ^{3}H-tyrosine are added to the cell cultures, synthesis of ^{3}H-acetylcholine and ^{3}H-dopamine is observed. Similarly the BE(2) line exhibits dopamine and acetylcholine synthesis (Biedler et al., 1978) despite the lack of detectable choline acetyltransferase activity in activity assay of the homogenates (Ross et al., 1980). These discrepancies may be related to the senstivity of the assay or to the heterogeneity of the cell population in the original line. No norepinephrine synthesis is detectable (Biedler et al., 1978), despite presence of DBH in both lines (Ross et al., 1980). The lack of intracellular DBH synthetic activty may be related to defective localization of the enzyme as has been suggested from analysis of primary neuroblastoma (see discussion section).

Analysis of clones derived from the SH line reveals the presence of cells in the population with different expression of neurotransmitter synthesizing enzymes. The SH-SY clone possesses high DBH, low TH and low CAT activity, while the SH-IN has detectable CAT, low TH and DBH. The SH-EP clone has no detectable adrenergic enzyme activity but some CAT activity (Ross et al., 1980). The ability to synthesize dopamine from ^{3}H-tyrosine is exhibited in the SY-IN and EP clones as is synthesis of acetylcholine from its precursor (Biedler et al., 1978).

The BE(2) clones also exhibit varation in the quantitative expression of enzyme. Tyrosine hydroxylase activity measured in homogenates ranges from 1.5 nmoles/hr/mg to 46.7 nmoles/hr/mg. DBH ranges from 0 to 9.6 nmoles/hr/mg protein. Dependent upon the proportions of each cell type in the original line in a given culture, the synthetic activity will vary.

The clones derived from a line may represent distinctive types

present in the tumor or variants which develop in culture. The SH line contained at least two distinctive cell types as revealed by karyotype analysis. A structurally abnormal chromosome was observed in 35% of the SH-EP cells examined but not in the thrice cloned SH-SY5Y (Ross et al., 1980).

Subclones of the EP cells retain the isochromosome marker 1q but exhibit morphologic interconversion from a flattened epithelial-like form to a neuroblast-like type. Subclones which morphologically transform to the neuroblast form also acquire adrenergic enzyme activity not detected in the epithelial types. The basis and significance of this bidirectional interconversion between the two cell types remains to be determined (Ross et al., 1980). It suggests, however, that significant clonal variation in enzyme activity may occur over time in culture and be responsible for a loss or gain in enzyme activity. Interconversion occured over several months time if cells were allowed to remain for long period between transfers.

In order to avoid variation due to the heterogenous cell population in the original lines, two clonal lines were selected for this work. The levels of enzyme activity in early vs. late experiments did not vary nor were morphological changes evident during the course of the experiments. The observed variations in morphology and enzyme activity are probably not due to selection of subpopulation or to the interconversion of cell types.

The SY5Y clone was selected for these studies since it exhibited a neuroblast morphology and was reported to respond to NGF by the elaboration of neurites (Perez-Polo et al., 1979). The cells had been characterized biochemically and shown to have low levels of TH and high DBH in fetal calf serum containing medium (Ross et al., 1980). Karyotype analysis (Biedler et al., 1980) and ultrastructural description (Barnes et al., 1980) of the parental lines had been

reported. It was of interest to determine if the morphological changes observed with NGF were accompanied by changes in enzyme activities.

The M17 clone was selected for its high tyrosine hydroxylase activity as a positive control for the enzymatic and immunocytochemical assays of this enzyme. Also, since the SY5Y basal levels of TH were so low, the effects of factors on this enzyme might not be observed. If the low basal levels were due to mutation in the gene for TH or asociated regulatory genes, no modulation would be possible. The M17 was selected as an alternative cell line which might prove useful in characterizing the effects of NGF and cyclic AMP on TH expression. This line has not been characterized previously in any medium other than that with serum supplementation.

No ultrastructural descriptions of the clonal populations have been reported. The parental lines exhibit ultrastrucutral features characteristic if early neuroblast stages with few dense core vesicles and neurites under standard culture conditions. The developmental stage of clonal populations was evaluated on the basis of enzyme expression and ultrastructural morphology. The influence of added NGF and cyclic AMP on these parameters was also studied.

DEFINED MEDIUM

The addition of serum to the culture medium is required to support the growth of most cells in culture, however the serum factors responsible are largely unknown. Recently, formulations of defined media have been developed which remove the serum requirement and replace it with hormonal and other factors. Not only can the complications of the unknown composition of serum be avoided, but in addition the medium exhibits selectivity in the cell type supported (Bottenstein & Sato, 1979; Barnes & Sato, 1980; Sato, 1980; Mather &

Sato , 1979). The removal of serum allows for a clearer evaluation of factor effects on neuronal expression in the neuroblastoma lines. A medium specific for rat neuroblastoma had been developed (Bottenstein & Sato, 1979) and has subsequently been shown to selectively support the growth of other neuronal but not nonneuronal cell types (Bottenstein, 1980; Zeevalk et al., 1982 submitted for publication).

Using the N2 defined medium, it was established that the SY5Y and M17 lines survived and retained the enzymes of interest (Lyser et al., 1980; Burmeister et al., 1980). Further work characterized the quantitative levels of tyrosine hydroxylase and/or dopamine-β-hydroxylase as compared to FCS supplementation. Also the ultrastructural features were studied and compared to those found in FCS cultures.

A defined medium may permit expression of a potentility or response to added factors that is antagonized or prevented by serum. The role of potentiating factors for a given response can be more clearly identified in defined medium. In this regard, the effects of both cyclic AMP and NGF on morphology, neurotransmitter synthesizing enzyme activities and ultrastructure were compared to those observed in 10% FCS supplemented medium.

MATERIALS AND METHODS

CELL LINES The SK-N-SH-SY5Y and SK-N-BE(2)-M17 human neuroblastoma cell lines were the generous gift of Dr. June Biedler (Sloan-Kettering Cancer Research Institute, New York). Some additional SY5Y cultures, which exhibit a morphological response to NGF, were obtained from Dr. Douglas Ishii (Columbia College of Physicians and Surgeons, New York). While the cultures differed with respect to morpholgical response to NGF, no significant variation in the levels of neurotransmitter synthesizing enzyme activity were observed. In addition, the cells responded similarly to defined medium and added factors with respect to TH and DBH activity.

SY5Y cultures from transfers T30-T46 were used for the experiments described. Cultures from T64-67 were used for some early determinations of growth in defined medium and determination of neurotransmitter synthesizing enzyme activity. No significant differences in levels of DBH were noted between the different cell passages used.

M17 cultures from T60-T80 were used for experiments discussed. No change over time was observed in overall culture morphology or levels of tyrosine hydroxylase activity.

All cell cultures were demonstrated to be mycoplasma free by the uridine phosphorylase method (Levine, 1972). The assay was generously performed by Dr. James Donegan (Hunter College of CUNY, New York).

MEDIA FORMULATIONS Basal medium for all conditions was Ham's F12/Dublecco's Modified Eagle's Medium (1:1, v/v).

10 % FCS This medium contained 45 % F12, 45%, DME and 10 % fetal calf serum (Microbiological Associates).

N2 The following ingredients were added to basal medium to give final concentrations indicated: insulin (Eli Lilley, Iletin U 100) 5 μg/ml, progesterone (Sigma, in 0.01% ethanol/F12:DME) 20 nM, putrescine (Sigma, diluted in F12:DME) 100 μM, sodium selenite (Bacto or Alfa chemical, diluted in F12:DME) 30 nM, transferrin (human, iron-free, Sigma, diluted in F12:DME) 100μg/ml. (Bottenstein & Sato, 1979). Bacto selenium provided for consistent cell growth while the Alfa Chemical product was sometimes toxic to cells.

N2A This medium contained the same factors as the N2, but selenium was eliminated.

FACTORS All factors for the defined medium were stored as 100 X stocks at 4°C and added fresh to medium just prior to use.

The following were added to 10 % FCS or N2 medium as indicated in the results section. The final concentration employed is noted.

cyclic AMP = N^6, O^{21}-dibutyryladenosine 3′:5′ cyclic monophosphate, Sigma, 1 mM, in F12:DME

8-bromo cyclic AMP = 8-bromoadenosine 3′:5′cyclic monophosphate, Sigma, 1 mM, in F12:DME

IBMX = 3-isobutyl-1-methylxanthine, 0.25 mM dissolved in dimethylsulfoxide and diluted 1:1 with basal medium prior to addition to culture medium

butyrate = butyric acid, Sigma, 1 mM

NGF Nerve growth factor from various sources was used. No differences in response of the cells was noted.

2.5Sβ-NGF was generous gift of Dr. Mark Bothwell (Princeton University, New Jersey). Someβ-NGF was also purchase from Collaborative Research (Waltham, Mass.). This was used at concentrations of 10-100 ng/ml as indicated.

7S NGF was generously supplied by Dr. J. Reginio Perez-Polo (University of Texas Medical Center, Galveston). The 7S NGF was used at concentrations of 250-500 ng/ml which is equivalent to 50-100 ng/ml β-NGF.

NGF stocks, at concentrations of 5, 10 and 100 μg/ml in 2 % bovine serum albumin, were stored frozen at -70^{o}C until use.

ANTIBODIES Rabbit antibodies raised against bovine adrenal trypsin-treated tyrosine hydroxylase, purified bovine adrenal dopamine-β-hydroxylase, preimmune sera and rabbit peroxidase-antiperoxidase complex were the generous gift of Dr. Tong Joh (Cornell University Medical School, New York). Antibodies were stored frozen at -70^{o}C in 20% goat serum 0.2 M Tris buffer pH 7.6.

Goat anti-rabbit IgG was obtained from Miles-Yeda, Ltd.. This was stored as frozen stock at -70^{o}C and diluted in 1 % normal goat serum in 0.2 M Tris-saline buffer pH 7.6.

3',3'-diamonobenzidine HCl was obtained from Baker chemical.

CELL CULTURE PROTOCOLS

STOCK CULTURES The SY5Y and M17 cultures were maintained in 45 % F12, 45 % DME, 10 % fetal calf serum in an atmosphere of 95 % air 5 % CO_2 at 37^{o}C. Cells were passaged weekly. Monolayers

were dissociated by incubation with 1 ml 0.02 % EDTA in Ca^{++}-Mg^{++}-free phosphate buffered saline. Complete medium was added (9ml) and cells triturated to break up clumps. Cell suspension, 1ml, was plated into 9 ml complete medium in T25 flask. Cultures were fed once at 3 days after transfer by a 70% change of medium.

LONG TERM GROWTH EXPERIMENTS Cells used for the study of effects of long term growth in N2 medium were passaged in experimental medium. Cells were dissociated in 0.02% EDTA in CMF-PBS and cell suspension diluted in experimental medium. Cells were plated onto serum coated T25 flasks. The serum coated flasks were prepared by incubating plates with 10% FCS medium at 37°C for 15-30 minutes. Plates were rinsed with two changes of serum free medium and were used immediately or stored at 4°C until use. Storage of coated plates up to two weeks did not affect cell attachment.

PREPARATION IF CELL CULTURES FOR DETERMINATION OF NEUROTRANSMITTER SYNTHESIZING ENZYME ACTIVITY Cells were plated out in F12/DME 10 % FCS for 24 hours to allow for attachment and then switched to experimental conditions on day 0 by complete replacement of the medium. The initial plating density used was 2-3 X 10^5 per 60 mm plate. Cell number was determined by hemocytometer counts of trypan blue excluding cells. All cultures were fed on day 2-3 of the experiment and harvested on day 4-5.

Cells were collected for enzyme assay by dissociation with 0.02% EDTA in CMF-PBS. Complete Dulbecco's PBS was added and cell suspension spun in clinical centrifuge at speed 6 (approximately 7500 rpm) for 5 minutes. Pellets were resuspended in PBS and recentrifuged. All solutions used during harvest were cold and cell suspensions were kept on ice. Pellets were frozen at -20°C until assayed. In some experiments cells were harvested in complete

PBS only and removed from the substrate by fluid stream jetted from a pasteur pipette. No differences in activity were noted with the two procedures and all data has been grouped together for averaging.

GROWTH CURVES - M17 Cells were plated into 35 mm tissue culture plates in F12/DME with 10 % fetal calf serum overnight to allow for attachment of the cells. Medium was completely replaced with experimental medium. This was considered day 0 of experiment. Cultures were fed again on day 3 of the experiment.

Counts were done in triplicate at each time point. Trypsin, 0.05 % in 0.02% EDTA was added to cultures for 3-5 munutes. The action of trypsin was stopped by addition an equal volume of soy bean trypsin inhibitor 0.05% in PBS. Cell suspension was diluted with PBS. Trypan blue, 0.4 % in 0.9% saline, was added to a final dilution of 1:6 to the cell suspension. Viable cell counts were determined on a hemocytometer.

The growth curves were plotted as log cell number vs. time to permit determination of population doubling time. Population doubling times were determined from three separate growth curves. These were averaged and compared for significant differences by Student's T-test.

GROWTH CURVES - SY5Y The data from experiments performed by Donald Burmeister were used to develop growth curves for SY5Y. Cells were plated out in 10% FCS medium at initial concentration of 1 X 10^5 cells /35 mm dish. After 24 hours, medium was completely removed, monolayers rinsed with F12:DME, and experimental medium added. Due to the problem of fragile attachment in N2 medium, cultures were not fed during the course of the experiment.

Cell counts were performed on triplicate cultures at each time point. Cells were dissociated in 0.02 % EDTA in Ca^{++},

Mg^{++}-free phosphate buffered saline containing 0.02 % trypan blue. The percent viability was determined for the first sample from each dish. For remaining counts, total cell count was enumerated since viability declined over time. The number of viable cells was determined from the percent viability and total cell counts for each plate. These procedures, while cumbersome, helped to insure single cell suspensions for counting. Other dissociation procedures did not allow for adequate dispersion of cell clumps to permit accurate determination of cell number (Burmeister, 1982).

The log of cell number was plotted vs. time and population doubling time determined from at least three separate experiments. These doubling times were averaged and compared by the Student's T test.

EFFECTS OF CELL DENSITY ON ENZYME LEVELS IN M17 Cells were plated in parallel to those used for growth curve. The initial plating density was 5×10^5 per 60 mm plate. Cells were switched on day 0 from 10 % FCS medium to experimental conditions by complete replacement of the medium. Cultures were fed at 2 day intervals for the duration of the experiment. Cells were removed from the dish by trituration with PBS, pelleted and stored in polystyrene freezer vials at -20^0C until assayed.

CELL CULTURES FOR ELECTRON MICROSCOPY Cultures were plated out in parallel to those on which enzyme levels were to be determined. Initial plating density of 5×10^5 was used and cells grown for 5 days prior to fixation. Medium was removed and 2.5% glutaraldehyde added directly to the monolayer to begin fixation.

CELL CULTURES FOR IMMUNOCYTOCHEMICAL STAINING Cells were plated onto polystyrene or glass coverslips. In cases indicated, the surface was coated with poly-D-lysine just prior to plating. To

prepare the coated surface 0.5 ml of 0.01 % poly-D-lysine solution in distilled water, sterilized by Millipore filtration, was added to cover slip for 15 minutes. Solution was removed and coverslips rinsed in sterile distilled water twice.

A 0.5 ml volume of the cell suspension, containing about 6×10^5 cells, was added to the cover slip and incubated for 1-2 hours to allow cells to attach to the substrate. Then 1.5 ml of medium was added to the culture dish. Cells were grown for 1-3 days before fixing.

The cultures in N2 were grown in experimental medium for one week prior to seeding onto cover slips. FCS cultures were plated onto both polylysine coated coverslips and uncoated tissue culture cover slips. The cells in N2 generally did not remain attached to serum coated substrates through the many rinses required for fixation and antibody staining. However, in a few cultures where sufficient numbers of cells were preserved on serum-coated substrates the immunocytochemical staining was similar to poly-D-lysine substrate. The growth on poly-D-lysine coated substrate did not interfere with immunocytochemical staining.

ASSAYS FOR NEUROTRANSMITTER-SYNTHESIZING ENZYMES

DOPAMINE-β-HYDROXYLASE

Dopamine-β-hydroxylase activity was assayed by a modification of the double enzyme assay of Molinoff, 1971.

Frozen cell pellets were homogenized in 5 mM potassium phosphate buffer, pH 7.0, with 0.2% triton X-100. Aliquots were removed for assay of cell protein and the remaining homogenate centrifuged at 3500-4000 rpm for 15 minutes. The supernatants were assayed for DBH

activity.

Optimal copper concentrations for each batch of cultures were determined by assaying activity over a range of $CuSO_4$ concentrations and deriving a concentration curve. The remaining samples were brought to similar protein concentrations and activity assayed at optimal $CuSO_4$. Sample copper curves for N2 and FCS are illustrated (Fig a).

To 50 µl of supernatant, cocktail containing 5 µl of $CuSO_4$ at appropriate concentration, 5 µl ascorbate 10.5 mg/ml, 5 µl fumarate 0.5 M, 4 µl pargyline 1.712mg/ml, 2.5 µl 1 M sodium acetate buffer pH 5.5, 1 µl catalase , 2.5 µl tyramine 0.03 M. The reaction was run for 30 minutes at 37°C.

The first reaction was stopped by addition of a cocktail containing 15 ul Tris 1M pH 8.6, 2.5 ul ^{14}C-SAM (S-adenosyl [^{14}C]methionine, New England Nuclear specific activity 53 mCi/mmole), 2.5 µl EDTA 5 mg/ml, 2.5 µl phenylethanolamine-N-methyltransferase and 2.5 µl H_2O. The PNMT reaction was run for 30 minutes at 37°C and stopped by the addition of 0.4 ml of 0.5 M borate buffer, pH 10.

The N-[^{14}C]-methyl octopamine formed was extracted into 6 ml of isoamyl alcohol:toluene (2:3, v/v) by vortexing for 10 sec. One ml of the organic phase was removed and 10 ml Aquassure added for liquid scintillation counting. In some cases 1 ml of organic phase was dried by evaporation of solvent under air stream in warm oven overnight. The residue was redissolved in absolute alcohol and Aquassure added for scintillation counting. The counts from either procedure were not significantly different.

Blanks consisting of homogenizing buffer were used in all assays

as well as internal standards in which 40 ng of octopamine was added to the reaction mixture.

The activity of DBH was determined from the conversion of octopamine to methyloctopamine per hour per mg of total cell protein.

TYROSINE HYDROXYLASE

Tyrosine hydroxylase activity was assayed by a modification of method of Coyle, 1972 (Joh et al., 1973).

The homogenization procedure was the same as described for DBH.

To 50 µl of homogenate supernatant 25 µl of cocktail was added which contained 7.5 µl sodium acetate 1 M pH 5.8, 5 µl tyrosine at 1×10^{-5} M (for SY5Y) or 2×10^{-4} M tyrosine, 1.5 µl H_2O, 5.0 µl ^{14}C-tyrosine (New England Nuclear), 5 µl DL-6-methyl-5,6,7,8-tetrahydropteridine (6-MPH_4) diluted in 3% mercaptoethanol to final concentration in reaction mixture of 10^{-3}M, and 1 µl catalase. Tubes were incubated at 30°C for 20 minutes. The reaction was stopped by addition of 1 ml 0.4 N perchloric acid containing 10 ug of L-DOPA. After centrifugation at 3500-4000 rpm for 15 minutes supernatants were removed and 6.5 ml of 2 % EDTA/0.35 M potassium phosphate monobasic (5:1.5, v/v) was added. Samples were stirred and rapidly brought to pH 8.7 by addition of sodium hydroxide. Samples were then poured over alumina oxide columns, washed with 20 ml of water. The ^{14}C-DOPA was eluted from the column with 0.5 M acetic acid. Aquassure, 15 ml, was added and radioactive product determined by liquid scintillattion counting. Conversion of ^{14}C-tyrosine to ^{14}C-DOPA was determined by comparison of radioactivity to that of cocktail.

Activity was expressed as nmoles ^{14}C-DOPA formed per hour

per mg total cell protein.

PROTEIN DETERMINATION Total cell protein of homogenate before centrifugation was determined by the Lowry Method (1951).

IMMUNOCYTOCHEMISTRY

SY5Y and M17 cells were immunocytochemically stained by the unlabelled antibody peroxidase-antiperoxidase method as described by Sternberger (1979).

Cells were fixed on culture cover slips by the addition of buffered formalin for 1/2 hour followed by several rinses and overnight incubation in phosphate buffered saline. Dehydration through a graded ethanol series, 50%, 70%, 95%, 100% was followed by rehydration 100%, 95%, 70%, 50% and Tris-saline (0.2M pH 7.6, 0.9 % NaCl). Coverslips were then transferred into 0.25 % triton X-100 in Tris-saline for 30 minutes. This was followed by two rinses in Tris-saline.

Cultures were then incubated in normal goat serum 1/30 dilution in Tris-saline for 30 minutes.

Incubation with primary antiserum was done overnight, usually for 24 hours, in cold. The dilutions of antisera used were: anti-DBH, 1:1000; anti-TH, 1:500, 1:1000, 1:2000; preimmune serum, 1:1000. M17 cells exhibited specific staining with TH antisera at all dilution while SY5Y failed to stain at any dilution.

After warming to room temperature, primary antiserum was removed and cultures rinsed in 1 % normal goat serum in Tris-saline. Incubation with the linking antiserum, goat anti-rabbit immumnogammaglobulin at 1:100 dilution was followed by four 5 minute

rinses in 0.2 M Tris-saline with 1 % normal goat serum..

Cultures were incubated in presence of rabbit antiperoxidase complex (PAP) diluted 1:100 in Tris-saline buffer with 1 % goat serum for 30 minutes at room temperature. This was followed by four 5 minute rinses in Tris-saline and two rinses in 0.2 M Tris-HCl pH 7.6.

Cover slips were immersed in a freshly prepared solution of diaminobenzidine hydrochloride 0.05% with 0.01% hydrogen peroxide in Tris-HCl buffer 0.2M pH 7.6 for 15 to 30 minutes. This was followed by several rinses of the cover slips in water. The cells were dehydrated through 50%, 70%, 95%, 100% ethanol. Coverslips were mounted in aqueous mounting medium (Aquamount).

For cultures sectioned after staining, cover slips were dehydrated, cut into small pieces and embedded in Epon. Alternatively, cover slips were floated cell side down on top of Epon and polymerized at 60°C for 24 hours. Cover slip plastic could usually be removed leaving cells embedded in Epon. This facilitated sectioning.

Thick sections, 3 μm, were cut to demonstrate intracellular localization of antibody reactivity. Some sections were counterstained with toluidine blue, 1% in 1% borax solution, to demonstrate nuclei.

ELECTRON MICROSCOPY

Cultures were fixed by addition of 2.5% glutaraldehyde in 0.1 M phosphate buffer pH 7.2 with $CaCl_2$ directly to the culture plate. Fixation in glutaraldehyde for one hour was followed by several rinses in 0.1M phosphate buffer pH 7.2 and postfixation with

osmium tetroxide 1% in phosphate buffer o.1M pH 7.2 for 1/2 hour. Cell cultures were rinsed several times in phosphate buffer and then dehydrated by passage through a graded series of ethanol, 50%, 70%, 95%, 100%. An Epon/ethanol mixture (1:1, v/v) was added for one hour and then drained completely from the plate. Fresh Epon was added and allowed to infiltrate overnight and polymerized at 60°C for 24 hours (Luft, 1961).

For sectioning parallel to the substrate, BEEM capsules were inverted and placed onto Epon-coated surface and filled with Epon. After polymerization, capsules could be snapped off removing the layer of cells and leaving plastic behind. For sectioning perpendicular to surface epon embedded monolayers were cut and re-embeded in flat embedding molds. Removal of the culture plastic facilitated sectioning.

Silver and gold sections were cut using a glass or diamond knife on a Porter-Blum MT2 Ultramicrtome and picked up on 200-300 mesh copper grids.

Staining with uranyl acetate was done during the dehydration. Uranyl acetate, 1%, was added to the 70 % ethanol and cultures stained for 1/2 hour. Counterstaining of sections was done with lead citrate (Reynolds, 1963).

Sections were studied with an RCA EMU-3H electron microscope routinely operated at an accelerating voltage of 100 kv using a 50 μm objective aperture.

LIGHT MICROSCOPY

Living cultures were viewed by phase contrast microscopy using a Nikon inverted phase scope.

Fixed cultures were studied on Leitz Ortholux microscope.

Sections of immunocytochemically stained cultures were photographed with the aid of an Omnicon image analyzer programmed for contrast enhancement.

RESULTS

GROWTH AND APPEARANCE OF SY5Y IN N2 MEDIUM

SY5Y cells survived and continued cycling in N2 medium. The growth rate of the population was slower than with serum supplementation. When plated at an initial cell density of 2-3 x 10^4 cells/cm^2, the cells continued exponential growth for up to six days in either N2 or FCS medium (Fig.1). The mean population doubling times determined from several such growth curve studies for cells in FCS and N2 medium were 43 and 74 hours respectively (Table 1). With no serum or hormone supplementation the population declines over the six day period (Fig. 1).

In N2 medium the saturation densities of the cultures at confluence were lower than those reached in cultures maintained with serum. The maximal density observed in FCS cultures was 1.7 x 10^5 cells/cm^2 as compared to maximal density of 1.0 x10^5 cells/cm^2 for N2 cultures. An accurate determination of the saturation density is difficult due to the fragile attachment of the cells to the substrate in N2 medium. Cells were easily dislodged from the substrate during any manipulations of the cultures, e.g. during medium changes, and many cells did not subsequently reattach. Cell counts in N2 medium were consistently lower than parallel FCS cultures. Stationary phase growth could not be maintained under N2 culture conditions. Scanning electron microscope observations suggested that the cell body had fewer sites of attachment to the substrate in N2 as compared to FCS medium. Stable attachment sites become localized mainly at the tips of processes in N2 medium (Burmeister et al., 1981).

Phase contrast microscopy of the cultures revealed changes in the morphology in N2. The cell bodies appeared to be of lesser diameter

and more rounded, perhaps due to the altered attachment to the substrate. Short and long processes extended from cells in FCS and N2 medium (Fig. 6), however, no significant difference in the number or length of processes was found (Burmeister, 1982). Cultures carried in N2 medium for up to 60 days, formed compact and dense clusters rather than forming a monolayer covering the entire available surface (Fig. 4). Cells appear to prefer attachment to other cells rather than to the serum-coated culture dish. The cells maintained in N2 medium became very "sticky" as indicated by difficulty in dissociation with mild procedures, i.e. EDTA and trituration, which allows for adequate dispersion of cells in FCS.

Alternatively, the cell clustering over the long term may indicate that nutritional factors elaborated by the cells provide for selective survival of the cells in close proximity to the cluster foci. Serum could supply similar factors which, when equilibrated throughout the medium, support growth over the entire area rather than in clusters.

Whether any ultrastructural alterations were induced by growth in N2 medium was studied by electron microscopy of the cell cultures. Cells grown in FCS or N2 conditions were qualitatively similar (Figs. 9, 10 & 11). These similarities do not preclude there being some quantitative changes, e.g. in the number of dense core vesicles or profiles of microtubule containing processes. Such quantitative comparisons have not been done on the ultrastructural level.

In SY5Y cells condensed chromatin was found in the nucleus underlying the nuclear envelope. Nucleoli were also prominent. The cytoplasm of SY5Y cells typically contained abundant free ribosomes and a few cisternae of granular endoplasmic reticulum. Rough endoplasmic reticulum, however, was not abundant in these cells under any medium condition. Mitochondria were numerous and Golgi apparati prominent in the cell body. Dense core vesicles were occasionally found in the cell

body most often singly or in pairs (Figs. 9, 10 & 11).

Of particular interest with respect to neuronal characteristics is the morphology of the processes observed by phase contrast microscopy. The cells elaborated processes in both N2 and FCS medium. The appearance of the processes of cells grown in FCS and N2 was similar as seen in longitudinal sections (Fig. 17 & 18) or in cross section (Fig. 20-25). The processes contained parrallel arrays of microtubules as a basic framework. Intermediate filaments, 10 nm, were found in a few processes but the arrangement of the elements in the processes was not representative of mature axons. The processes generally contained ribosomes, though the number varied. Mitochondria, endoplasmic reticulum and dense core vesicles were also found along the length of the processes. Growth cones, the presumptive area of neurite elongation and membrane addition, could be found in favorable sections at the ends of the processes (Fig. 17 & 18). The growth cone areas contained many cytoplasmic organelles including ribosomes, rough endoplasmic reticulum, mitochondria, dense core vesicles and microtubules. The most distal portion of the growth cone consisted mainly of membranous elements.

Dense core vesicles, the putative storage granules for catecholamines in sympathetic neurons, were also observed in the cell bodies and processes of the SY5Y cells in N2 and FCS medium (Figs. 32 & 35). In processes the dense core vesicles were found in small clusters though no localization into varicosities or arrangement suggestive synaptic organization was observed. Though not present in abundant numbers, dense core vesicles were frequently seen in routine scanning of sections and they were easily found in all samples studied. The mean size of the vesicles was 100 nm, with a size range of 70-150 nm. The presence of dense core vesicles in SY5Y is surprising since few were observed in the parental SH line (Barnes et al., 1981). Dividing cells contained dense core vesicles also (Fig. 16).

Whether these dense core vesicles actually contain catecholamine could not be determined by standard electron microscopic observations. Preliminary attempts at histochemical staining using a chromate-dichromate technique, specific for catecholamine, indicate that there may be catecholamine present. Fluoresent techniques, i.e. formaldehyde induced floruescence of cultures, gave ambiguous results, so the question of catecholamine content remains open. The chromate dichromate technique has shown the dense core vesicles observed in cultured human fetal sympathetic ganglia to contain catecholamine. Chromate positive vesicles were observed in sympathetic ganglion cultures maintained in FCS or N2 medium with NGF for up to forty days (Zeevalk et al., 1982, submitted for publication).

LEVELS OF NEUROTRANSMITTER SYNTHESIZING ENZYMES

SY5Y cells were assayed for levels of the neurotransmitter synthesizing enzymes tyrosine hydroxylase (TH) and dopamine-β-hydroxylase (DBH) by measurement of activity in cell homogenates. Log phase cells maintained in N2 medium for four days had two-fold higher DBH specific activity than cells in FCS medium (Table 2).

Cells maintained in FCS medium exhibited a decrease in DBH specific activity as the cells approached confluency. The difference was statisically significant (Table 2). In FCS stationary phase growth was observed at a cell density of 2×10^5 cells/cm^2. Cells maintained for comparable periods or longer in N2 medium failed to exhibit a significant decrease in DBH activity.

These findings indicated that a differentiated function, i.e. expression of neurotransmitter synthesizing enzyme, can be maintained in serum-free supplemented medium. However, the growth and behavior of the cells under these conditions may not be identical to that observed

in FCS. With respect to DBH specific activity a small but statistically significant elevation over levels measured in FCS was stimulated at 4 days after cells were switched to growth in N2 medium. Moreover, the increase was maintained when cultures were passaged in the defined medium (Table 2). The time course of the increase cannot be determined as measurments earlier than 4 days have not been done.

In contrast to the results obtained for DBH activity, the level of TH specific activity measured in cell homogenates was lower for cultures grown in N2 than those in 10% FCS medium (Table 3). The detectable activity in both cases, however, was extremely low and at the limits of sensitivity for the assay. Detection of activity required the most sensitive assay conditions, i.e. 10^{-5} M tyrosine and 10^{-3}M 6-methyl-tetrahydropteridine, the cofactor required for enzyme activity. No activity was detected under assay conditions where the substrate concentration was 2×10^{-4}M or cofactor at 10^{-4}M.

Many of the cultures assayed from either medium failed to exhibit detectable <u>in vitro</u> activity of TH despite the presence of cellular protein as determined by Lowry assay of the homogenate. The low and variable levels of enzyme activity may in part be due to the kinetics of the enzyme. As the Vmax for TH is very low, substantial amounts of protein are required for <u>in vitro</u> activity to be measured. DBH, in contrast, exhibits a high Vmax, and therefore activity is more easily detected by <u>in vitro</u> activity assay. The results presented suggest that the SY5Y cells contain very low levels of TH under FCS and N2 culture conditions. Tests for statistical significance were not performed due to the highly variable data obtained from the assays.

IMMUNOCYTOCHEMICAL STAINING WITH ANTIBODIES TO TH AND DBH

SY5Y cells displayed very faint reactivity with antibody raised against partially purified bovine adrenal TH (Figs. 68b & d). Pale cytoplasmic staining barely better than with preimmune serum was observed throughout the cell. These results were consistent with the low activity measured by in vitro assay of cell homogenates and indicate that the amounts of TH present in the SY5Y were at the limits of detection by both assays.

DBH-like immunoreactivity was localized in the nucleus as well as in the cytoplasm (Figs. 68 e & h; 70 c). The dark peroxidase reaction product could be seen throughout the nucleoplasm in 3 um sections of the stained cells (Fig. 70 d). Whether the staining represented reaction specific to DBH was unclear. Preimmune serum from the same rabbit failed to produce such staining (Fig. 68a). The DBH antiserum preparation may have reacted with some non-specific antigen in addition to DBH.

EFFECTS OF CYCLIC AMP IN N2 AND FCS MEDIA

The addition of cyclic AMP to either N2 or 10 % FCS medium lengthed the population doubling time for SY5Y. The doubling time in N2-cyclic AMP medium was 189 hours as compared to 74 in N2. The population doubling time in 10% FCS was increased to 65 hours as compared to 43 hour doubling time in 10 % FCS. These are both significantly different from the controls (Table 1).

The morphology of the cells in the presence of cyclic AMP was altered dramatically in N2 but remained virturally unchanged in FCS medium. Extensive neurite outgrowth developed within 2-3 days of treatment with either dibutyryl-cyclic AMP or 8-bromo-cyclic AMP in N2 medium (Figs 6b & 8e). The neurites elaborated with cyclic AMP

treatment were long and slender. The number of cells exhibiting processes longer than 100 μm increased from 8.4 to 23 % in N2-cyclic AMP medium. The number of cells with 100 um processes was 8.0 and 7.2 % for FCS and FCS-cyclic AMP, respectively (Burmeister, 1982).

The extensive outgrowth of neurites in response to cyclic AMP suggested the possibility of novel expression of more neuronal processes in the presence of this agent. However, the neuritic ultrastructure of cells grown in N2-cyclic AMP for five days was similar to that observed under control conditions. Despite the increased numbers of long processes, more mature neuronal profiles were not found. Cells in N2-cyclic AMP and FCS-cyclic AMP exhibited typical content of cytoplasmic organelles, similar to that found in controls (Figs 10 & 14; 11 & 15). A typical process from a cell grown in presence of cyclic AMP contained microtubules and ribosomes (Fig. 19).

CYCLIC AMP AND ENZYME EXPRESSION

DBH specific activity was stimulated by cyclic AMP in both FCS and N2 medium. The increase relative to the appropriate control was two- to three-fold in each case (Table 4). The increase was observed whether cyclic AMP levels were elevated by addition of the dibutyryl cyclic AMP or with isobutylmethylxanthine (IBMX), a phosphodiesterase inhibitor. The maximal increase was observed when IBMX (0.25 mM) was included along with cyclic AMP (1 mM), either the 8 bromo or dibutyryl analogue.

Sodium butyrate, tested as a control for the dibutyryl analogue, produced an elevation of DBH activity in N2 but not FCS medium (Table 4). The total cell protein, however, was significantly lowered indicating toxic or growth inhibiting effects of butyrate at 1 mM concentration. 2 mM butyrate was lethal to the cells. Total cell

protein in paired cultures was reduced from 1.0 mg/culture to 0.15 mg/culture in N2 vs N2-butyrate (1mM). The total cell protein in FCS vs. FCS-butyrate (1mM) was reduced from 1.6 mg/culture to 0.45 mg/culture. The action of sodium butyrate on DBH activity may be due to its known effect on intracellular cyclic AMP or to its effect on cell division. Butyrate had a more severe effect on level of cell protein than dibutyryl cyclic AMP while its effect on DBH specific activity was less than that of dibutyryl cyclic AMP. Since the effects found with dibutyryl cyclic AMP can be mimicked by the 8-bromo analogue the role for butyrate in mediating the effects on morphology and enzyme levels seems unlikely. The influence of the 8 bromo-cyclic AMP analogue or its breakdown products on cell division in SY5Y has not been determined, therefore, a primary effect on growth which secondarily causes changes in morphology and enzyme levels cannot be conclusively eliminated as the basis for the observed effects.

The addition of cyclic AMP and IBMX to SY5Y cells in N2 medium enhanced TH specific activity measured in homogenates of the cell cultures. The level of TH specific activity approached that measured in FCS cultures (Table 3). As cells in N2 medium have barely detectable levels of TH, stimulation of the activity to that found in FCS could indicate a deficiency of the N2 medium with respect to maintaining the expression of the enzyme. The difficulty in detecting TH activity in N2 medium seemed to be altered by the addition of cyclic AMP. Though the absolute values for TH specific activity for cells maintained in N2-cyclic AMP remained variable, demonstration of activity was more consistent than in N2 alone. In other words, few cyclic AMP cultures assayed failed to exhibit some detectable TH activity. However, the addition of cyclic AMP to N2 medium allows for a level of enzyme activity, albiet very low, comparable to that found in FCS. The TH activity of cultures maintained in FCS is not further elevated by the addition of cyclic AMP to the medium (Table 3).

The effects of cyclic AMP on growth, morphology and expression of neurotransmitter synthsizing enzymes were not necessarily correlated. Inhibition of growth per se was not sufficient to account for all effects of cyclic AMP. The slowing of the growth rate by growth in N2 medium or by inclusion of butyrate did not lead to responses of the same magnitude as with cyclic AMP. Inhibition of growth by serum removal with no hormonal supplementation did not lead to increased numbers of neurites (Burmeister, 1982). In FCS medium, cyclic AMP caused a slowing of the doubling time, an elevation of DBH specific activity but no increase in numbers of long neurites. In N2 medium, cyclic AMP caused a slower growth rate as well as enhancing the numbers of long neurites and increasing the specific activity of DBH. The slowing of growth rate may contribute in part to morphological and enzymatic effects of cyclic AMP in N2 medium, but did not appear to be sufficient to account for all the effects observed.

EFFECTS OF NERVE GROWTH FACTOR

As nerve growth factor exerts important influence on the development of sympathetic neurons and some work indicates that cyclic AMP may be involved in mediating some of the intracellular actions of the factor, the effects of exogenous NGF on SY5Y cells were assayed and compared to those of cyclic AMP.

The population doubling time for SY5Y cells grown in the presence of nerve growth factor (NGF) at a concentration of 100ng/ml remained the same in 10% FCS medium but was significantly longer in N2 medium (Figure 3). The response of cells to NGF in N2 medium with respect to growth rate was highly variable. In all cases, however, the population doubling time was much longer being virtually stopped in the extreme case. The mean population doubling time was calculated using those values in the range of 100-200 hours (Table 1). The source of the variability has not been determined but the inhibitory trend, while

varying in magnitude, was consistent. NGF did not exert an inhibitory action on SY5Y growth rate when FCS was included in the medium. The mean population doubling times for FCS and FCS-NGF were not significantly different (Figure 3b; Table 1).

The morphology of the cells, however, was influenced by NGF only in the presence of serum and not in N2 medium (Figure 5). The percent of cells exhibiting long processes greater than 100 um increased from 7.2 to 15% when NGF was added to FCS medium while in N2 and N2-NGF medium the percent of cells with 100 um processes was the same, 8.4% as compared to 9.1% (Burmeister, 1982). The morphological effects of NGF did not correlate with the effects on population growth.

On the ultrastructural level, no significant alterations in cell structures were found (Figs. 12-13). The processes elaborated in the presence of NGF resembled those found in N2 or FCS (Figs. 27 & 31). Dense core vesicles were also observed in cells treated with NGF (Figs. 34 & 36). As with the cyclic AMP effect on neurite outgrowth, no novel structures were found despite a quantiative increase in neurite outgrowth.

Cultures were prepared by several different protocols to assay the possible short and long term effects of NGF on neurotransmitter synthesizing enzymes and these are described in legends of the tables. Cells passaged and maintained in the presence of 50 ng/ml NGF for up to three weeks exhibit no change in DBH levels over time (Table 5-6). A pair of cultures maintained in presence of 100 ng/ ml NGF for five days, a time at which morphological effects are maximal, failed to exhibit increases in DBH activity (Table 6). Neither increasing the duration of NGF exposure nor increasing the concentration to 100 ng/ml was sufficient to elicit changes in DBH.

No detectable levels of TH activity were found in homogenates from

cultures grown in presence of NGF. Cultures maintained for 5 days in the presence of 50 ng/ml NGF had no detectable TH activity despite total protein levels comparable to control cultures in N2 or FCS. Whether this represents a significant decline in enzyme activity cannot be determined due to the low and highly variable response of the control cultures (Table 3).

NGF elicits maximal morphological changes in SY5Y cells at concentrations from 100 ng/ml to 1 µg/ml (Burmeister, 1982; Sonnefeld & Ishii, 1982; Perez Polo et al., 1979). Significant morphological responses are observed at 50 ng/ml (Sonnefeld, personal communication). Since enzyme induction in other systems requires levels of NGF higher than necessary to elicit morphological responses, the posibility of NGF effects on TH and DBH in SY5Y at higher concentrations of the factor remains a possibility.

The effects of NGF were not correlated with altered growth rate. Also the NGF effects on morphology and growth rate were different from those observed with cyclic AMP. NGF enhances the quantitative expression of neurites only in presence of serum and this is not associated with slowing of the growth rate. Rather, when the growth rate is slowed as in N2-NGF medium, no increase in the percent of process bearing cells is observed. Cyclic AMP in contrast influences neurite outgrowth only in N2 medium. The actions of cyclic AMP and NGF differed with respect to requirements for additional factors.

GROWTH AND APPEARANCE OF M17 IN DEFINED MEDIUM N2

The mean population doubling time for SK-N-BE(2)-M17 (M17) was significantly lower for cultures grown in N2 as compared to 10% serum supplementation (Table 7). Cells exhibited logarithmic increases in cell number for six days when initial plating densities were

approximately 1-3 x 10^4 cells/cm^2. The maximal cell density reached in N2 medium was 2.8 x 10^5 cells/cm^2 as compared to 3.5 x 10^5 cells/cm^2 in 10 % FCS medium (Fig.38). The cells in FCS appeared to be past confluence and were probably entering stationary phase growth at the latter density. The rate of increase in cell number was declining. As with SY5Y, M17 cells in N2 medium were more easily dislodged from the substrate than FCS cultures. Loss of cells during medium addition and removal under these conditions prevented an accurate determination of saturation density. Some cells in F12/DME with no supplementation remained viable over the 6 day period but do not grow at a rate comparable to N2 or FCS (Figure 38). The persistance of viable cells under this condition may be due to residual serum factors.

The M17 cells exhibited a large flat morphology in FCS medium. The nucleus and nucleoli were clearly visible (Figs. 42c, 43c, 44b). After five days in N2 medium the cells appeared to be of lesser diameter and more rounded but remained flattened and spread over the surface. While some outgrowth of processes occurred in both media, long neurites were rare. No quantitative assays have been performed with the M17 cells to evaluate changes in cell size, shape or neurite outgrowth. No obvious qualitative changes followed tranfer of the cells into the N2 defined medium (Figs. 42a, 43a, 44a). Addition of the N2 ingredients to 10% FCS medium did not significantly alter the cellular morphology (Figs. 42e & 43e).

The M17 ultrastructure resembled that of early neuroblasts and no evidence for expression of more mature phenotype was obtained for cells grown in either N2 or FCS medium. The neurite profiles resemble early neuronal processes and no synaptic junctions were observed. The ultrastructure under the two conditions appeared qualitatively similar. The nuclei were immature in appearance, containing patches of condensed chromatin in nucleoplasm as well as underlying the inner surface of the

nuclear envelope. Nucleoli were present in the nucleus. Cytoplasmic protrusions observed in cross sections of the nucleus were probably due to irregular highly indented shape of the nucleus (Figs. 45 & 49).

The cytoplasm of the M17 cells was rich in free ribosomes and moderate amounts of rough endoplasmic reticulum were scattered in the cell body. Mitochondria were abundant (Figs. 45 & 46). Golgi apparati were extensive in the cell body especially in the perinuclear region (Figs. 46, 49, 50). Numerous vesicles were observed in the region of the Golgi including clear, coated (Fig. 46) and dense core varieties (Fig. 49). Golgi were also prominent in the proximity to centrioles. Microtubules were abundant in the area surrounding the centrioles and appeared to be radiating from the centriolar area (Figs. 46, 47, 48).

Dense core vesicles were numerous in the M17 cells in both N2 (Figs. 65-67) and FCS medium (Figure 45 and 62-64). These vesicles were found both scattered and in clusters in the cell body, along cytoplasmic extensions of the cells and at the tips of such processes. Dividing cells also contained dense core vesicles (Fig.51). The size of the majority of dense core vesicles was 70 to 150 nm, but a few vesicles in 200 nm range were found. The mean size of dense core vesicle population as determined from measurement of 100 vesicles was found to be 130 nm. The core typically occupied about 70% of the vesicle diameter. The proportional area of the vesicle filled by dense material was calculated to be 0.68 ± 0.11. A few vesicles had very small cores and larger clear area surrounding core, but the majority had the appearance described above. Some irregularly shaped vesicles, ovals or crescents, containing dense material were found. Such structures may be profiles of endoplasmic reticulum which were cut in cross or tangential section and contained some dense osmophilic material in the cisternae.

The processes observed in electron micrographs of the M17 had microtubules as the main structural feature. In FCS (Figs. 52-54) and N2 (Figs. 56-58) the processes contained numerous microtubules and occasional 10 nm filaments. Some cross sections revealed processes or regions of processes of small diameter and containing mainly filaments. These filaments were difficult to measure accurately but appear to be in the size range of actin filaments (Figs. 53 & 58). Some neuritic profiles had a reduced ribosomal content as compared to the cytoplasm (Figs. 55-58) while others contained some endoplasmic reticulum, ribosomes, mitochondria and dense core vesicles(Figs.52-54 ; Figs. 55-61).

This survey of M17 ultrastructure indicated that some structures found in differentiated neuronal cells were also expressed in the M17. While the content of the dense core vesicle could not be determined with standard glutaraldehyde-osmium fixation of cells, their presence suggested that the cells may synthesize and store catecholamines. Biochemical data supported this hypothesis (see below). Dense core vesicles were rarely found in analysis of the parental BE(2) line during log or stationary phase growth (Barnes et al., 1981) despite presence of active enzyme (Ross et al., 1981) and catecholamine synthetic ability (Beidler et al., 1978). The M17 clone presents a more promising model for further investigation of catecholamine synthesis and storage.

TYROSINE HYDROXYLASE ACTIVITY IN M17

The specific activity of TH in M17 cells cultured in N2 medium was slightly lower than FCS controls (Table 8). Total cell protein was reduced, probably due to slower growth rate of the culture. Analysis of cultures over a 9-12 day growth period demonstrated an increase over time in TH specific activity for cells grown in FCS but not N2 (Table 9). Even when cell protein levels were comparable, TH

activity in the N2 medium was significantly lower. The increase with cell density observed in FCS cultures was small but statistically significant. Cells in N2 exhibited a small but statistically significant decline in TH specific activity in older cultures of higher density.

The specific activity of TH in M17 was consistently higher in FCS cultures than in N2. TH activity is increased during stationary phase growth (Ross, personal communication). The trend toward higher TH levels with increased cell protein was noted (Table 9). This increase was not found in N2 cultures exhibiting protein levels of up to 2.2 mg/culture (Table 9).

Cultures grown in FCS medium to which the N2 ingredients were added exhibited levels of total cell protein and TH specific activity comparable to FCS cultures (Table 8). This suggested that factor(s) in the N2 formulation did not specifically inhibit growth or TH activity but rather that the N2 medium lacked serum factors which allow for fuller expression of active enzyme and more rapid cell growth.

IMMUNOCYTOCHEMICAL STAINING OF M17

M17 cells exhibit very intense cytoplasmic staining with antibody prepared against highly purified, trypsin-treated bovine adrenal tyrosine hydroxylase (Figs. 69 e-h; 70 b-c). Section (3 um) through stained cultures revealed absence of peroxidase reaction product in the nuclear region (Figs. 71e-f). The intense staining correlated with the levels of TH detected by <u>in vitro</u> activity assay.

Immunocytochemical staining with antibodies against bovine adrenal dopamine-β-hydroxylase resulted in both nuclear and cytoplasmic reaction. The nucleus appeared more darkly stained than the cytoplasm (Figs 69 b-d;). The antibody penetrated and interacted with

nuclear antigen as seen in 3 μm sections of the stained cells (Fig. 70 a-b). Treatment with preimmune sera did not result in cell staining (Fig. 69a). The basis for the nuclear staining has not been determined.

EFFECTS OF CYCLIC AMP ON M17

The growth of M17 cells in presence of cyclic AMP was slightly accelerated in FCS medium but remained unchanged in N2 medium (Table 7). As the cells approached confluence the cyclic AMP cultures exhibited a sharp decline in cell numbers as compared to the controls which entered stationary growth where a steady cell number was maintained for a time (Figure 39). The decline in cell numbers may be related to generation of toxic products resulting from the metabolism of the dibutyryl analogue. For example, the accumulation of butyrate in the medium could cause such a decline. All determinations for levels of enzyme were carried out on log phase cells.

The morphology of the cells in medium containing cyclic AMP was similar to controls. Short irregular processes are numerous under all conditions. The cell diameter appeared to be decreased slightly as compared to control cultures (Figs. 43-44).

The elevation of intracellular cyclic AMP by addition of the dibutyryl analogue, the phophodieaterase inhibitor IBMX, or a combination of the two agents caused a significant increase in specific activity measured in N2 cultures (Table 10). TH specific activity approached or exceeded that measured in FCS cultures. The total cell protein in the N2-cyclic AMP cultures did not vary significantly from the N2 controls. The addition of sodium butyrate (1mM) to N2 medium elevated TH activity, but also significantly lowered total cell protein in the N2 medium. The elevation of TH activity with cyclic AMP and IBMX were not accompanied by a reduction in population growth rate (see

Table 7).

Addition of cyclic AMP and IBMX to FCS medium stimulated a small but not statistically significant increase in specific activity of TH. There was also a slight but statistically significant increase in total cell protein. The addition of sodium butyrate to FCS medium produced a consistent decrease in total cell protein accompanied by an increase in TH activity (Table 10). A reduction in population growth rate was not observed when cyclic AMP and IBMX were added to FCS medium (Table 7).

EFFECTS OF NGF ON M17

The population doubling times and growth curves for M17 cells maintained in the presence of NGF at a final concentration of 50 ng/ml did not vary from those found in FCS or N2 controls (Table 7; Fig. 40). The morphology of the cells in the presence or absence of NGF in FCS or N2 medium was essentially the same as the corresponding control (Fig.42). Addition of the N2 factors along with NGF to FCS medium did not alter the observed morphology (Fig. 42).

TH specific activity in cell homogenates from N2 or FCS cultures with NGF did not exhibit significant differences from the controls. Total cell protein was not affected by NGF at a concentration of 100 ng/ml (Table 11). No synergistic action of the N2 supplements with NGF and serum factors was found when these were added to FCS medium (Table 11). NGF at 100 ng/ml included with N2 medium did not restore TH activity to the level observed in FCS medium nor did it stimulate any increased TH activity when administered to cultures in either medium over the course of 5 days.

FIGURES

PLATE I.

Fig. 1. Growth Curves of SY5Y cells in N2, FCS and F12/DME F12/DME medium. Cell counts were done on triplicate cultures for each time point. The numbers represent viable cells per 35 mm dish $\pm$ S.E.M. as determined by hemocytometer counts of trypan blue excluding cells.

Population doubling times were determined from the linear regression line.

Correlation coefficients (r):

FCS = 0.99

N2 = 0.98

F12/DME = 0.80

Fig. 1

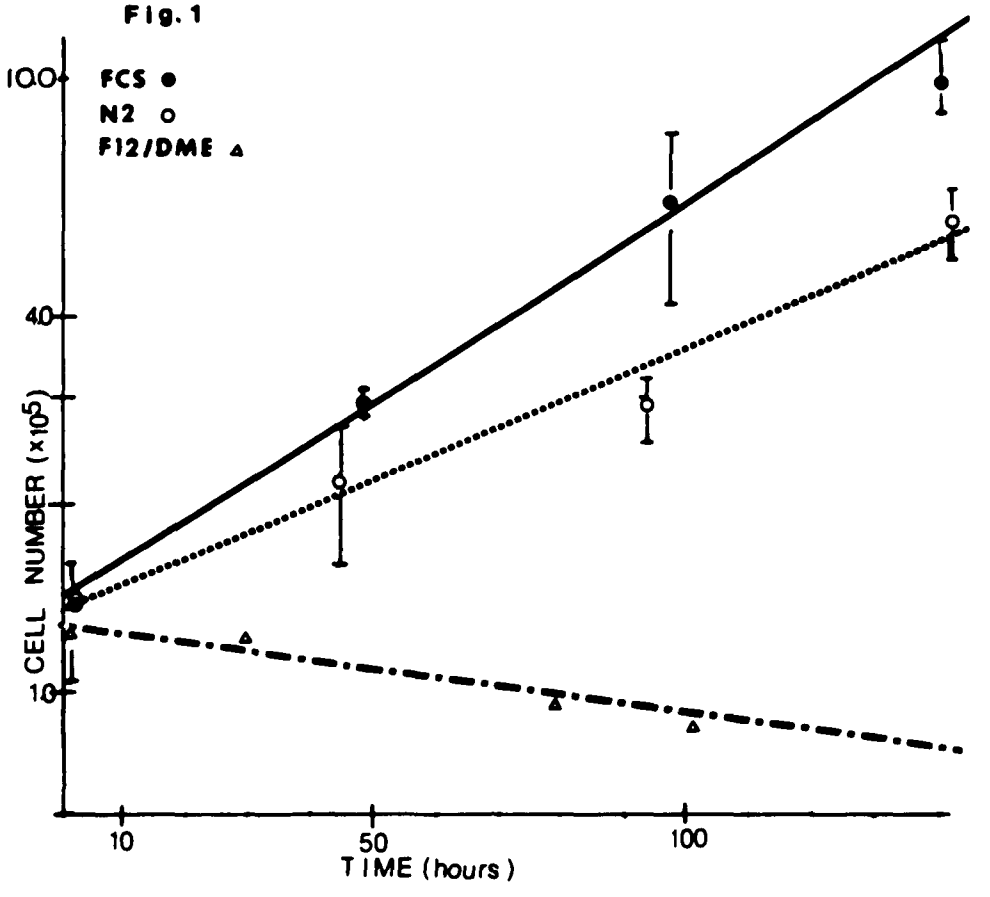


PLATE II.

Fig. 2. Growth Curve of SY5Y cells in N2 vs. N2-cyclic AMP and FCS vs. FCS cyclic AMP. The cyclic AMP was added to a final concentration of 1 mM and IBMX at 0.25 mM was added as a phosphodiesterase inhibitor. Cell counts and plating via same protocol as described in Fig. 1.

Population doubling times were determined from the linear regression lines.

Correlation coefficients (r):

FCS = 0.99

FCS-cyclic AMP = 0.97

N2 = 0.99

N2-cyclic AMP = 0.95

Fig. 2a

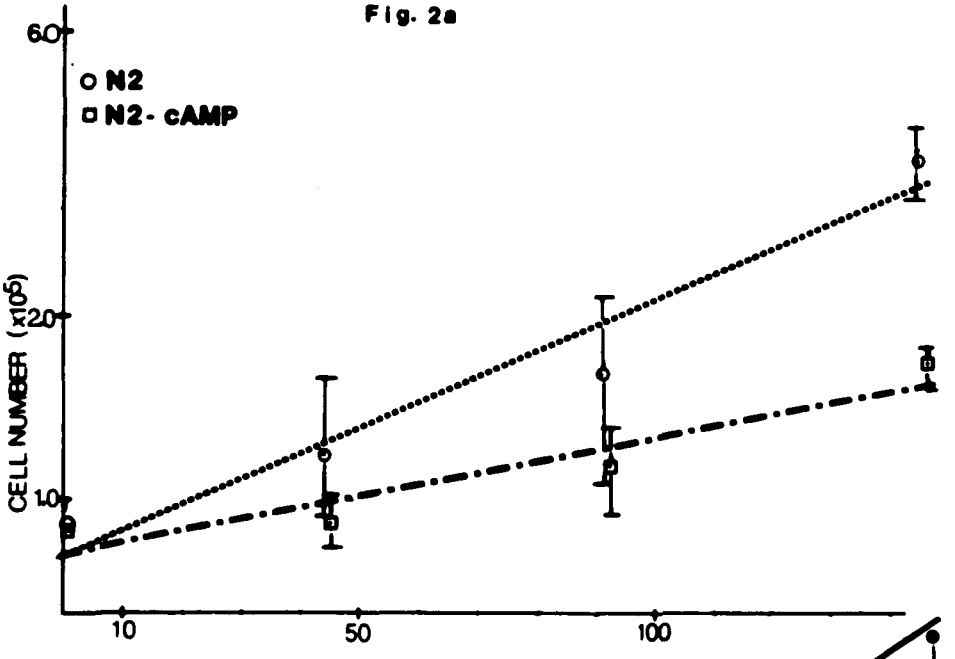

6.0
○ N2
□ N2- cAMP
CELL NUMBER (x10⁵)
2.0
1.0
10
50
100

Fig. 2b

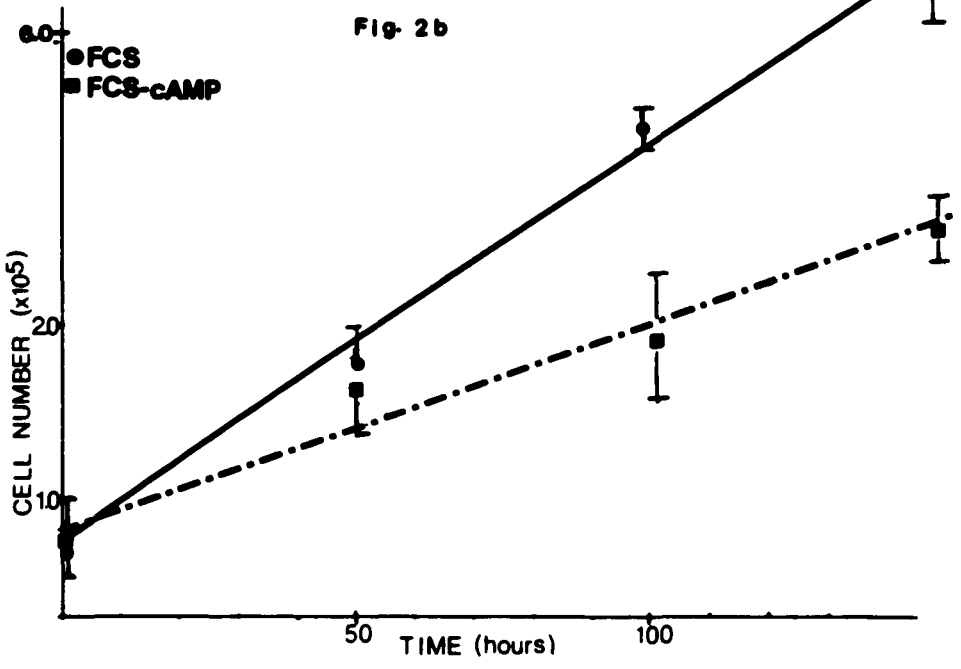

6.0
●FCS
■ FCS-cAMP
CELL NUMBER (x10⁵)
2.0
1.0
50
TIME (hours)
100

PLATE III.

Fig. 3. Growth Curve of SY5Y cells in N2, N2-NGF, FCS and FCS-NGF. NGF, 7S, was used at a final concentration of 500ng/ml.

Population doubling times were determined from the linear regression lines.

Correlation coefficients (r):

FCS = 0.99

FCS-NGF = 0.99

N2 = 0.98

N2-NGF = 0.85

Fig. 3a

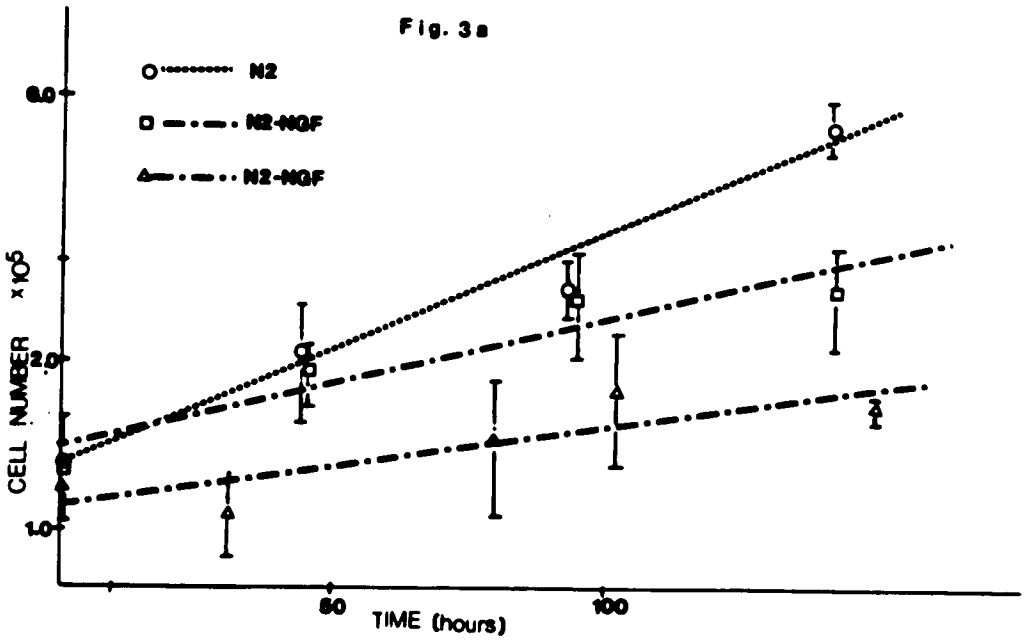

N2
N2-NGF
N2-NGF
6.0
2.0
1.0
CELL NUMBER x10⁵
80
100
TIME (hours)

Fig. 3b

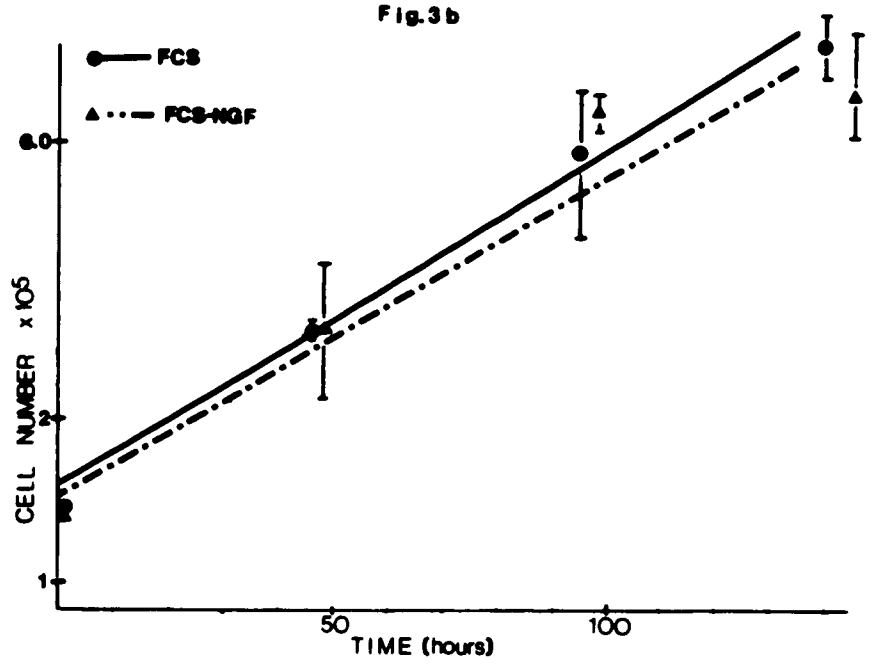

FCS
FCS-NGF
6.0
2
1
CELL NUMBER x10⁵
50
100
TIME (hours)

PLATE IV.

Fig. 4. Phase contrast micrographs of SY5Y cells in various culture media.

a. Cultures maintained in 10% FCS for one week. Note the flattened appearance of the cells and the extension of short cytoplasmic processes.

b. Culture also maintained in 10% FCS for one week since previous transfer. Note the presence of some cells with a more rounded appearance.

c. Culture maintained N2 medium for four weeks. Cells were plated at each transfer onto a serum-coated substrate in defined medium.

d. Culture maintained in N2A medium for one month. Cells were plated at each transfer onto serum coated substrate in defined medium.

X 300

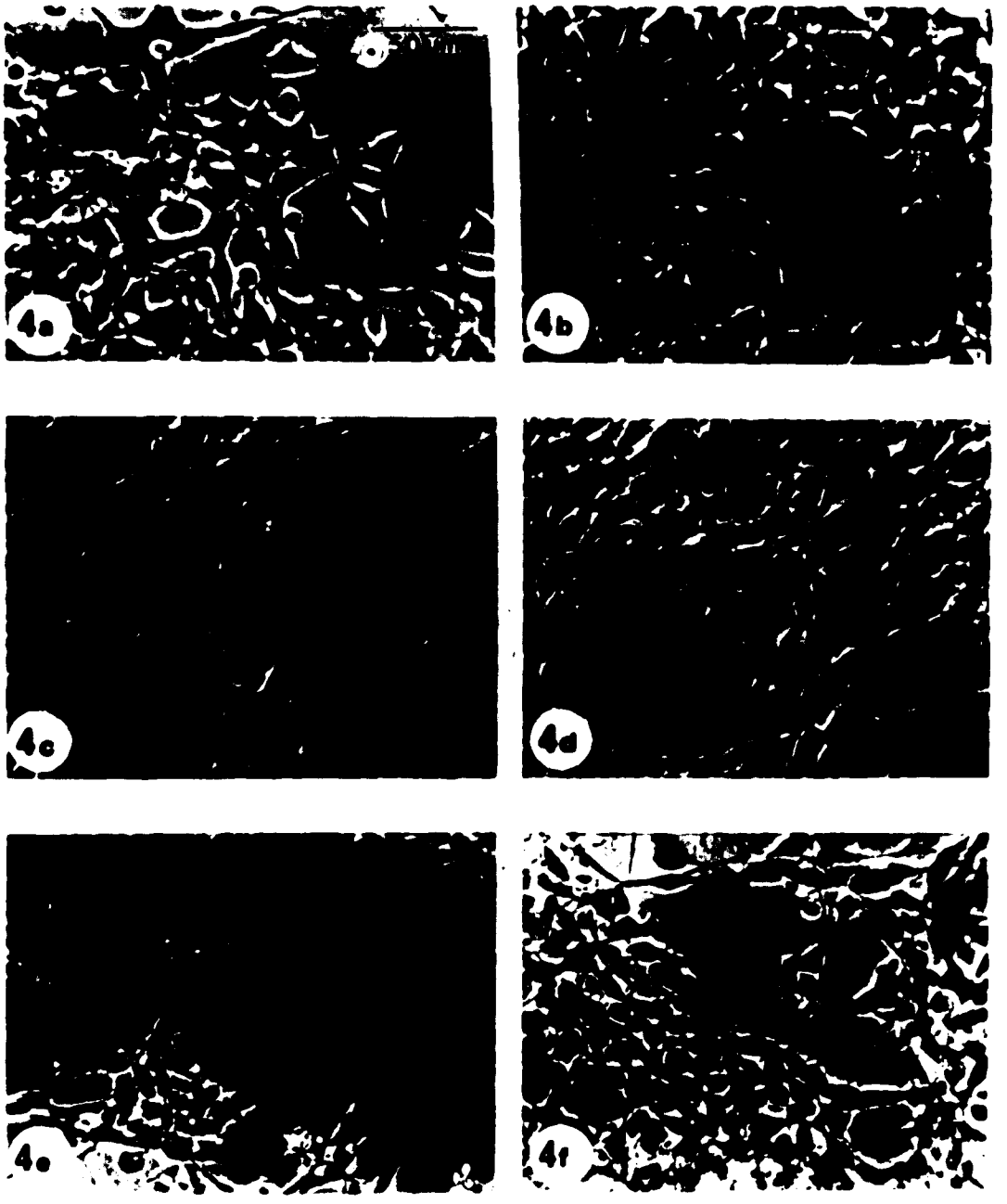

4a
4b
4c
4d
4e
4f

PLATE V.

Fig. 5. Phase contrast microscopy of SY5Y in FCS and N2 medium with and without NGF.

a. Culture maintained in 10 % FCS medium for one week since transfer.

b. Culture maintained in 10 % FCS to which NGF was added at final concentration of 50ng/ml. NGF was supplied as 2.5S β-NGF.

c. Culture maintained for one week in N2 medium. For all N2 cultures cells were initially plated out into 10% FCS medium for 24 hours and then switched to defined medium by complete replacement of the medium. Cultures were fed every 2-3 days for the course of the experiment.

d. Culture maintained for one week in N2 medium to which β-NGF was added to a final concentration of 50ng/ml.

e. Cultures maintained in 10 % FCS medium to which the N2 factors had been added. Cells were initially plated into 10% FCS medium and fed with experimental medium at 24 hours.

f. Culture maintained in 10 % FCS medium to which N2 factors and NGF were added. The final concentration of 2.5S β-NGF was 50 ng/ml.

X300

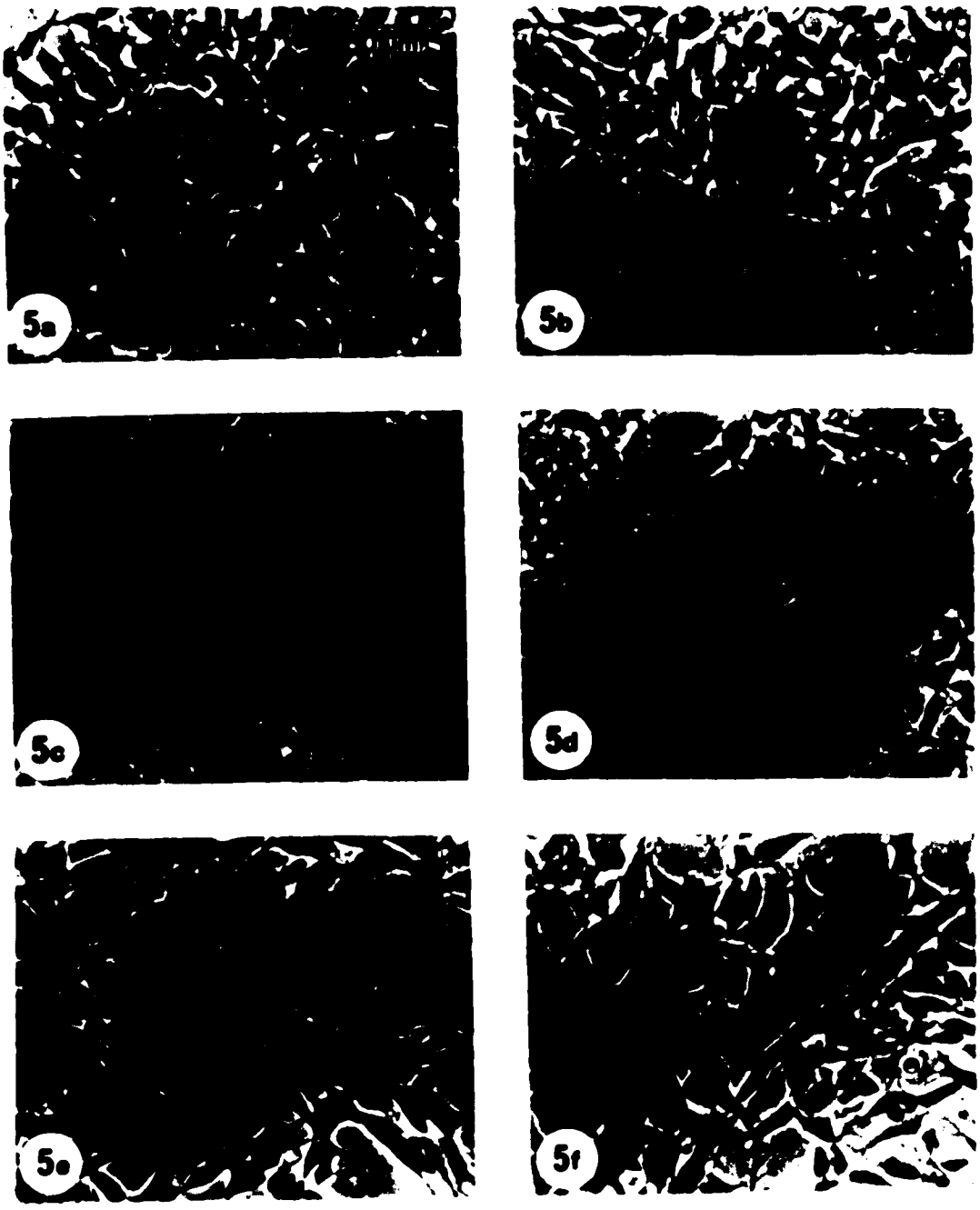

5a
5b
5c
5d
5e
5f

PLATE VI.

Fig. 6. Phase contrast micrographs of SY5Y cells in presence of cyclic AMP. All cultures were plated into FCS for 24 hours and then switched to experimental condition by complete medium replacement. Cultures were photographed at one week.
a. Culture in N2 medium. There are numerous small processes and a few longer processes evident in this part of the culture.
b. Culture in N2 medium to which cyclic AMP and IBMX have been added. The number of long processes is significantly increased over that observed under other culture conditions.
c. Culture in 10 % FCS to which N2 factors were added. Cell morphology resembles that observed in 10 % FCS.
d. Culture in 10 % FCS to which cyclic AMP and IBMX were added. Short and long processes are found at about same frequency as in 10% FCS.
e. Culture in 10 % FCS. A few short processes extend from the cells.
f. Culture in 10 % FCS to which cyclic AMP and IBMX have been added. A few long processes are observed in these cultures at about the same frequency as in 10 % FCS.

X300
IBMX = isobutylmethylxanthine
cyclic AMP = dibutyryl cyclic AMP

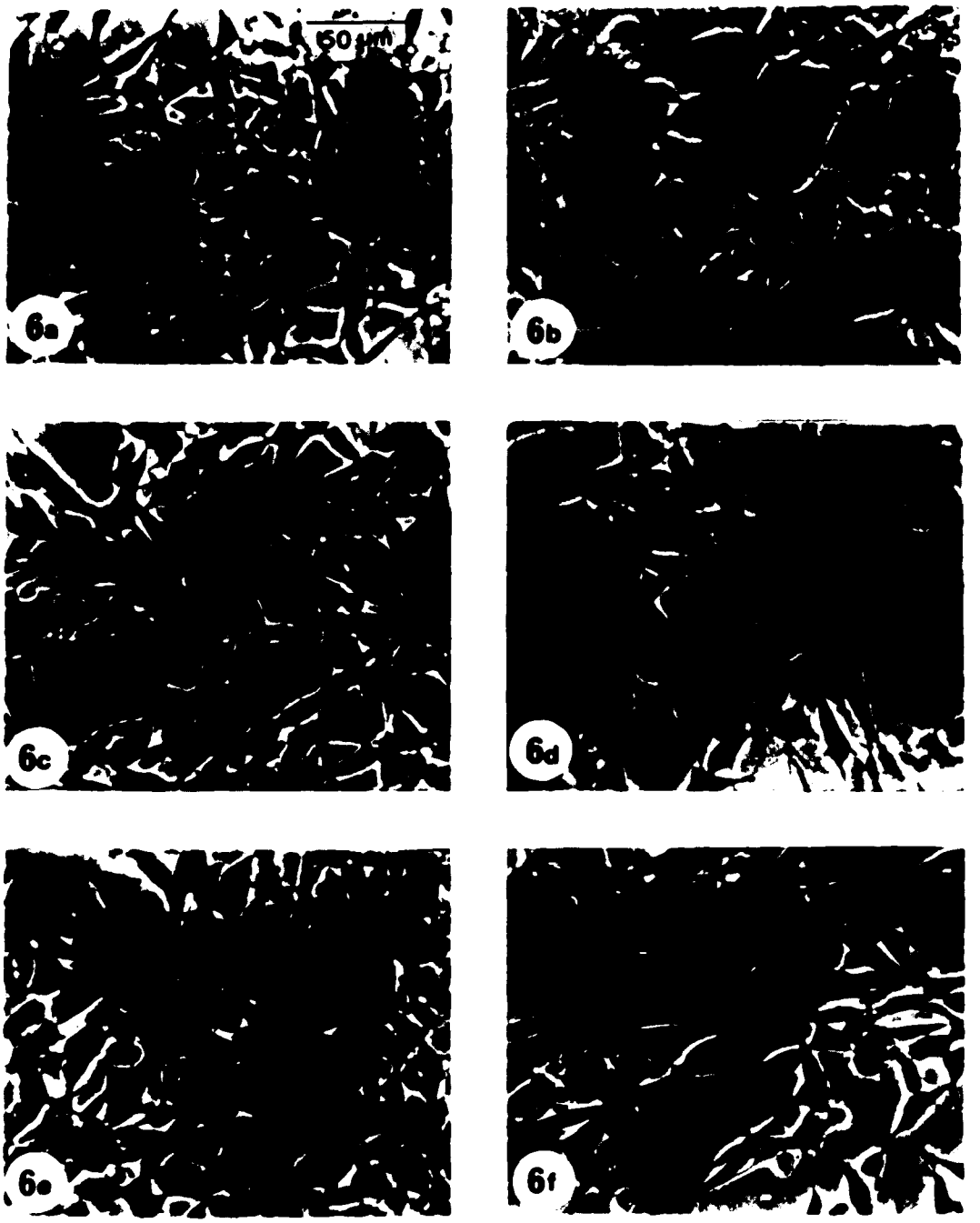

50 µm
6a
6b
6c
6d
6e
6f

PLATE VII.

Fig.7. Controls for cyclic AMP effect on morphology as viewed by phase contrast microscopy.
a. SY5Y culture in 10 % FCS with cyclic AMP and IBMX. Cells exhibiting either a flattened or rounded morphology are present in the culture.
b. SY5Y culture in 10 % FCS with cyclic AMP. Overall morphology of cells is not significantly different from 10 % FCS controls.
c. SY5Y culture in 10 % FCS with IBMX. Overall morphology is not significantly different from 10 % FCS controls.
d. SY5Y culture in 10 % FCS with 1 mM butyrate. Cell numbers were reduced. Cultures contain many flattened cells with granular appearance to the cytoplasm which may be related to toxicity of butyrate.
e. SY5Y culture in N2 with 1 mM cyclic AMP and 0.25 mM IBMX. Note presence of long processes on most cells in this field.
f. SY5Y culture in N2 with 1 mM cyclic AMP. Numerous long processes are present in cultures.
g. SY5Y culture with IBMX. Some long processes are elaborated by the cells but response was less dramatic than with cyclic AMP or cyclic AMP plus IBMX.
h. SY5Y culture in N2 medium with 1 mM butyrate. Note the difference in cell shape and the lack of long slender processes observed with cyclic AMP. Cell numbers are severely reduced at this concentration of butyrate.

X 300

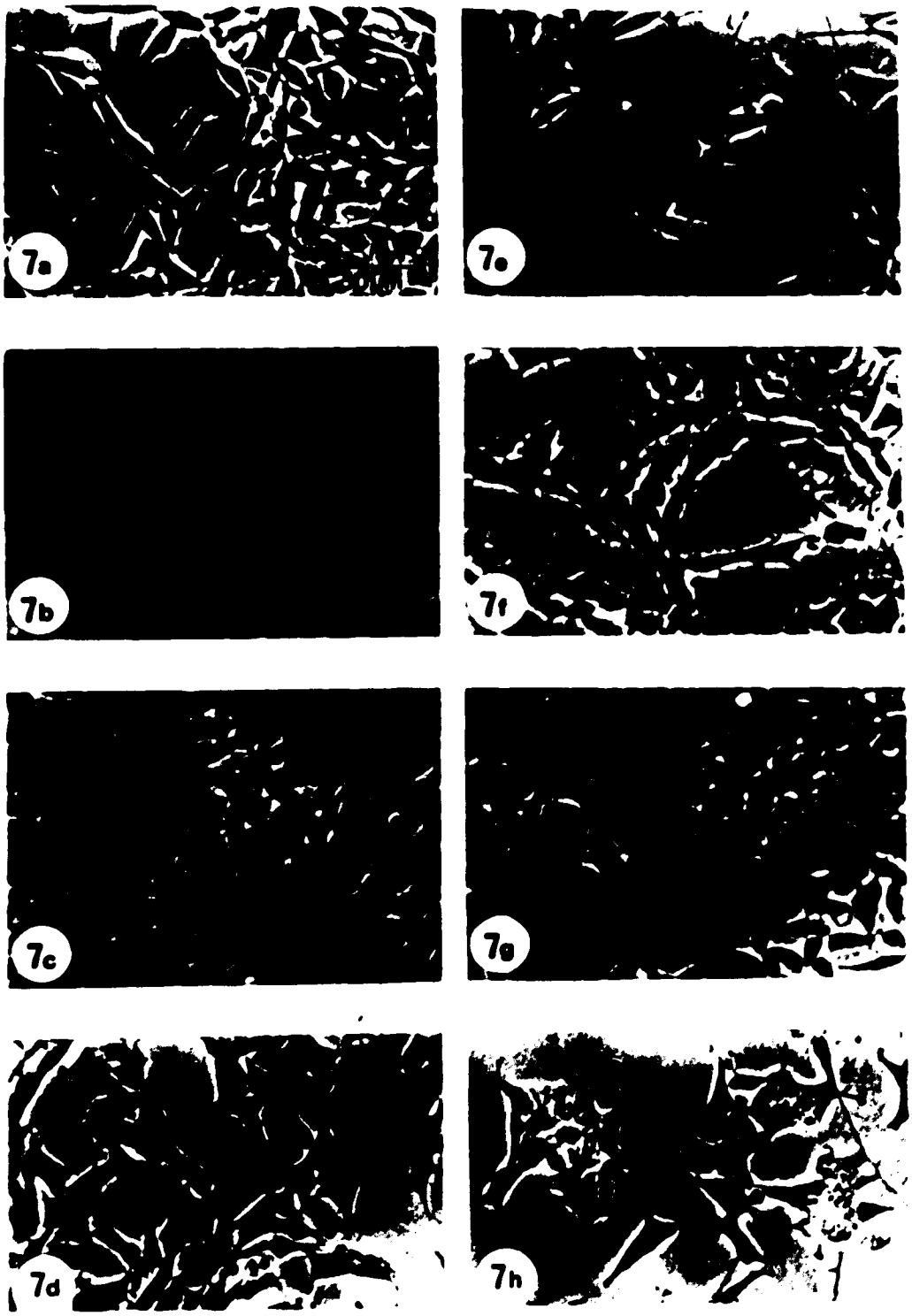

7a
7e
7b
7f
7c
7g
7d
7h

PLATE VIII.

Fig. 8. Phase contrast morphology of SY5Y in presence of 8-bromo cyclic AMP analogue.
The morphological effects of this analogue were indistinguisable from those observed with dibutyryl cyclic AMP.
a. SY5Y culture in 10 % FCS medium.
b. SY5Y culture in 10 % FCS medium with 8-bromo cyclic AMP and IBMX.
c. SY5Y culture in 10 % FCS medium with 8-bromo cyclic AMP.
d. SY5Y culture in N2 medium.
e. SY5Y culture in N2 medium with 8-bromo cyclic AMP and IBMX.
f. SY5Y culture in N2 medium with 8-bromo cyclic AMP.

X 300

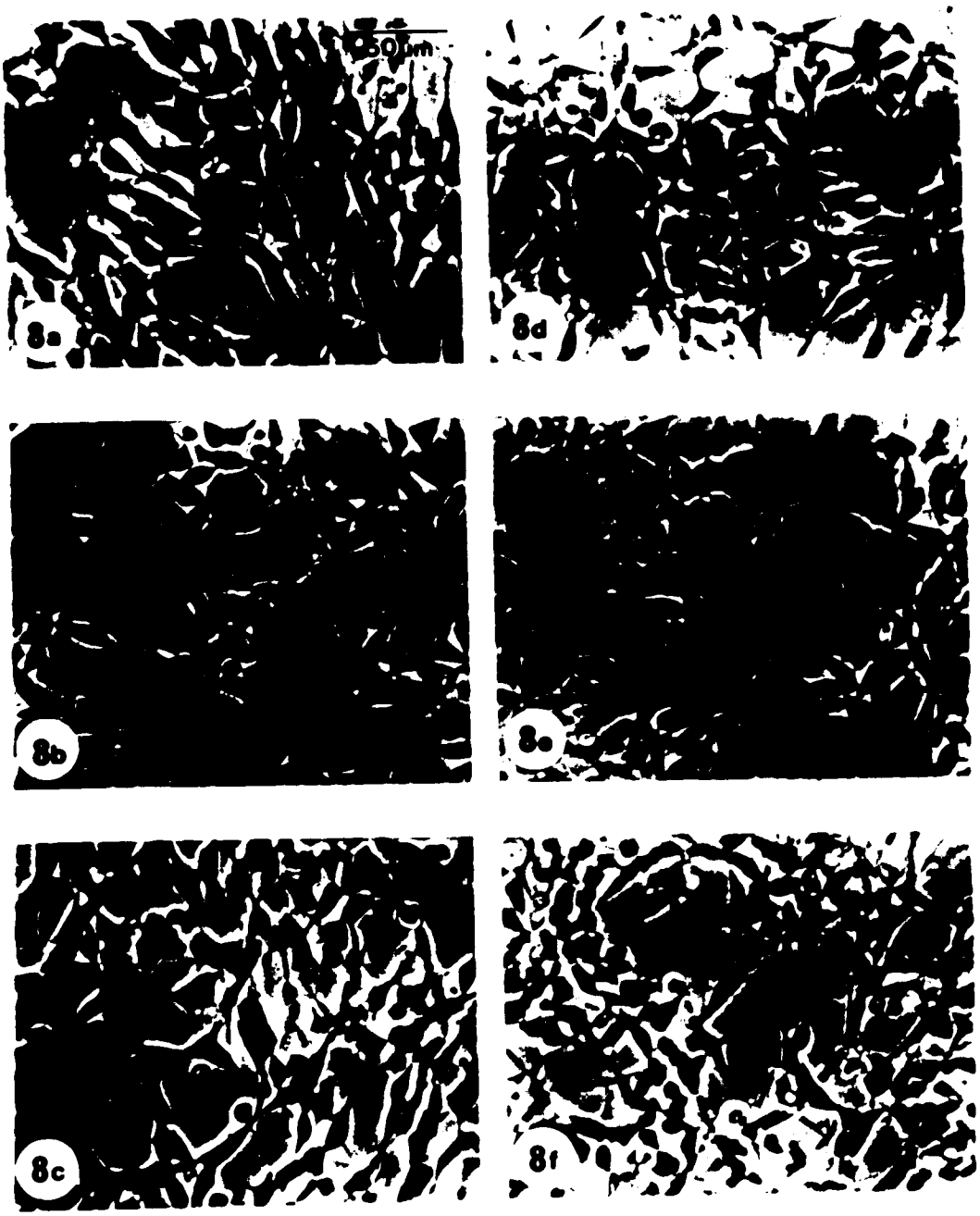

50µm
8a
8d
8b
8e
8c
8f

PLATE IX.

Fig. 9. Electron micrograph of SY5Y culture maintained in N2 medium for one week. The culture was sectioned perpendicular to the culture substrate which is indicated by the arrows. The cytoplasm contains free ribosomes, scattered rer, prominent golgi, dcv, mitochondia. Cell processes contain numerous mt, an occasional dcv, mitochondria and some ser. A GC can be seen spread along the substrate.

X 15,000

KEY: dcv = dense core vesicle
g = Golgi apparatus
GC = growth cone
m = mitochondria
mt = microtubule
n =nucleolous
Nu = nucleus
rer = rough endoplasmic reticulum
ser = smooth endoplasmic reticulum


1.0μm
apv

PLATE X.

Fig. 10. SY5Y cell maintained in N2 medium for one week. The cytoplasm typically contains prominent Golgi apparatus, numerous mitochondria, scattered rer, and an occasional mt or dcv was observed. The nucleus contains condensed chromatin most prominent as a rim underlying the nuclear envelope.

X 18,800

KEY: g = Golgi apparatus
mt = nicrotubule
Nu =nucleus
rer = rough endoplasmic reticulum

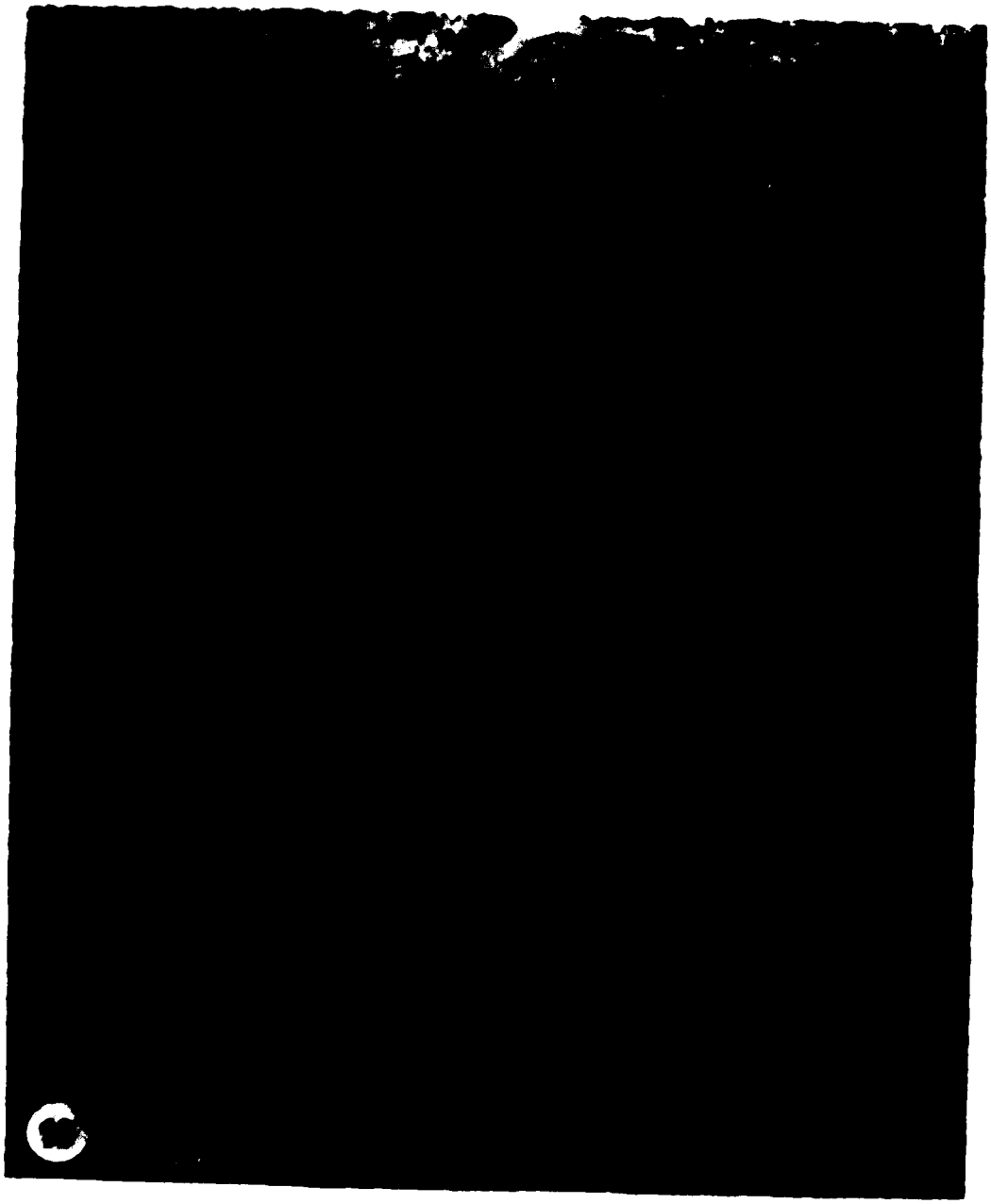

C

PLATE XI.

Fig.11. SY5Y cell in FCS medium. The culture substrate is indicated on the lower left. Cytoplasm contains numerous mitochondria, prominent Golgi, some rer, numerous free ribosomes, dcv. The nucleus contains patches of condensed chromatin, as a rim underlying the nuclear envelope. A nucleolous is prominent in this section.

X 12,800

KEY: dcv = dense core vesicle
g = Golgi apparatus
n = nucleolous
Nu = nucleus

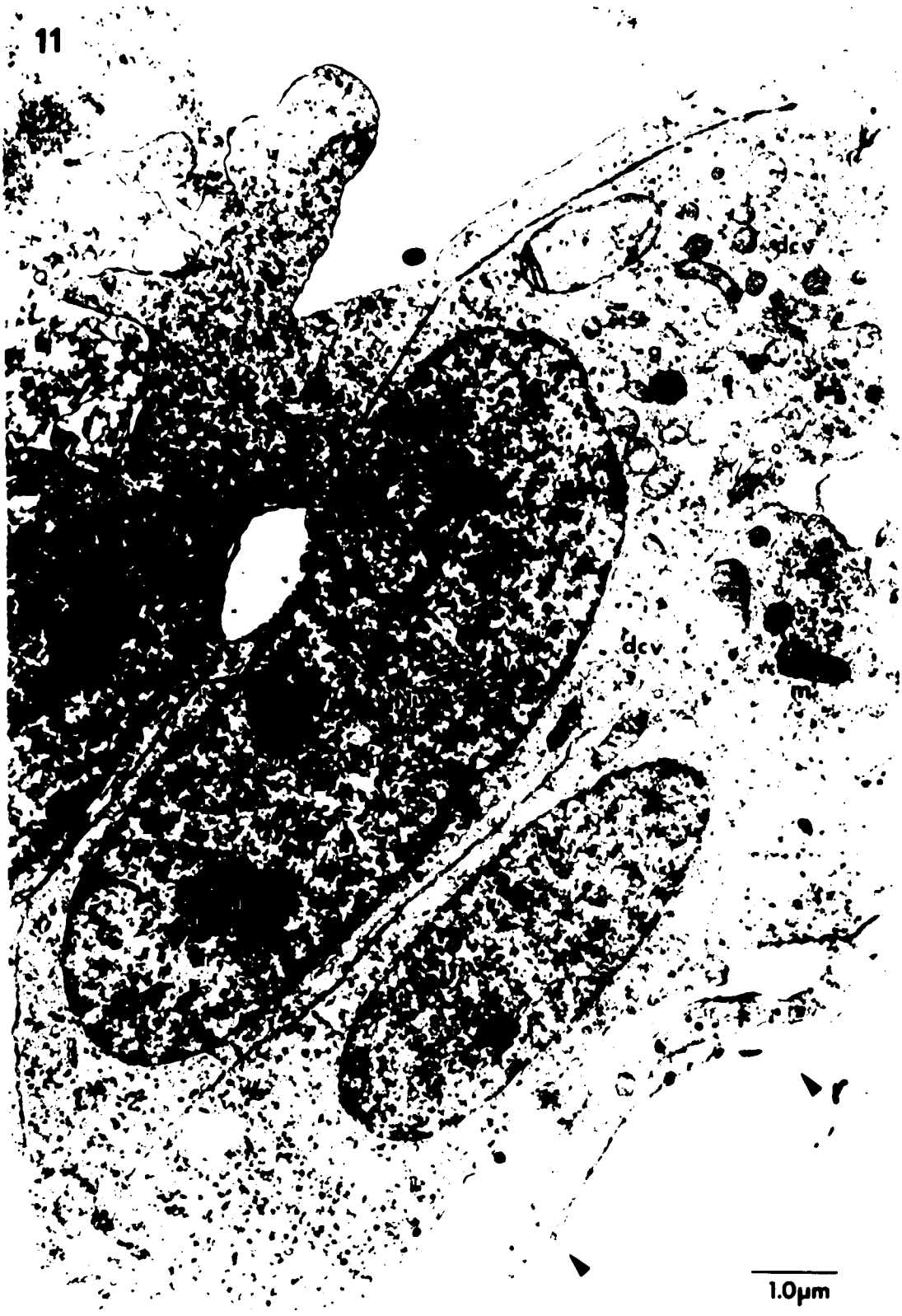

11
dcv
g
dcv
m
1.0μm

PLATE XII.

Fig.12. SY5Y cell in N2-NGF medium. The cytoplasm contains typical organelles including Golgi, rer, dcv, mitochondria, ribosomes. A cross section through a process (p), reveals its main component to be microtubules. Numerous dcv are apparent in upper right indicated by the arrows.

X 10,400

Fig. 13. SY5Y cell in 10 % FCS-NGF medium. The cytoplasmic organelles are similar to those discussed for cells in 10 % FCS medium. Note the dcv in region of extensive Golgi.

X 16,300

KEY: cv = coated vesicle
dcv = dense core vesicle
g = Golgi apparatus
Nu = nucleus
p = process


12
1.0μm


1.0μm
13

PLATE XIII.

Fig. 14. SY5Y cell from N2-cyclic AMP culture. The cells exhibit organelle content similar to that found in N2 cultures. The mitochondria are particularly numerous in this section and rer very prominent in an almost Nissl pattern. A centriole cut in longitudinal section is apparent. The nucleus contains a thin rim of condensed chromatin under the nuclear membrane and a nucleolous is present.

X 14,100

Fig. 15. SY5Y cell from 10 % FCS-cyclic AMP culture. The ultrastructure observed is essentially the same as in 10 % FCS. Arrow indicates and area from which microtubules appeared to be radiating perhaps from centriolar region not included in the plane of section. Mitochondria are numerous.

X 6500

KEY: c = centriole
g = Golgi apparatus
m = mitochondrion
Nu = nucleus
rer = rough endoplasmic reticulum


14
1.0μm


15
1.0μm

PLATE XIV.

Fig. 16. Section through a dividing cell in SY5Y culture maintained in N2 medium for one week. Mitochondria, er, and dcv are seen scattered in the periphery of the cell with patches of condensed chromatin located more centrally. The dcv indicated by arrows are enlarged in upper left insert. A cross section of a microtubule containing narow process ia seen between two adjacent cells (p).

X 16,900
X 27,600 (upper insert)

KEY: dcv = dense core vesicle
er = endoplasmic reticulum
p = process

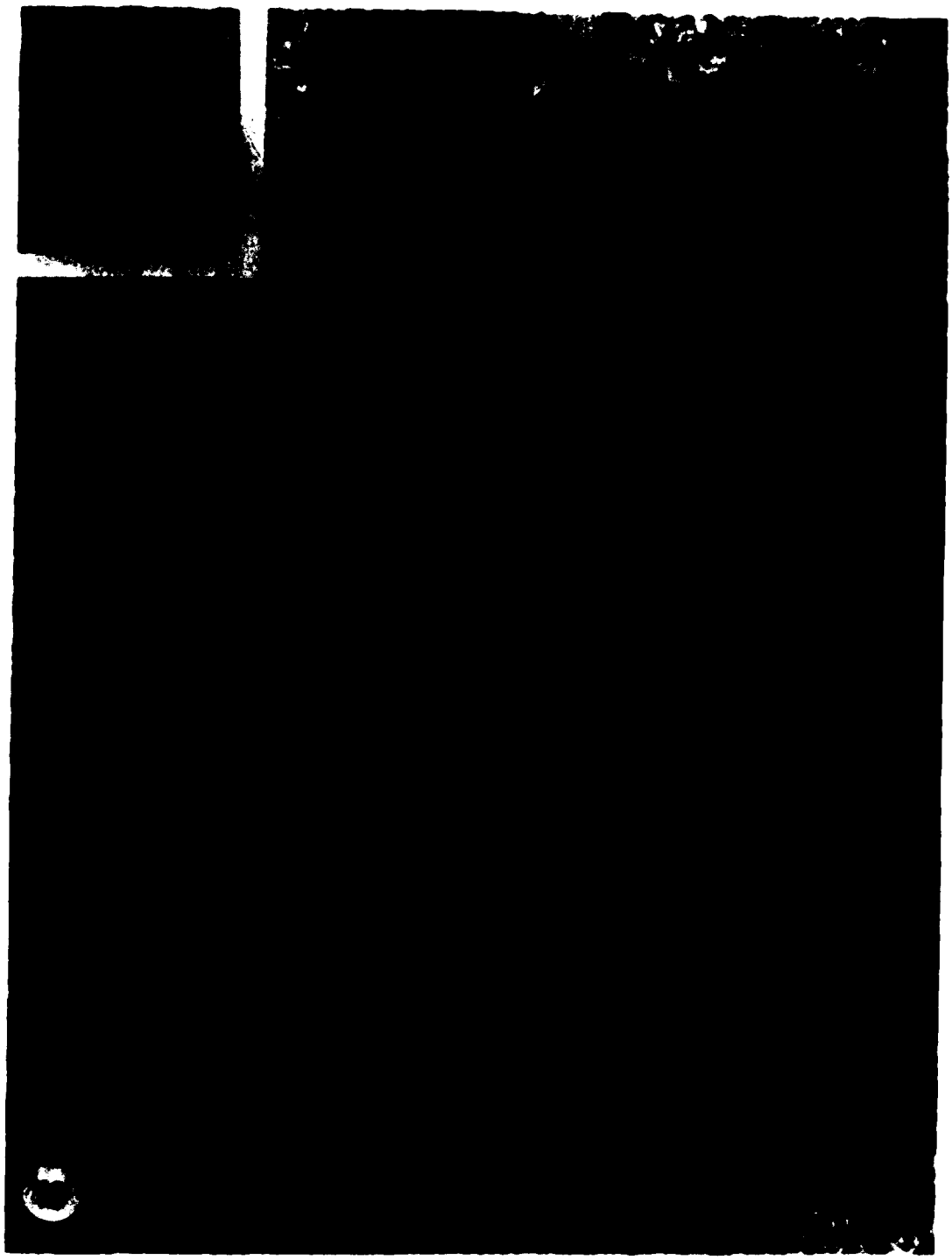


PLATE XV.

Fig. 17. Longitudinal section through a part of SY5Y cell process from a culture maintained in N2 medium. Note the parallel arrays of mt, and the large expansive area at the most distal part of tthe process captured in this section. This resembles intermediate area of growth cone in that it contains ribosomes and a few scattered mt. The ribosomal content along the shaft of the process appeared to be reduced while the growth cone area was rich in ribosomes. A dcv is seen in the process, another in growth cone area and two are seen in the adjacent cell body.

Fig. 18. Longitudinal section of process of SY5Y cell from a culture maintained in 10 % FCS. Note the presence of longitudinal microtubules along the short extent of the process which is seen as it emerges from the cell body and expands at the distal end into a growth cone. Areas of membrane expansion are seen budding from the growth cone area and continuity of the membrane is evident in lower such expansion. A process cut in cross section contianing numerous microtubules are also seen (p).

X13,800

KEY: dcv = dense core vesicle
GC = growth cone
mt = microtubule
m = mitochondrion


18
0.5μm


17
dcv
GC
0.5μm

PLATE XVI.

Fig. 19. Longitudinal section of SY5Y process from culture maintained in N2-cyclic AMP for one week. Process is seen as it emerges from the cell body and at its distal end expands into growth cone. The most distal aspect of the growth cone was not captured in this section. The growth cone area depicted is rich in organelles including rer, Golgi, scattered mt and ribosomes. The shaft of the process contains mt, some ribosomes, a few dcv, a mitochondrion. Cross sections of several processes are also seen in this section, two of which are enlarged as indicated with asteriks. Note the numerous microtubules in parallel array and reduced numbers of ribosomes.

X 13,200

X 29,000 (inserts)

KEY: dcv = dense core vesicle
g = Golgi apparatus
GC = growth cone
m = mitochondrion
mt = microtubules

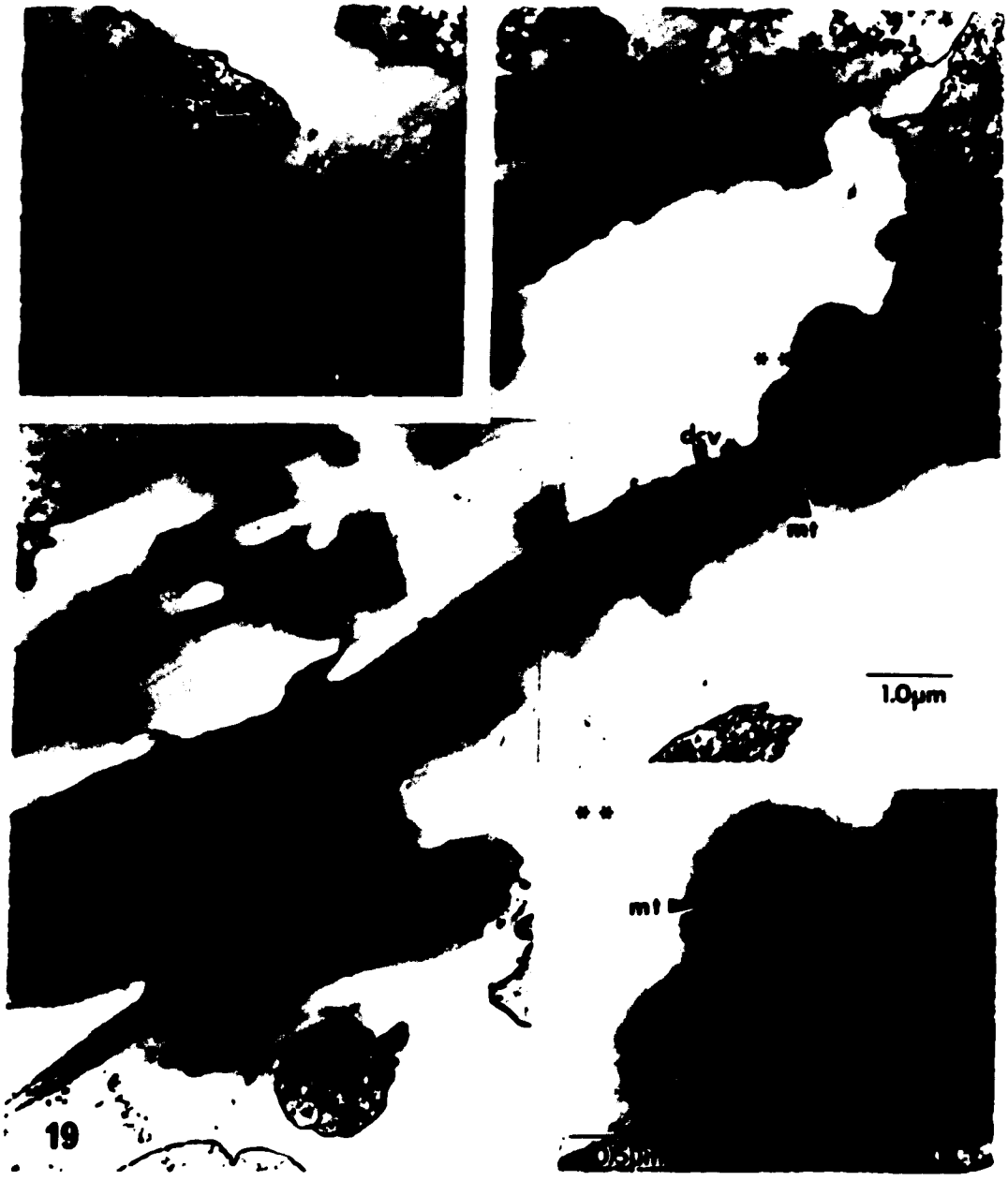

dcv
mt
1.0μm
mt
19

PLATE XVII.

Cross sections through processes from SY5Y cultures maintained in N2 medium for one week.

Fig. 20. Note the high density of microtubules aligned in parallel. A few ribosomes are present but content is less than seen in cell body. A large mitochondrion is present and two smaller double membraned organelles which may be dense core vesicles or sections through a narrow part of a mitochondrion. The process exhibits fuzzy material which is not well resolved but probably is due to presence of filamentous material.
X 46,600

Fig. 21. The density of the microtubules is less in this process than in fig. 20. The ribosomal content is more conspicuous as well. Cross sections through filamentous elements which measure in the range of 5 (f) to 10 nm (if) are indicated with arrows.
X 46,600

Fig. 22. Microtubules are numerous amongst several organelles present in proecess including mitochondrion, some rer, and filamentous material.
X 41,400

Fig. 23. Process in which filaments are numerous. Arrows indicate cross and longitudinal sections through filaments measuring about 10 nm. Microtubules are most numerous around the perimeter of the process while filaments are scattered throughout the diameter. The section was cut perpendicular to the plane of substrate which is indicated by asterik on lower left.
X 63,000

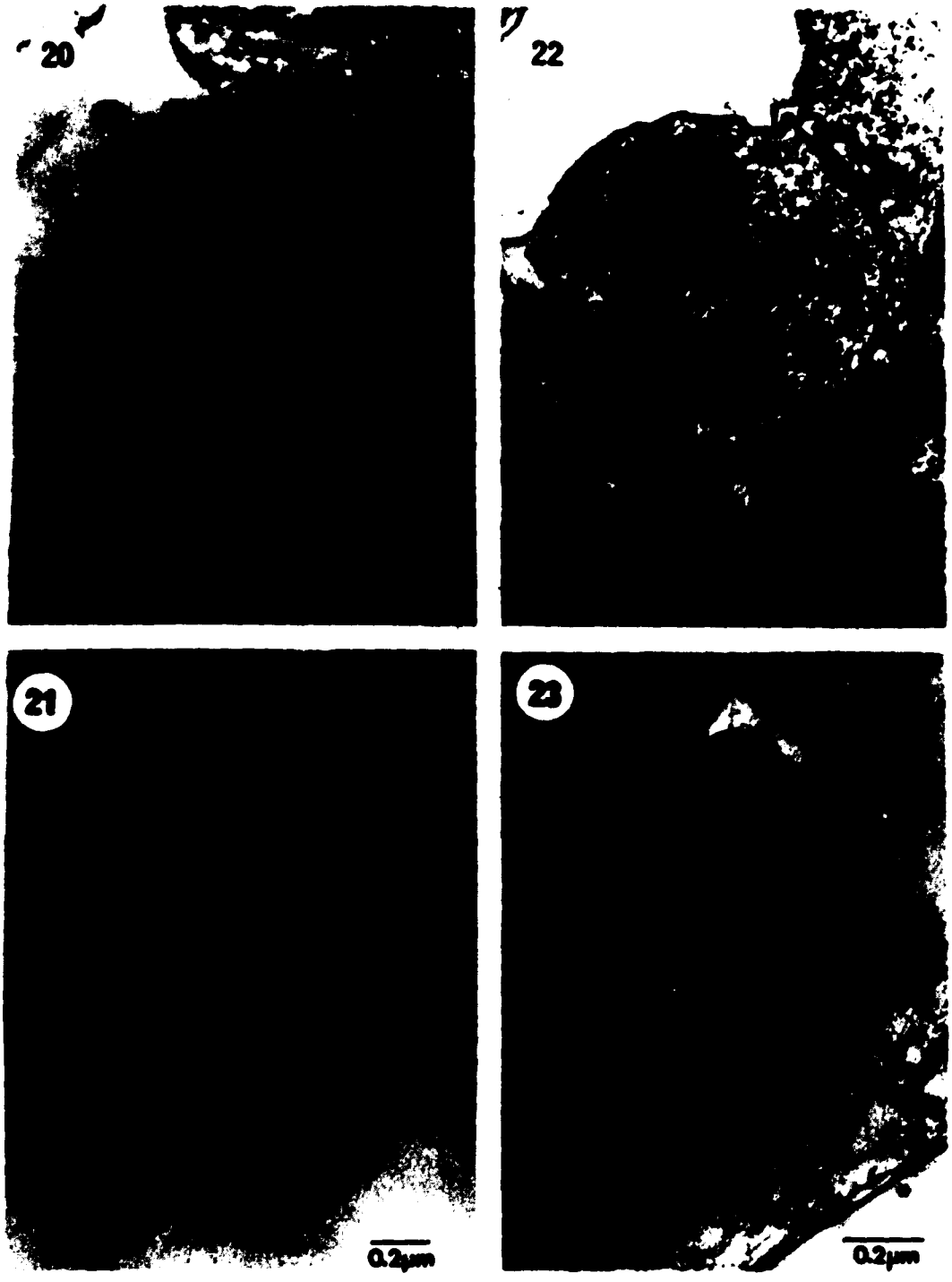

20
22
21
23
0.2μm
0.2μm

PLATE XVIII.

Cross sections of processes of SY5Y cells in 10 % FCS medium. No significant changes in ultrastructure were found when cyclic AMP or NGF were added to the medium for one week.

Fig. 24. Processes contain high density of microtubules. Some er and a few ribosomes in clusters are also seen. X 33,800

Fig. 25 Processes or portions of processes of narrow diameter containing numerous microtubules. Filamentous material is also evident but not well resolved. X 44,500

Fig. 26. Processes of SY5Y cells which were maintained in 10 % FCS-cyclic AMP for one week. Contents of the process include mt, er, mitochondria, dcv and ribosomes. Dense core vesicle indicated measures 100 nm. X 22,600

Fig. 27. Process of SY5Y cell from culture maintained in 10 % FCS-NGF. Microtubules are numerous and ribosomes, mitochondria and filamentous material are also present. X 35,900

KEY: dcv = dense core vesicle
er = endoplasmic reticulum
mt = microtubule
r = ribosomes

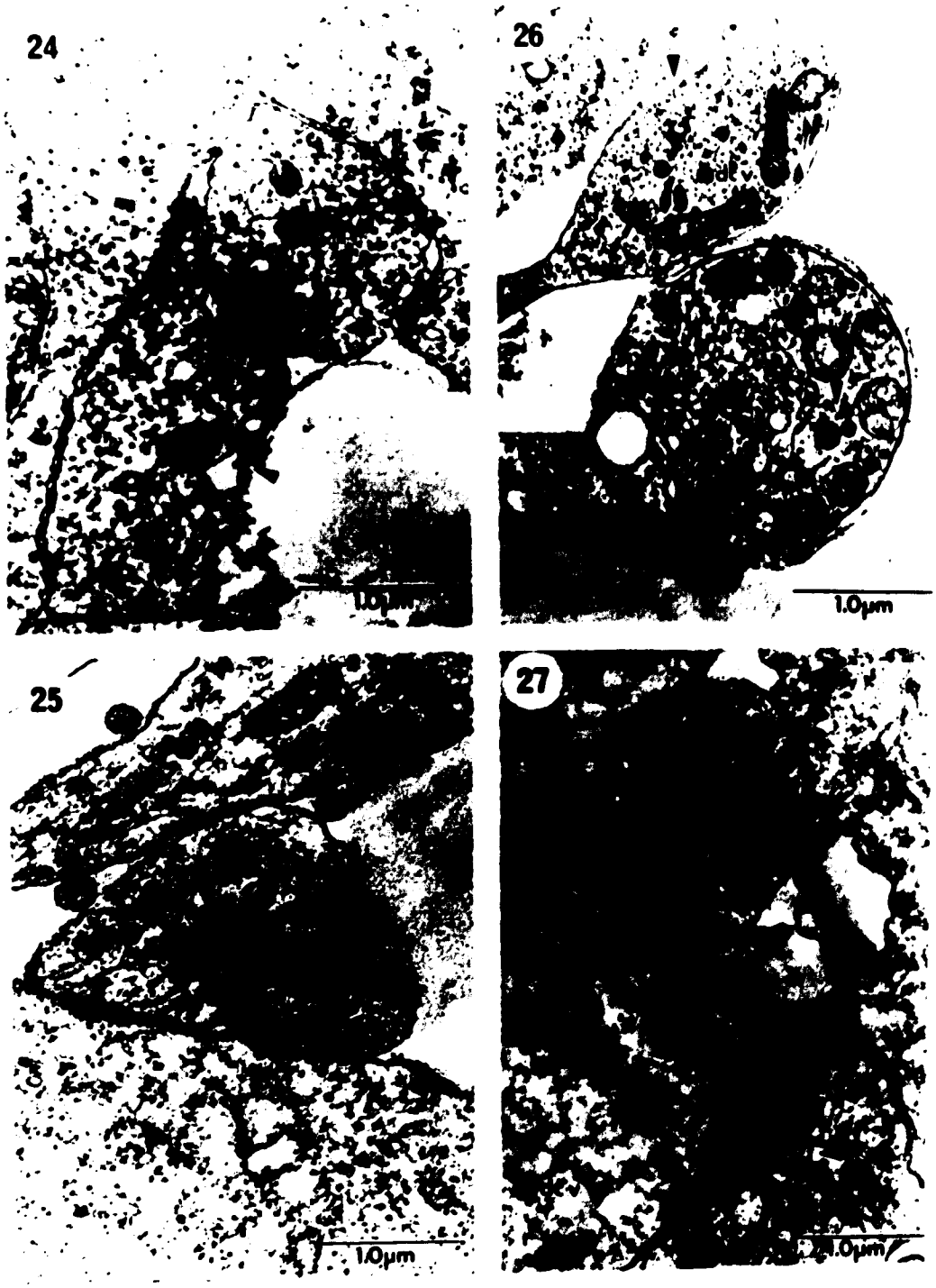

24
1.0μm
26
1.0μm
25
1.0μm
27
1.0μm

PLATE XIX.

Cross sections of processes observed in N2 medium. No significant alterations in basic ultrastructure were found with NGF or cyclic AMP treatment.

Fig. 28. Processes in N2 medium exhibiting numerous microtubules. Cisternae of er are seen cut in cross section. Ribosomal content is reduced as compared to cytoplasm of the adjacent cell.
X38,400

Fig. 29. Processes in N2 medium which exhibit filaments as well as microtubules. Mitochondria and some ser are also seen.
X 44,500

Fig. 30. Processes of SY5Y cells from culture maintained in N2-cyclic AMP medium. Similar in ultrastructure to those observed in N2 medium.
X 68, 500

Fig. 31. Process of SY5Y cell from N2-NGF culture. Contains numerous microtubules some ser and mitochondria but ribosomal content is reduced.
X 57,000

KEY: er = endoplasmic reticulum
ser = smooth endoplasmic reticulum
m = mitochondrion
mt = microtubule

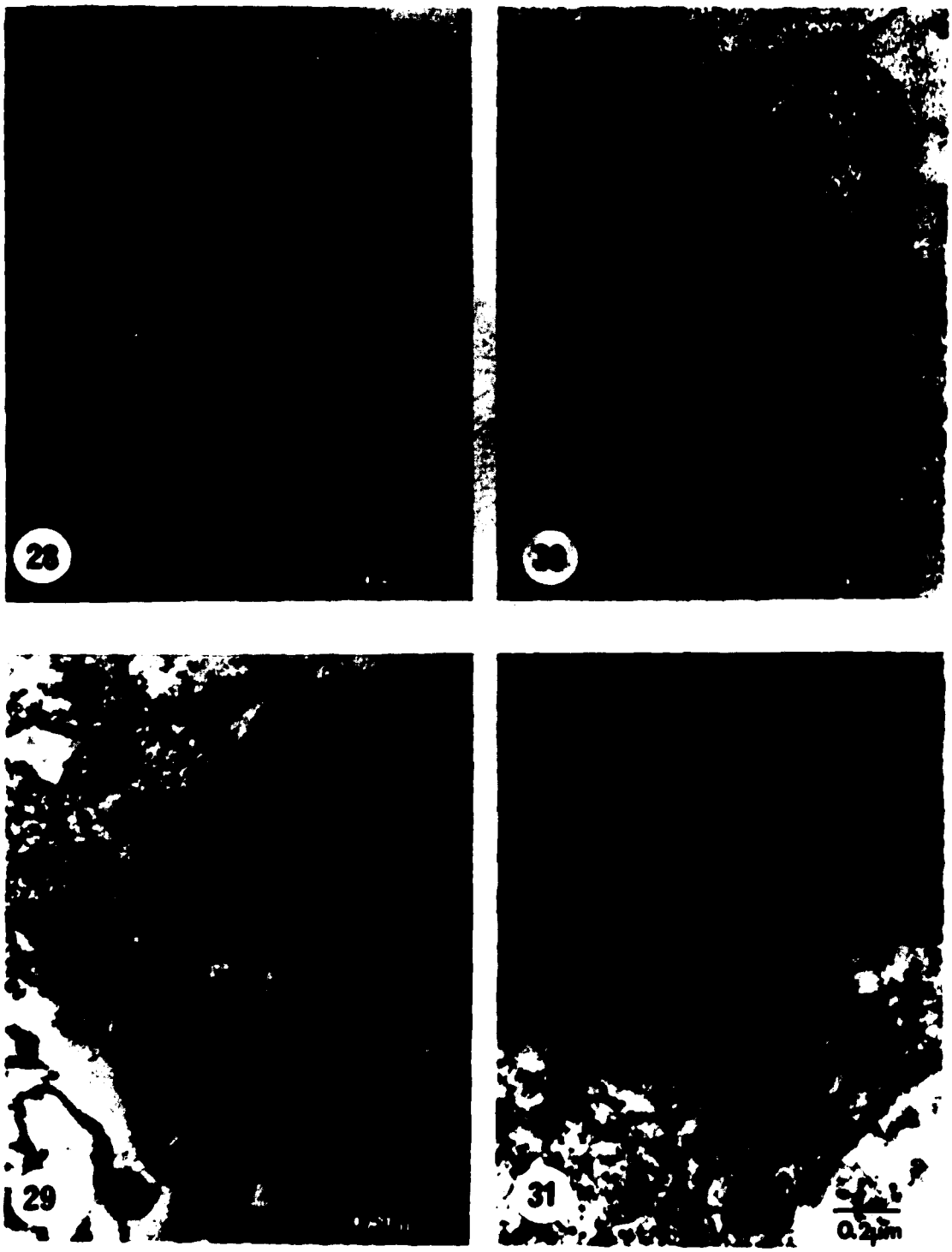

28
30
29
31
0.2μm

PLATE XX.

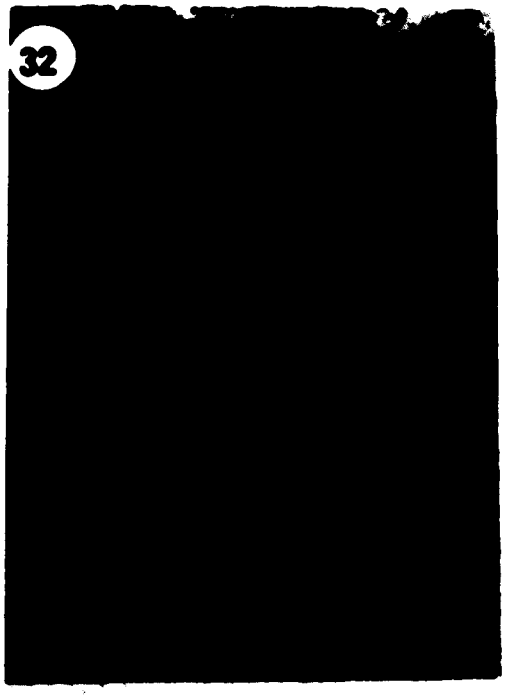
Presence of dense core vesicles in SY5Y cells maintained in N2 medium.

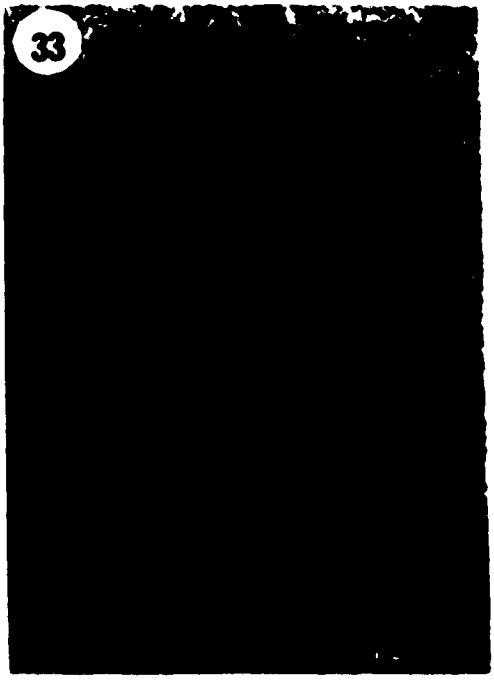
Fig. 32. SY5Y cells maintained in N2 medium for one week. Numerous dcv are evident in a region that may be a growth cone but its relation to process was not apparent in this plane of section. The area consists mainly of membranous elements and only a few ribosomes. The arrow indicates a dcv of 100 nm diameter.
X 23,500

Fig. 33. SY5Y cells maintained in N2-cyclic AMP medium for one week. The dcv are numerous in a cytoplasmic area also rich in ribosomes and mitochondria. Arrow indicates a dcv of 70 nm diameter. Some large dcv are also seen in this section and measure in 200 nm range. A small process (p) is seen interposed betweem two cells.
X 24,800

Fig. 34. SY5Y cells maintained in N2-NGF medium for one week. Numerous dcv are seen along the length of the process which also contains mt, ribosomes and coated vesicles. The arrow indicates a vesicle with a diameter of 100 nm. The adjacent cell body also contains a pair of dcv.
X 19,700

KEY: cv = coated vesicle
dcv = dense core vesicle
mt = microtubule
r = ribosome


32


33


C

PLATE XXI.

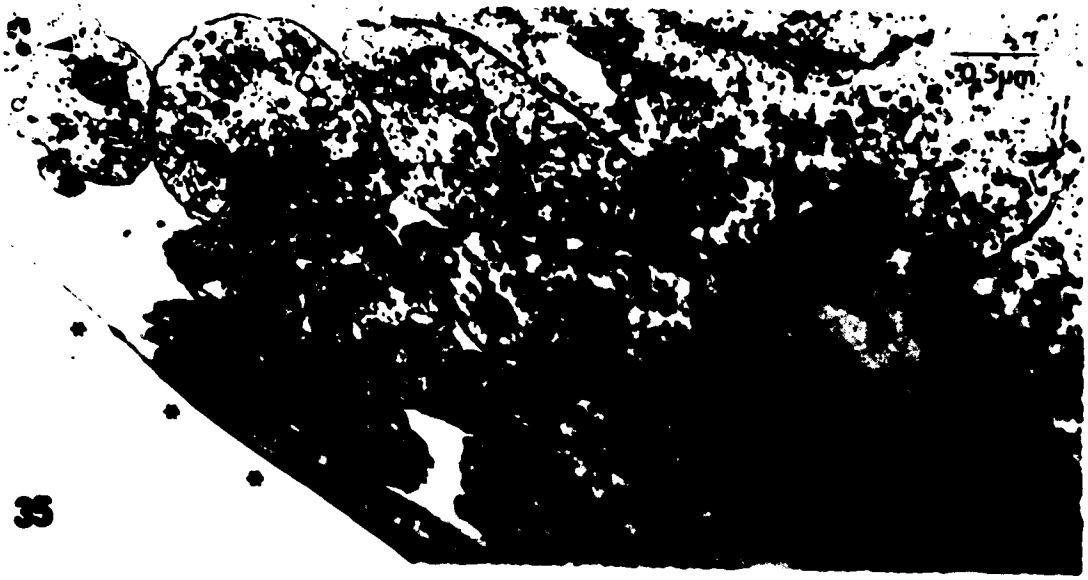
Presence of dense core vesicles in 10 % FCS medium.

Fig. 35. SY5Y culture maintained in 10 % FCS for one week. Cells are seen along the substrate as sections were cut perpendicular to the substrate. Several dcv are seen in various cells. One vesicle of 100 nm diameter is indicated with an arrow. Vesicles of varying sizes and with differences in density of core are evident. X 23,500

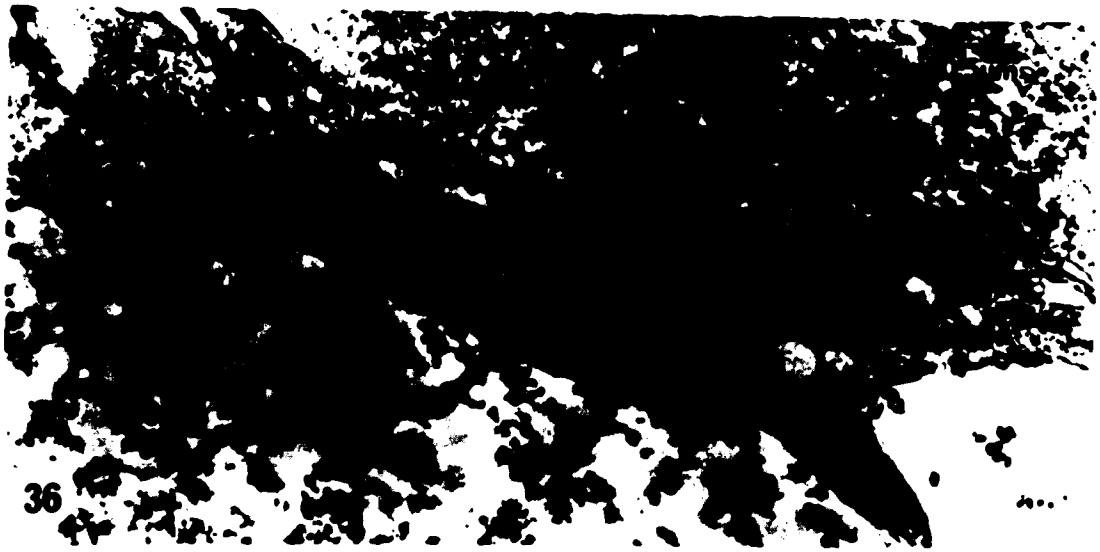
Fig. 36. SY5Y culture maintained in 10 % FCS-NGF medium. The section was cut parallel to the substrate and this cytoplasmic area appears spread over the surface and may be part of a growth cone. Numerous mitochondria are present in this area as well as ribosomes, dcv and a few clear vesicles. Vesicle indicated by arrow is approximately 100 nm in diameter.
X 18,800

Fig. 37. SY5Y cultures maintained in 10 % FCS-cyclic AMP for one week. The section was cut parallel to the culture substrate and numerous dcv of varying sizes are seen. A vesicle of 100 nm diameter is indicated by the arrow.
X 32,000

KEY: dcv = dense core vesicle



35



36

37

PLATE XXII.

Fig. 38. Growth curves of M17 cells in N2, FCS and F12/DME medium. Cell counts were done on triplicate cultures for each time point. The numbers represent viable cells per 35 mm dish $\pm$ S.E.M. as determined by hemocytometer counts of trypan blue excluding cells.

Population doubling time was determined from the linear regression lines whose correlation coeficients were:

FCS $r = 0.99$

N2 $r = 0.98$

F12/DME $r = 0.80$

Fig. 38

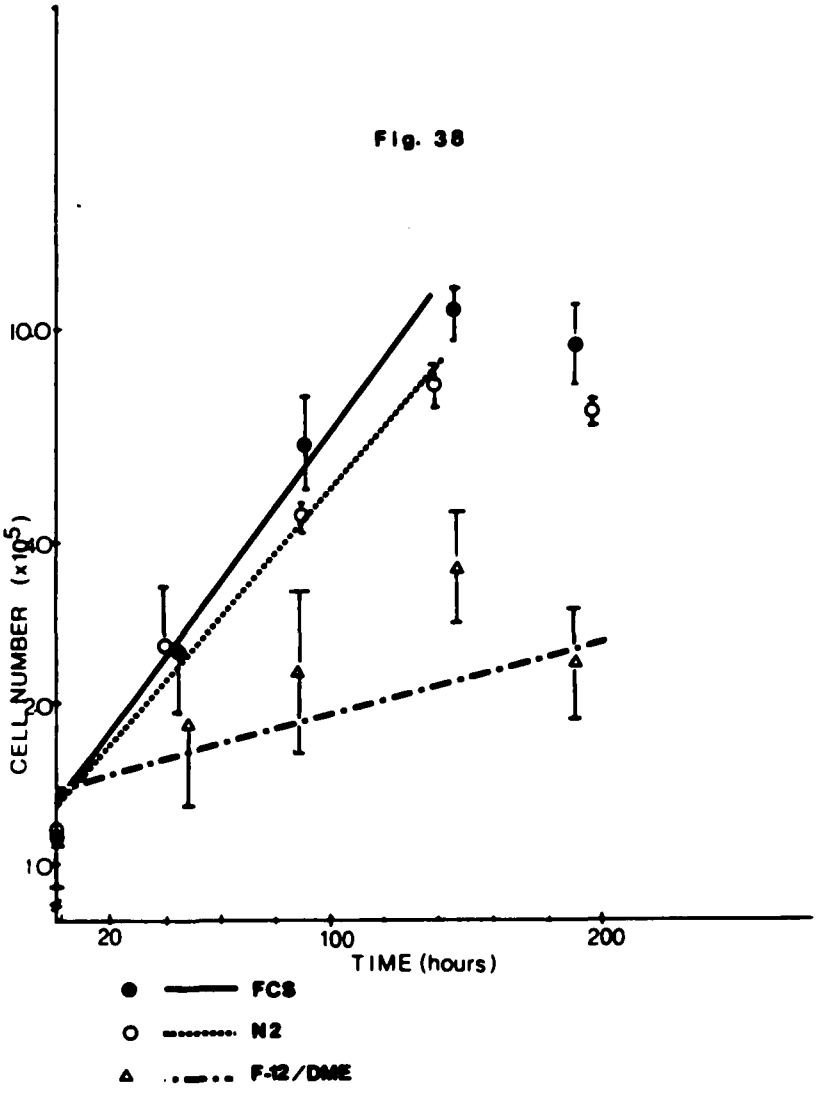

100
40
20
10
CELL NUMBER (x10^5)
20
100
200
TIME (hours)
FCS
N2
F-12/DME

PLATE XXIII.

Fig. 2. Growth curves of M17 cells in N2 vs. N2-cyclic AMP and FCS vs. FCS cyclic AMP. The cyclic AMP was added to a final concentration of 1 mM and IBMX at 0.25 mM was added as a phosphodiesterase inhibitor. Cell counts and plating via same protocol as described in Fig. 38.

Population doubling times were determined from the linear regression lines whose correlation coefficients were:

N2 $r = 0.99$

N2 cyclic AMP $r = 0.95$

FCS $r = 0.99$

FCS cyclic AMP $r = 0.97$

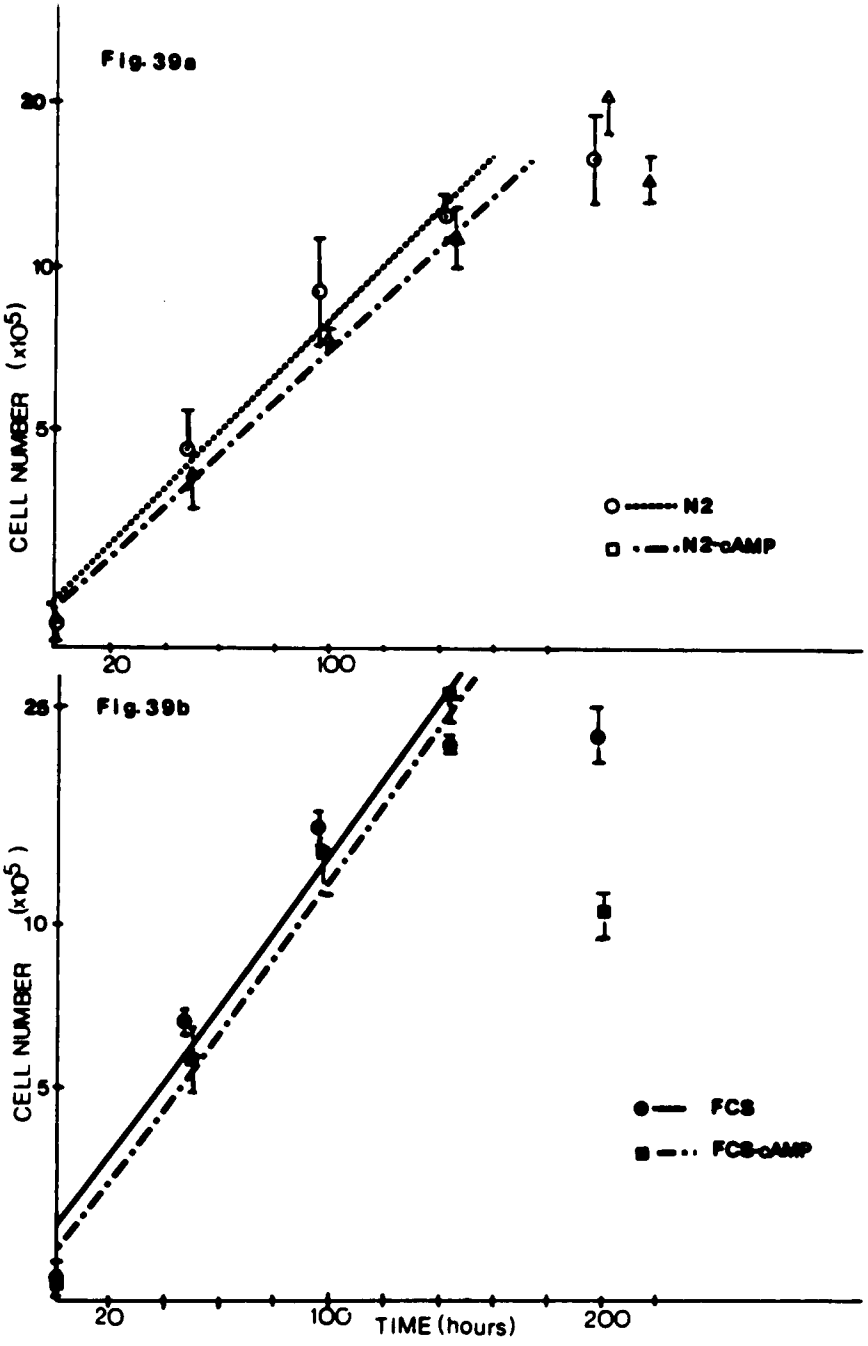


Fig. 39a
20
10
5
CELL NUMBER (x10⁵)
20
100
N2
N2-cAMP
Fig. 39b
25
10
5
CELL NUMBER (x10⁵)
20
100
200
TIME (hours)
FCS
FCS-cAMP

PLATE XXIV.

Fig. 40. Growth Curve of M17 cells in N2, N2-NGF, FCS and FCS-NGF. NGF, 7S, was used at a final concentration of 250ng/ml. Cell counts and plating as described in Fig. 38.

Population doubling times were determined from the linear regression lines whose correlation coefficients were:

N2 $r = 0.98$

N2-NGF $r = 0.85$

FCS $r = 0.99$

FCS-NGF $r = 0.99$

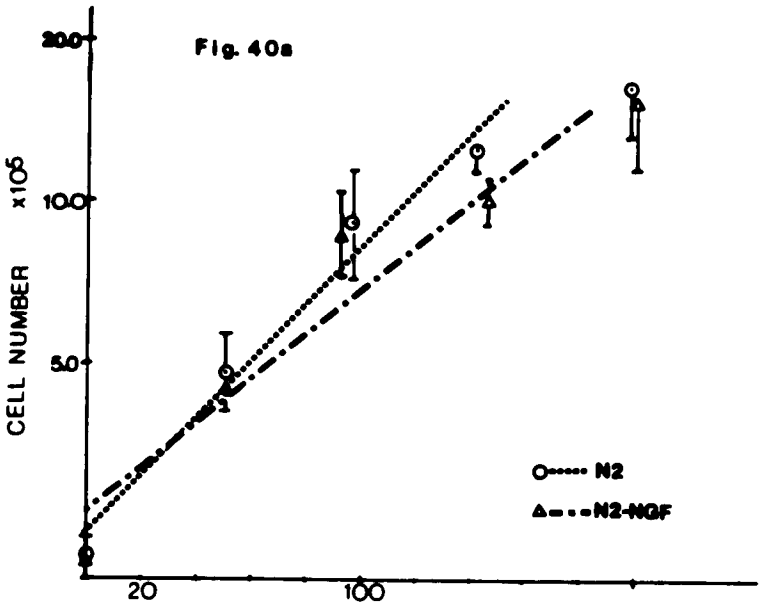


Fig. 40a
20.0
10.0
5.0
CELL NUMBER x10⁵
20
100
N2
N2-NGF

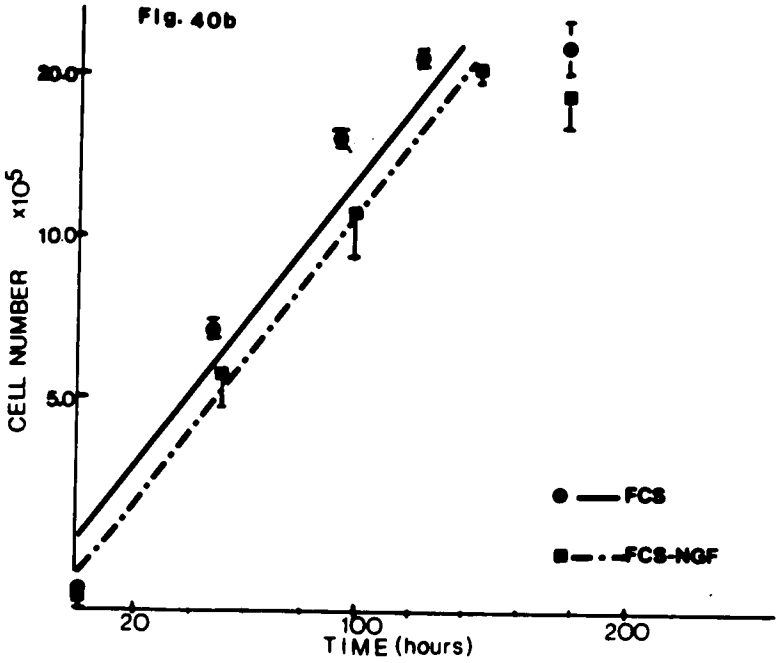


Fig. 40b
20.0
10.0
5.0
CELL NUMBER x10⁵
20
100
200
TIME (hours)
FCS
FCS-NGF

PLATE XXV.

Phase contrast microscopy of M17 cells in 10 % FCS and N2 media. All cultures were initially plated into 10 % FCS medium and switched to experimental medium 24 hours later. Cultures were photographed after five days growth in experimental medium.

Fig.42a. M17 culture in N2 medium. Cells appeared to be of lesser diameter than in 10 % FCS and exhibit a few processes.

Fig. 42b. M17 culture in N2-NGF medium. Cells are similar in morphology to N2 controls.

Fig. 42c. M17 culture in 10 % FCS medium. Note the large and flattened cell morphology. A few processes are evident.

Fig. 42d. M17 culture in 10 % FCS-NGF. Same large flattened morphology as in 10 % FCS. Nuclei have prominent nucleoli. A long thick process is seen in center of the field.

Fig. 42e. M17 culture in 10 % FCS to which N2 factors have been added. Cells resemble those in cultures carried in 10% FCS. A few thin processes are seen.

Fig. 42f. M17 culture in 10% FCS medium to which N2 factors and NGF have been added. Cells appears similar to ones in 10 % FCS-NGF.

NGF = 50 ng/ml B-NGF

X 300

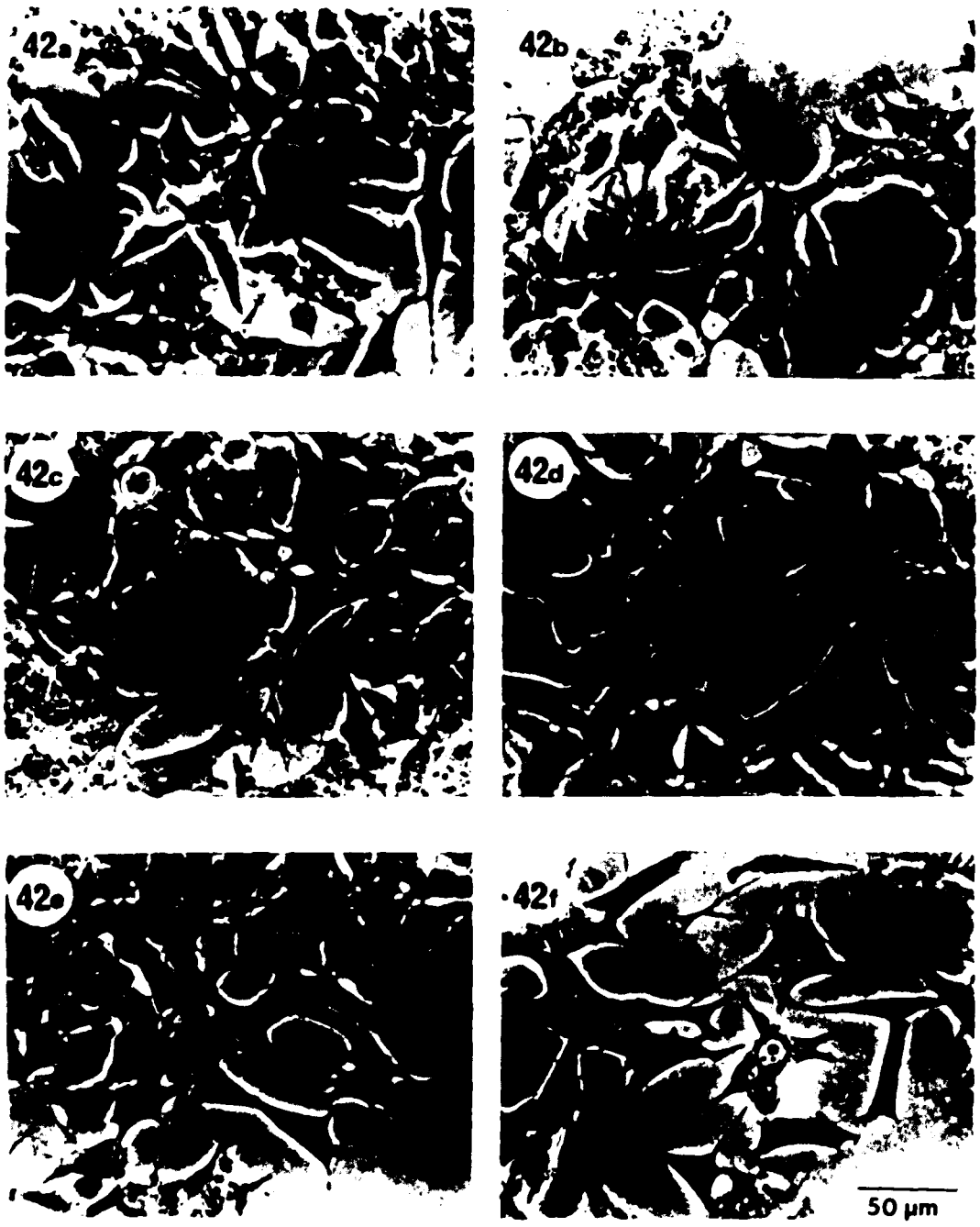

42a
42b
42c
42d
42e
42f
50 µm

PLATE XXVI.

Phase contrast microscopy of M17 cultures maintained in N2 and 10 % FCS. All cells were initially plated into 10% FCS medium and switched at 24 hours to experimental medium. The cultures were photographed after five days in experimental medium.

Fig. 43a. M17 culture in N2 medium.

Fig. 43b. M17 culture in N2 medium to which cyclic AMP and IBMX had been added. No change in morphology at five days was observed.

Fig. 43c. M17 culture in 10 % FCS medium.

Fig. 43d. M17 culture in 10 % FCS medium to which cyclic AMP and IBMX had been added. No change in morphology as compared to controls was observed at five days.

Fig. 43e. M17 culture in 10 % FCS medium to which the N2 had been added. This field contains some very large and flattened cells. The nucleoli are apparent in the nuclei.

Fig. 43f. M17 culture in 10 % FCS medium to which the N2 and cyclic AMP and IBMX had been added. Some cells in the population appeared to have reduced diameter. Long thick process with several branches runs across center of the field.

X 300

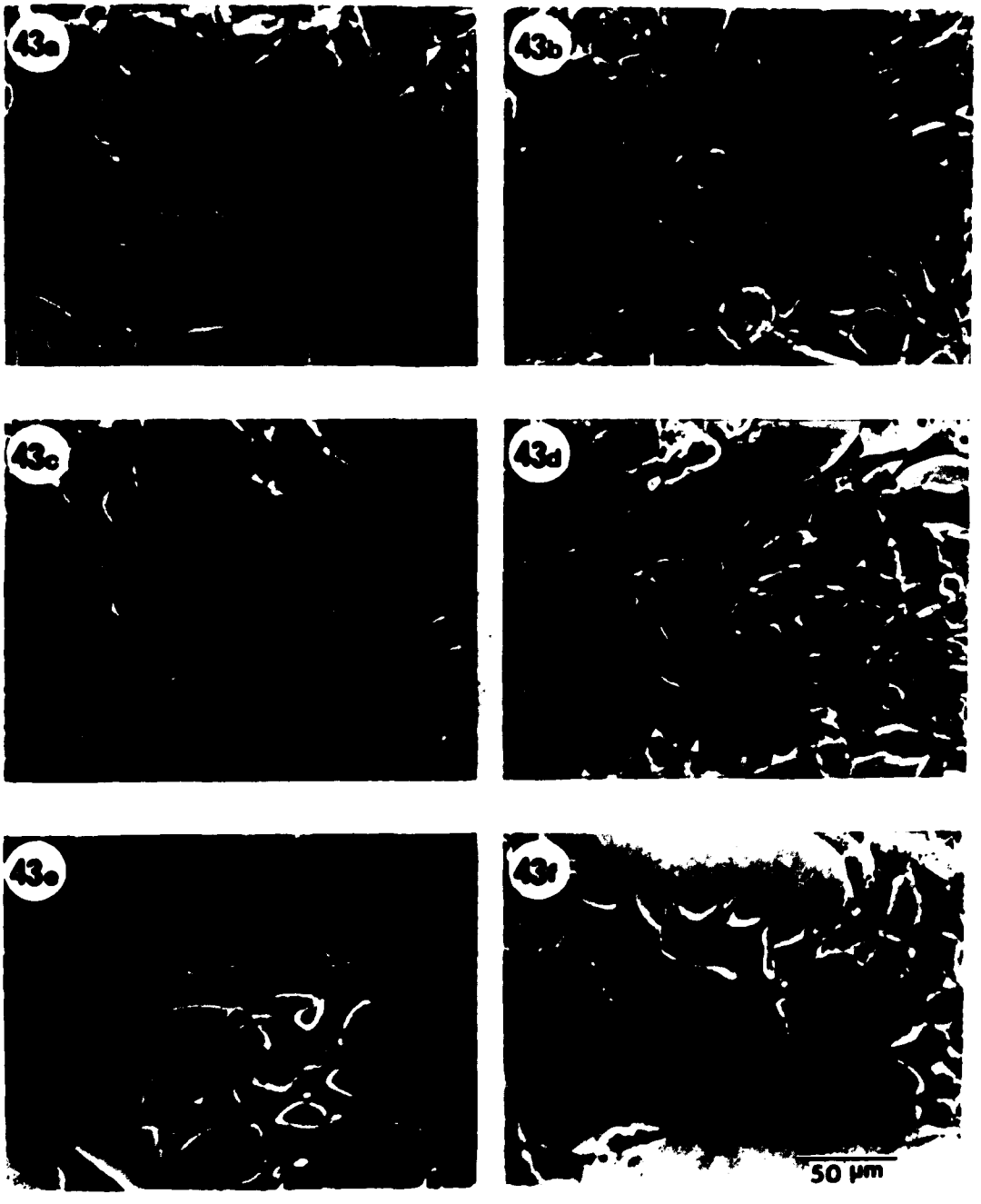

43a
43b
43c
43d
43e
43f
50 µm

PLATE XXVII.

Phase contrast microscopy of M17 cultures with cyclic AMP, IBMX or butyrate added to N2 or FCS medium. No significant alterations are apparent under these conditions.

Fig. 44a. M17 culture in N2.

Fig. 44b. M17 culture in 10 % FCS.

Fig. 44c. M17 culture in N2-cyclic AMP.

Fig. 44d. M17 culture in 10% FCS-cyclic AMP.

Fig. 44e. M17 culture in N2-IBMX.

Fig. 44f. M17 culture in 10 % FCS-IBMX.

Fig. 44g. M17 culture in N2-butyrate.

Fig. 44h. M17 culture in 10 % FCS-butyrate.

X 300

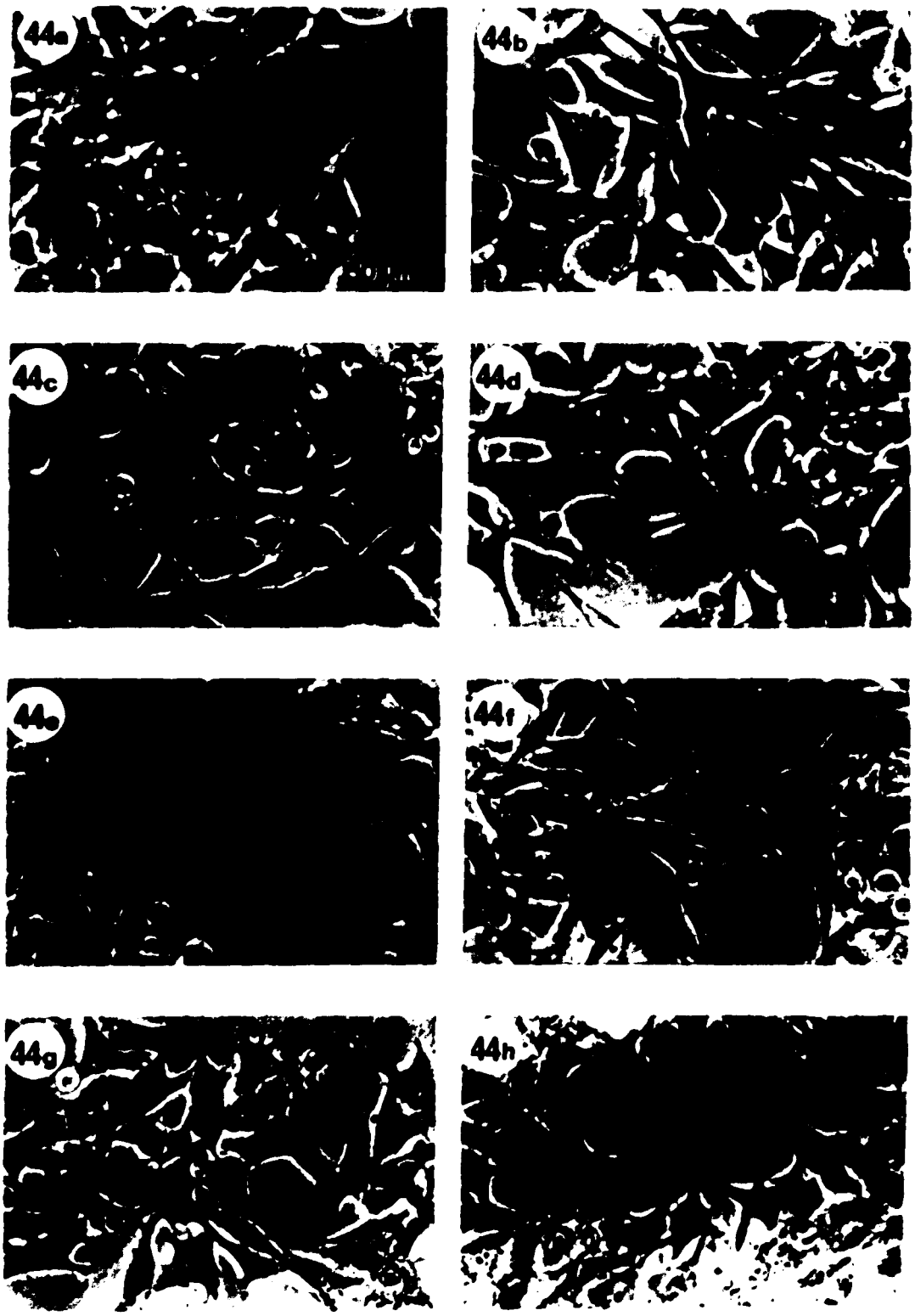

44a
44b
44c
44d
44e
44f
44g
44h

PLATE XXVIII.

Fig. 45. Electron micrograph of M17 cell from 10 % FCS culture. Note presence of dcv of varying sizes. including vesicles of 100 nm diameter(arrows). Cytoplasmic protrusions into the nucleus (*) are frequently observed. Nucleolar material and condensed chromatin are found in most nuclei.

X 14,300

KEY: cv = coated vesicle
dcv = dense core vesicle
n = nucleolous
Nu = nucleus


45
1.0μm

PLATE XXIX.

Fig.46. Area of cytoplasm exhibiting extensive Golgi, numerous coated vesicles in vicinity of the Golgi, a pair of centrioles from which numerous microtubules radiate.

X 23,500

KEY: C = centriole
cv = coated vesicle
g = Golgi apparatus
m = mitochondrion
Nu = nucleus
rer = rough endoplasmic reticulum


46
1.0μm

PLATE XXX.

Centriolar areas of M17 cells in 10 % FCS medium. Note the abundance of microtubules in the area as well as extensive Golgi and vesicluiar elements.

Fig. 47. Longitudinal section through pair of centrioles.

X 43,800

Fig. 48. Cross section through centriole.

X 43,800

KEY: C = centriole
er = endoplasmic reticulum
g = Golgi
mt = microtubules
np = nuclear pore

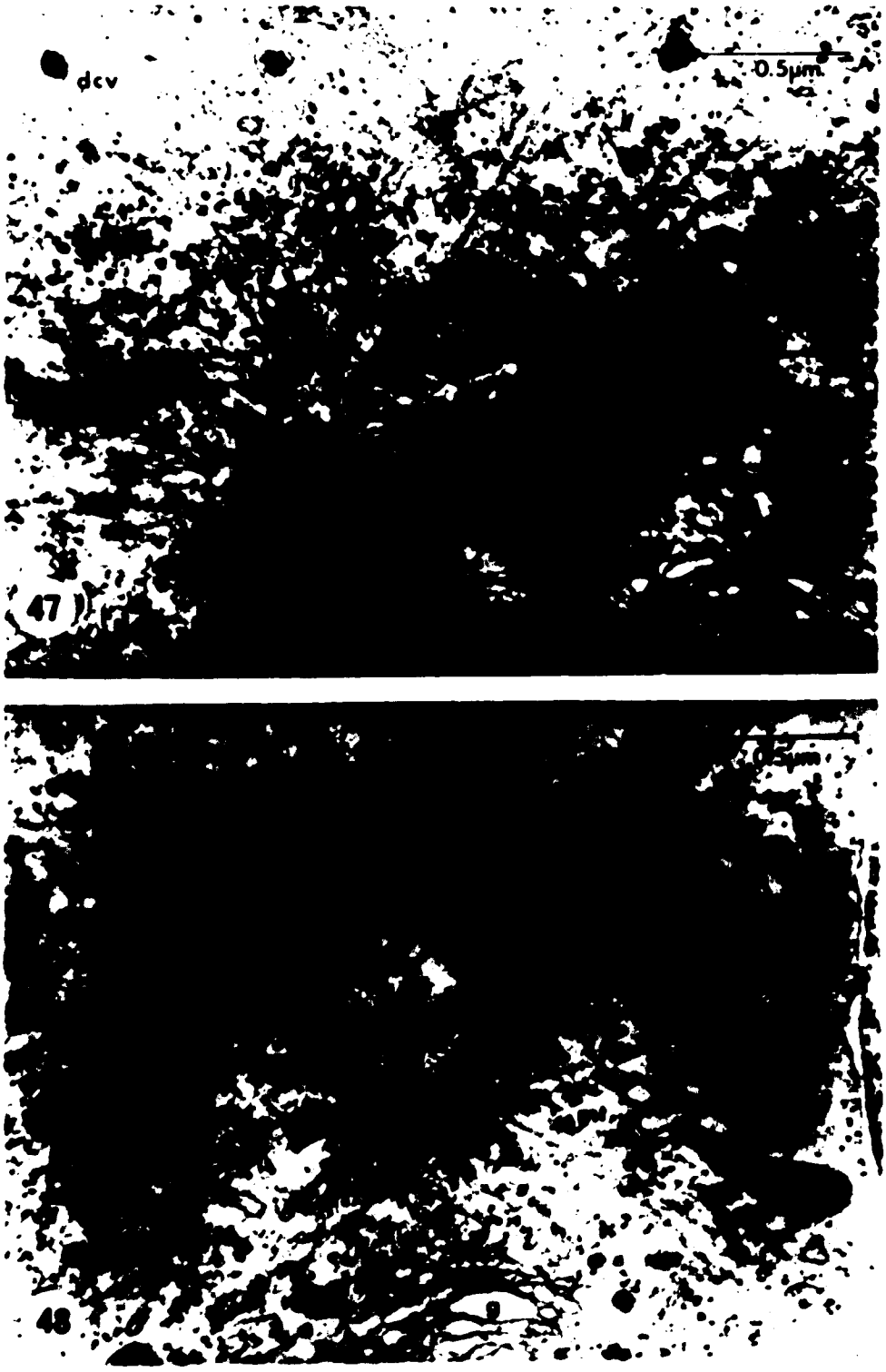

dcv
0.5µm
47
48

PLATE XXXI.

High power micrographs of extensive Golgi apparati present in M17 in 10 % FCS medium. Note the numerous veiscular elements present in this region including dense core, coated and clear vesicles. Ribosomal content is rich and mitochondria numerous.

Fig. 49. M17 culture, 10 % FCS. X 43,500

Fig. 50. M17 culture, 10 % FCS. X 43,500

KEY: cv = coated vesicle
dcv = dense core vesicle
g = Golgi apparati
m = mitochondrion
Nu = nucleus
v = vesicle

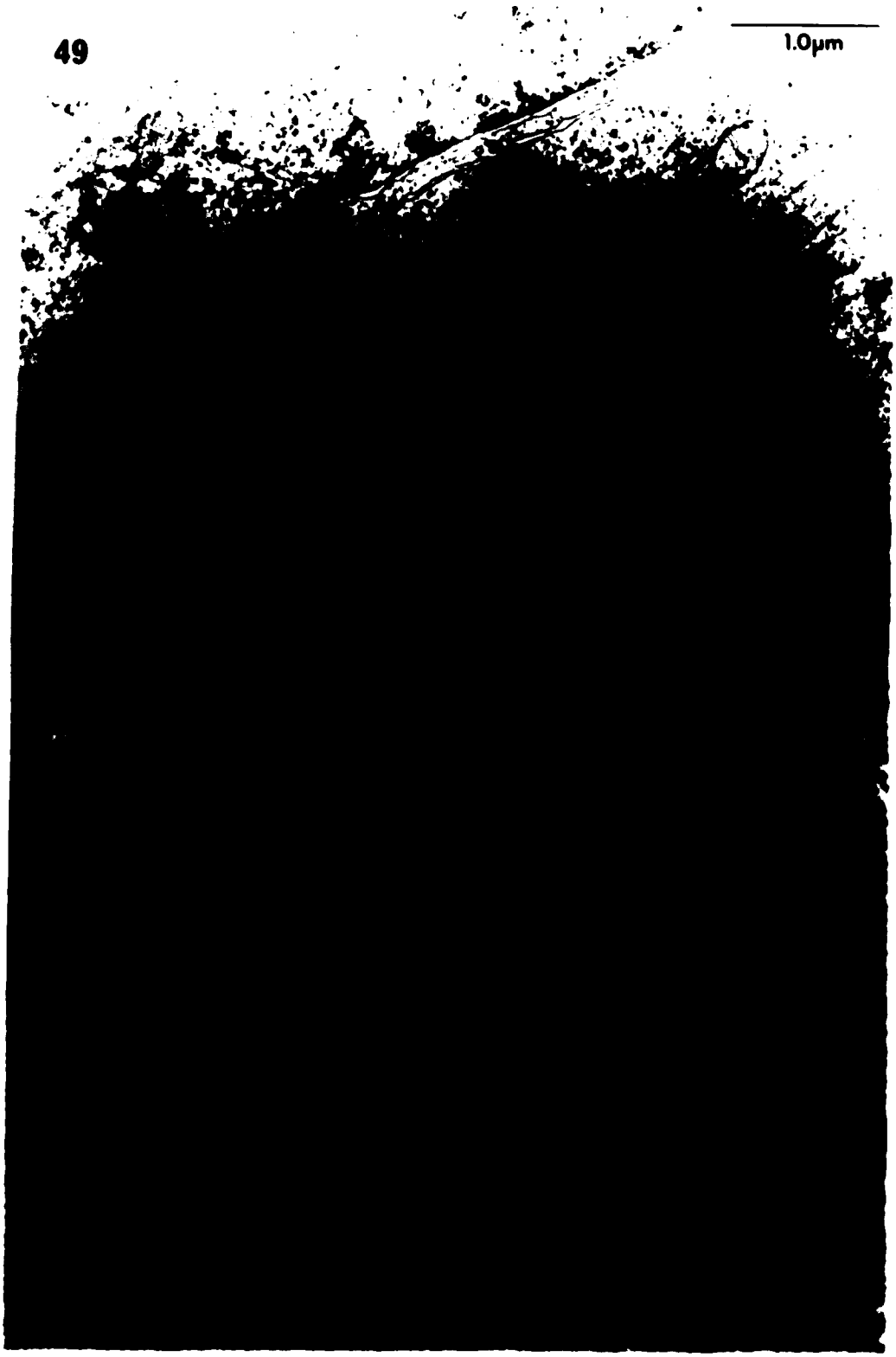

49
1.0µm

PLATE XXXII.

Fig. 51. Dividing cell in M17 culture maintained in N2 medium for one week. Membraneous organelles, rer, ser are concentrated at the periphery of the cell while chromatin occupies central region. Dense core vesicles are present (indicated with arrows, one pair enlarged in insert). A membranous mound filled with clear vesicles (*) is seen.

X 7300

KEY: c = centriole
dcv = dense core vesicle
rer = rough endoplasmic reticulum

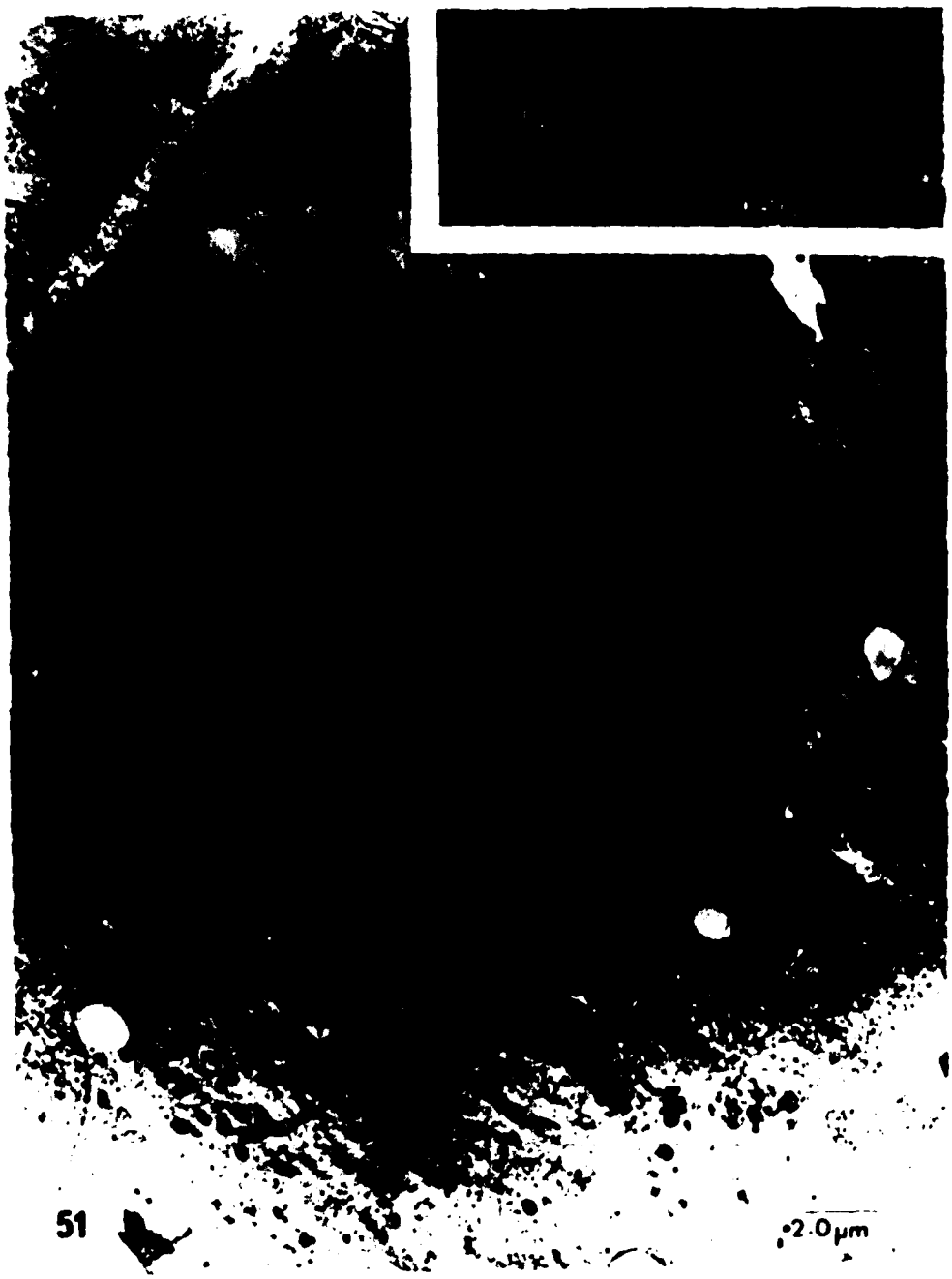

51
2.0μm

PLATE XXXIII.

Fig. 52. Portion of process as it emerges from the cell from an area with extensive Golgi, and rich in vesicles. Microtubules in parallel array with numerous ribosomes, mitochondria, er, some filamentous material scattered along process and a dcv.

X 16,900

KEY: cv = coated vesicles
dcv = dense core vesicles
er = endoplasmic reticulum
g = Golgi
m = mitochondrion
mt = microtubule


52
1.0µm

PLATE XXXIV.

Longitutudinal sections through areas rich in microtubules and filaments.

Fig. 53. Shaft of M17 process composed mainly of filamentous material with little microtubular content in this section of the process.

X 32,000

Fig. 54. Several areas where microtubules are in parallel arrays and associated with areas of reduced ribosomal content probably portions of cellular processes.

X 24,800

KEY: cv = coated vesicle
dcv = dense core vesicle
f = filament
mt = microtubule


53
0.5μm


54
0.5μm

PLATE XXXV.

Processes formed by M17 cells in 10 % FCS medium and N2. The processes are rich in microtubules and intermediate filaments are also observed. A few processes of small diameter or narrow portions of a process consisting of mainly filamentous elements are also noted (*).

Fig. 55. M17 culture in 10 % FCS. X 60,300

Fig. 56. M17 culture in N2. X 90,000

Fig. 57. M17 culture in N2. X 46,300

Fig. 58. M17 culture in N2. X 57,500

KEY: if = intermediate filament
mt = microtubule
Nu = nucleus
rer = rough endoplasmic reticulum


55
0.1μm


57
mt
0.1μm


56
0.1μm


58
0.1μm

PLATE XXXVI.

Processes observed in M17 cells containing many cytoplasmic organelles in addition to numerous microtubules. Note the presence of mitochondria, ribosomes, rough endoplasmic reticulum, and dense core vesicles.

Fig. 59. Several sections through small and large diameter processes. M17 culture in 10 % FCS.

X46,600

Fig. 60. Processes with numerous ribosomes, microtubules, rer. M17 culture in 10 % FCS.

X46,600

Fig. 61. Processes with numerous ribosomes, er and mt. M17 culture 10% FCS.

X50,000

KEY: dcv = dense core vesicle
m = mitochondrion
mt = microtubule
rer = rough endoplasmic reticulum


59
0.5μm


60
0.5μm


61
0.1μm

PLATE XXXVII.

Fig. 62. M17 culture in 10 % FCS. Area contains numerous dense core vesicles of varying sizes. Also rich in ribosomes, mitochondria and rer. Some dense core vesicles are 990 to 100 nm in diameter (arrows).

× 29,000

KEY: dcv = dense core vesicle
m = mitochondria
mt = microtubule
Nu = nucleus
rer = rough endoplasmic reticulum

PLATE XXXVIII.

Areas containing large numbers of profiles with dense material in cisternae or vesicles. Vesicles of 100 and 130 nm indicated with arrows. Numerous other cytoplasmic organelles are present.

Fig. 63. M17 culture in 10 % FCS.

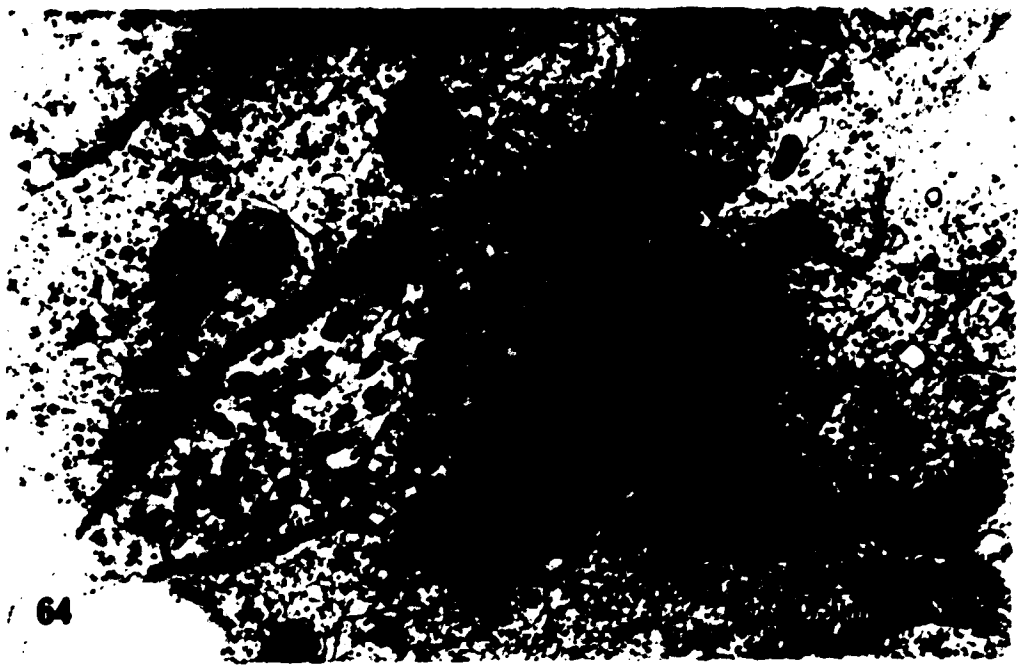
X 41,400

Fig. 64. M17 culture in 10 % FCS. Note the presence of many irregularly shaped vesicles which may be sections through cisternae of endoplasmic reticulum.

X 26,200

KEY: m = mitochondrion
mt = microtubule
f = filaments

63



64

PLATE XXXIX.

Areas containing large numbers of dense core vesicles from M17 cultures maintained in N2 medium.

Fig. 65. Numerous vesicles in area which appears to have a cellular extension spread along the surface. Microtubules are seen in the connecting region and vesicles in a bleb. A membranous mound is also seen (*). Section was cut parallel to the substrate.

X 23,500

Fig. 66. Numerous dense core vesicles including small dcv of 100 nm diameter indicated by arrow. Section cut parallel to the substrate.

X 20,700

Fig. 67. Dense core vesicles in process near the substrate. Small vesicles of 100 nm diameter are indicated by the arrows. Section was cut parallel to the surface.

X 27,600

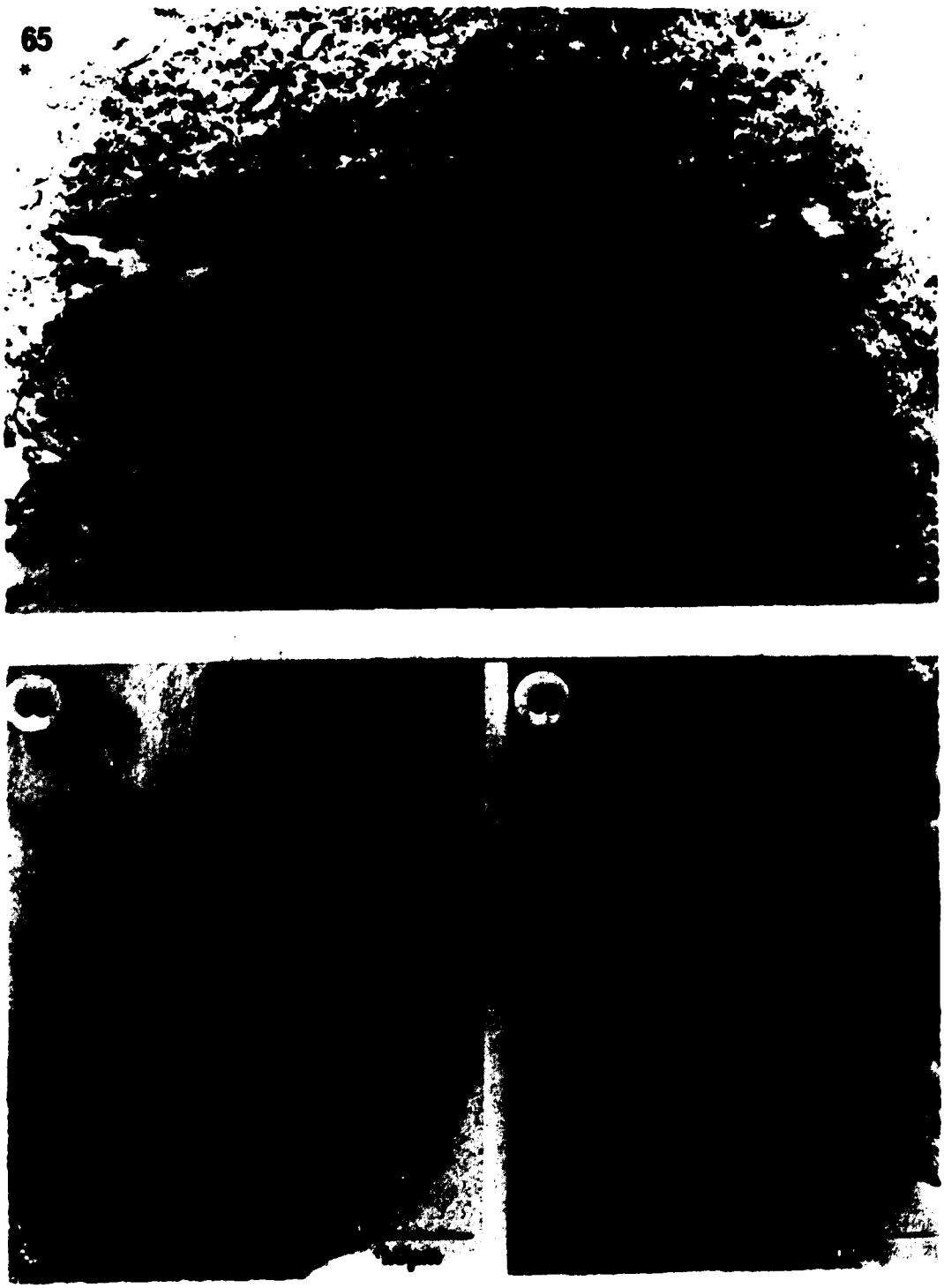

65

PLATE XL.

Immunocytochemical staining of SY5Y with antibodies vs. tyrosine hydroxylase and dopamine-β-hydroxylase using PAP method.

Fig. 68a. SY5Y, N2 medium, polylysine substrate, preimmune serum.

Fig. 68b. SY5Y, 10 % FCS medium, tissue culture polystyrene substrate, preimmune serum.

Fig. 68c. SY5Y, N2 medium, polylysine-coated substrate, anti-TH serum.

Fig. 68d. SY5Y, 10 % FCS medium, tissue culture polystyrene substrate, anti-TH serum.

Fig. 68e. SY5Y, N2 medium, polylysine-coated substrate, anti-DBH serum.

Fig. 68f. SY5Y, 10% FCS, polylysine-coated substrate, anti DBH serum.

Fig. 68g. SY5Y, 10 % FCS, polylysine-coated substrate, anti-DBH serum.

Fig. 68h. SY5Y, 10 % FCS, tissue culture polystyrene substrate, anti-DBH serum.

X 800

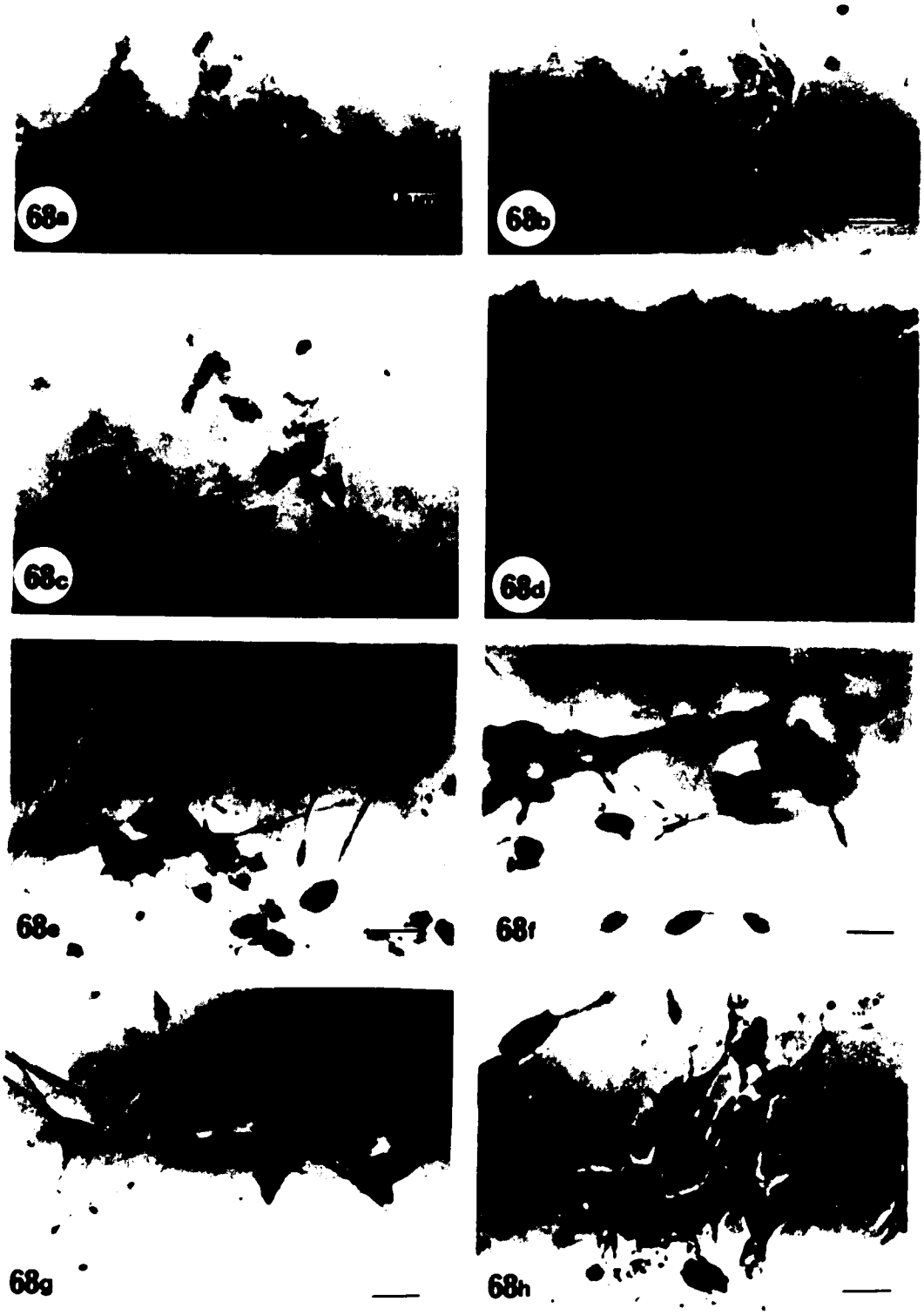

68a
68b
68c
68d
68e
68f
68g
68h

PLATE XLI.

Immunocytochemical staining of M17 with antibodies vs. tyrosine hydoxylase (TH) and dopamine-β-hydroxylase (DBH) using PAP method.

Fig. 69a. M17, 10 % FCS, tissue culture polystyrene substrate, preimmune serum.

Fig. 69b. M17, N2 medium, polylysine-coated substrate, anti-DBH serum.

Fig. 69c. M17, 10 % FCS, tissue culture polystyrene substrate, anti-DBH serum.

Fig. 69d. M17, N2 medium, polylysine-coated substrate, anti-DBH serum.

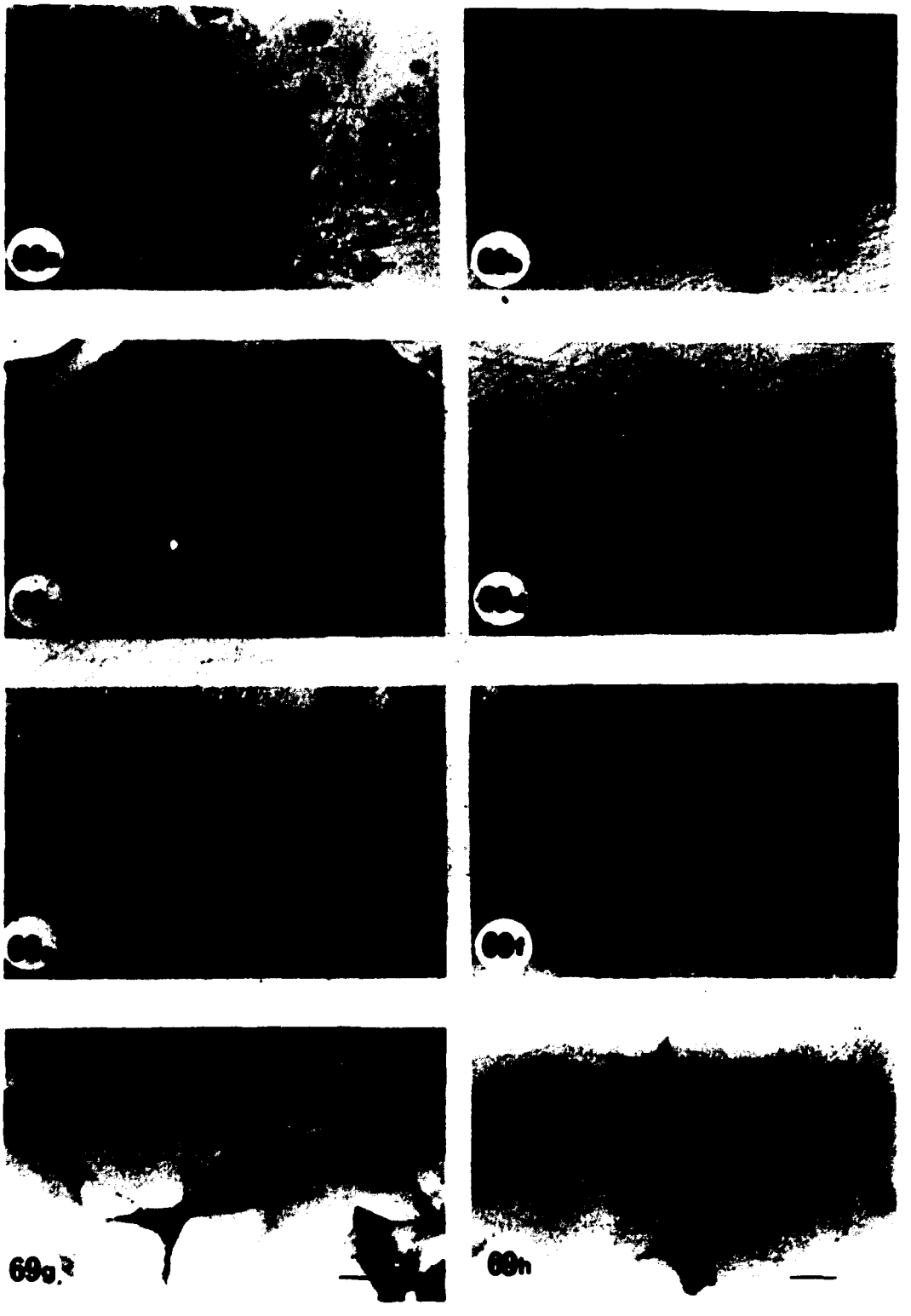
Fig. 69e. M17, 10 % FCS, polylysine-coated substrate, anti-TH serum.

Fig. 69f. M17, 10% FCS, tissue culture polystyrene substrate, anti-TH serum.

Fig. 69g. M17, 10 % FCS, polylysine-coated substrate, anti-TH serum.

Fig. 69h. M17, N2 medium, polylysine-coated substrate, anti-TH serum.

X 800



69g, h

PLATE XLII.

Sections of immunocytochemically stained cultures, PAP method.

Fig. 70a. M17 culture, 10 % FCS, stained with anti-DBH serum.
X 800

Fig. 70b. 3 µm section cut through M17 culture, post-staining. Note the dark DBH immunoreactivity throughout nucleoplasm as well as in cytoplasm. X 1400

Fig. 70c. SY5Y culture, 10 % FCS, stained with anti-DBH serum.

X 800

Fig. 70d. 3 µm section through SY5Y culture, post staining. Note the DBH immunoreactivity in nucleoplasm and cytoplasm.

X 1400

Fig. 70e. SY5Y culture, 10% FCS, stained with anti-TH serum.

X 800

Fig. 70f 3 µm section through SY5Y culture, post staining. Faint TH immunoreactivity present in cytoplasm, nucleus appears dense.

X 1400

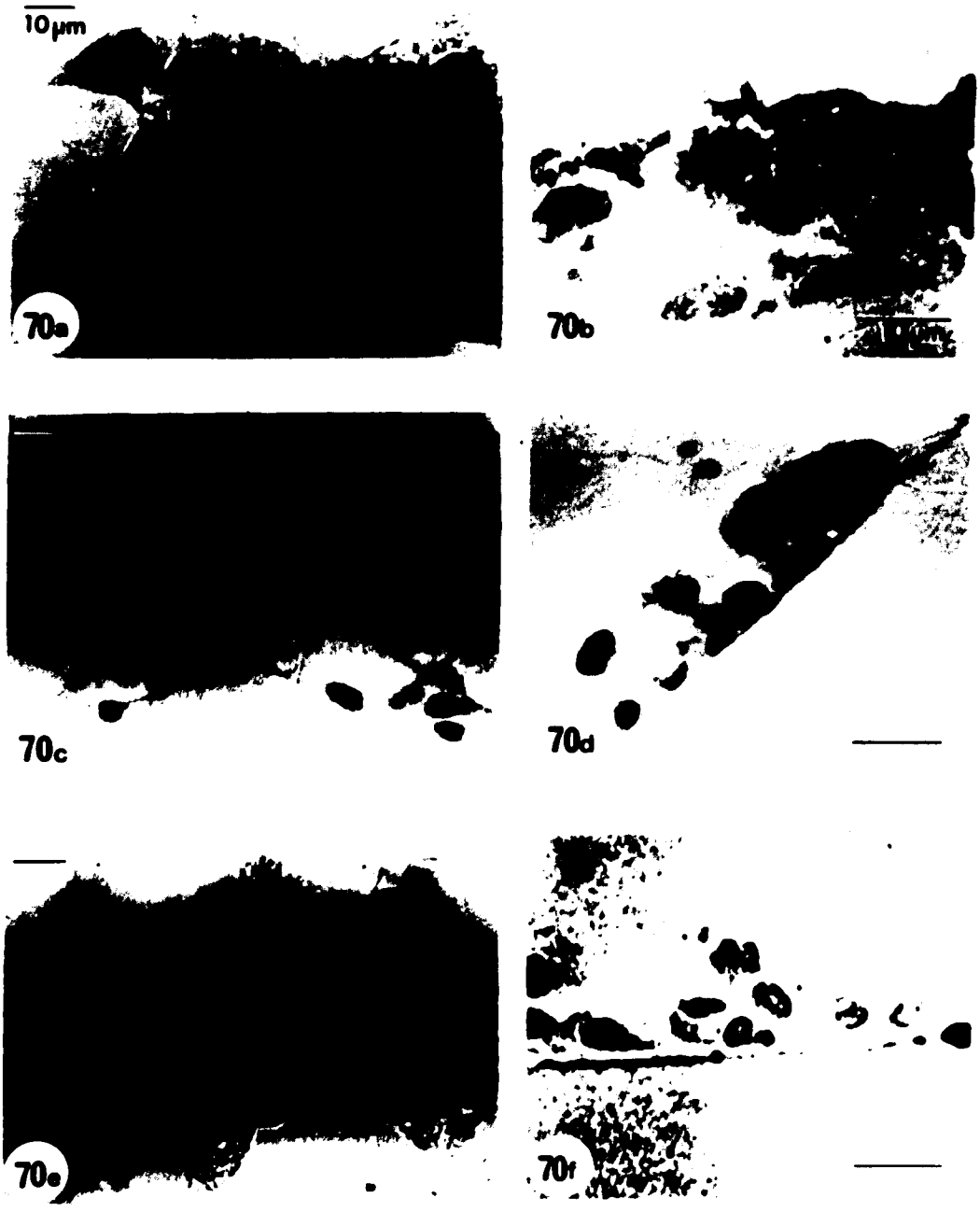

10μm
70a
70b
70c
70d
70e
70f

PLATE XLIII.

Sections of M17 cultures treated with anti-TH antibody and stained using PAP method.

Fig. 71a. M17 culture, 10 % FCS, preimmune serum.

X 200

Fig. 71b. M17 culture, 10 % FCS, anti-TH serum.

X 200

Fig. 71c. M17 culture, 10% FCS, anti-TH serum.

X 800

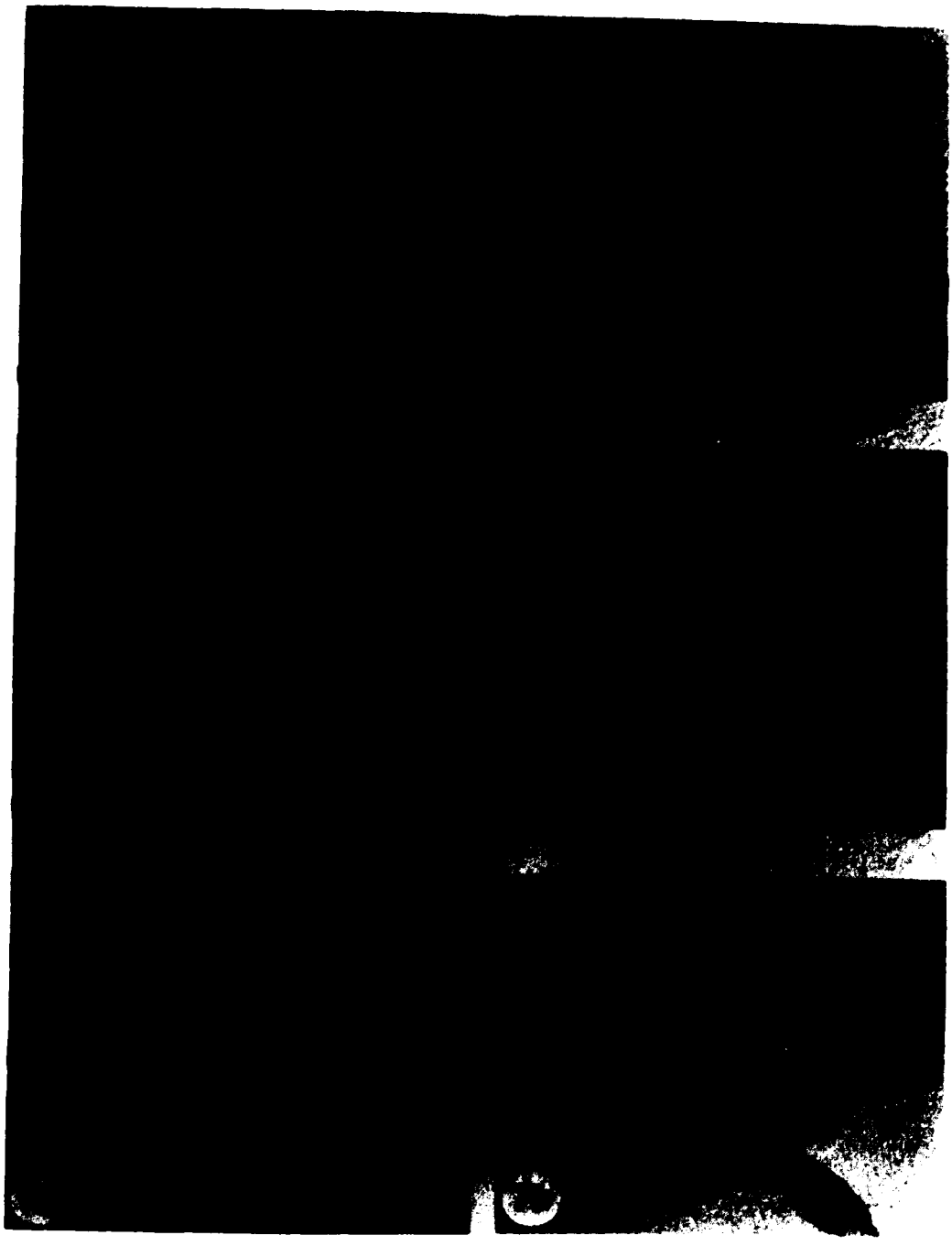
Fig. 71d. 3 µm section through M17 culture treated with anti-TH serum, counterstained with toluidine blue. Note presence of dividing cell.

Fig. 71e. 3 µm section adjacent to that in 71d. Note dark reaction product in cytoplasm and clear areas corresponding to the nuclei.

X 1400

Fig. 71f. 3 µm section of M17 culture previously reacted with anti-TH antibody. Note the dark PAP reaction product in cytoplasm and absence of reaction in nucleus. Dividing cell rounded up containing intense reaction product.

X 1400



TABLES

TABLE NUMBER 1.

SY5Y POPULATION DOUBLING TIMES

MEDIUM	N	MEAN POP. DOUBLING TIME ± S.E.M.
10% FCS	3	43 ± 2.9
N2	5	74 ± 3.8***
10% FCS +dbcAMP +IBMX	4	65 ± 7.0*
10% FCS+ NGF	4	47 ± 2.6
N2	5	74 ± 3.8
N2 +dbcAMP +IBMX	4	189 ± 21.8***
N2+ NGF	4	144 ± 23.7*

NOTES. dbcAMP concentration was 1.0 mM, IBMX 0.25 mM.
NGF concentration was 50 ng/ml 2.5S NGF.

* $p < 0.05$
*** $p < 0.001$

TABLE NUMBER 2.

THE EFFECTS OF FCS and N2

ON SY5Y DOPAMINE -β- HYDROXYLASE ACTIVITY

MEDIUM	N	DBH SPEC. ACTIVITY ± S.E.M.
N2	11	11.51 ± 0.55
10% FCS	7	6.90 ± 0.40***
N2 4-5 days	11	11.51 ± 0.55
N2 7 days & long term	5	9.20 ± 1.50
10% FCS 4 days	7	6.90 ± 0.40
10% FCS 7 days	7	4.80 ± 0.16***

NOTES. Specific activity is expressed as nmoles octopamine converted to methyloctopamine per hour per mg total cell protein.

*** $p < 0.001$.

TABLE NUMBER 3.

THE EFFECT OF FACTORS

ON SY5Y TYROSINE HYDROXYLASE ACTIVITY

MEDIUM	N	TYROSINE HYDROXYLASE SPEC. ACT. ± S.E.M.
N2	6	0.009 ± 0.002
N2 + dbcAMP + IBMX	8	0.017 ± 0.002
N2 + NGF	5	n.d.
N2 + IBMX	3	n.d.
N2 + dbcAMP	2	0.010
10% FCS	5	0.023 ± 0.005
10% FCS + dbcAMP + IBMX	6	0.018 ± 0.005
10% FCS + dbcAMP	1	0.006
10% FCS + IBMX	1	0.013
10% FCS + NGF	4	n.d.

NOTES. Specific activity expressed as nmoles of tyrosine converted to DOPA per hour per mg total cell protein.
n. d. not detectable, activity less than twice blank.

dbcAMP concentration was 1 mM, IBMX 0.25 mM.
NGF concentration was 50 ng/ml 2.5S NGF.

TABLE NUMBER 4.

THE EFFECTS OF CYCLIC AMP

ON SY5Y DOPAMINE -β- HYDROXYLASE ACTIVITY

MEDIUM	N	DBH SPEC. ACT. ± S.E.M.
N2	11	11.51 ± 0.55
N2 +dbcAMP +IBMX	10	21.58 ± 0.86***
N2 +8bromo cAMP	3	19.04 ± 0.45***
N2 +dbcAMP	2	18.97 ± 0.69***
N2 +butyrate	2	16.35 ± 0.35**
10% FCS	7	6.90 ± 0.40
10% FCS +dbcAMP	5	15.23 ± 1.05***
10% FCS +8bromo cAMP +IBMX	4	18.71 ± 1.65***
10% FCS +dbcAMP +IBMX	2	13.45 ± 0.42**
10% FCS +butyrate	2	4.95 ± 1.00

NOTES. Specific activity expressed as nmoles octopamine converted to methyloctopamine per hour per mg total cell protein.

** $p < 0.01$
*** $p < 0.001$

TABLE NUMBER 5.

THE EFFECTS OF NGF

ON SY5Y DOPAMINE -β- HYDROXYLASE ACTIVITY

MEDIUM	TIME DAYS	N	CELL DENSITY x 10^4/cm	DBH SPEC. ACT.
N2A	6	1	----	4.96
	14	2	7.10	6.64
	21	1	3.60	6.97
	23	1	6.40	6.43
N2A +NGF	6	1	----	6.85
	14	2	6.40	7.54
	21	2	6.40	8.92
	23	1	6.80	9.46
FCS	6	1	----	5.18
	14	2	14.30	4.28
	21	2	9.64	5.06
	23	2	17.10	4.71
FCS +NGF	6	1	----	5.54
	14	2	10.70	4.67
	21	2	10.70	7.39
	23	1	14.30	6.70

NOTE: NGF = β-NGF at 50 ng/ml

TABLE NUMBER 6.

THE EFFECTS OF NGF

ON SY5Y DOPAMINE -β- HYDROXYLASE ACTIVITY

MEDIUM	N	NGF CONC. mg/ml	TIME	DBH SPEC. ACT. ± S.E.M.
N2A	5	---	1-4 wks	6.67 ± 0.61
N2A +NGF[a]	7	50	1-4 wks	8.38 ± 0.38*
N2	5	---	1 wk	9.20 ± 1.50
N2 +NGF[a]	7	10	1 wk	8.10 ± 1.51
N2	2	---	5 d	10.01 ± 0.37
N2 +NGF[b]	2	100	5 d	6.67 ± 0.89
10% FCS	7	---	1-4 wks	4.80 ± 0.16
10% FCS +NGF[a]	7	50	1-4 wks	5.62 ± 0.48

NOTES. a. NGF supplied as 2.5 S β-NGF.
b. NGF supplied as 7 S NGF to give concentration equivalent to amount of β-NGF indicated.

Specific activity is expressed as nmoles octopamine converted to methyloctopamine per hour per mg total cell protein.

* $p < 0.05$

TABLE NUMBER 7.

M17 POPULATION DOUBLING TIMES

MEDIUM	N	MEAN POP. DOUBLING TIME ± S.E.M.
FCS	3	43 ± 0.0
N2	3	55 ± 2.1***
FCS +dbcAMP +IBMX	3	39 ± 1.4*
FCS +NGF	3	47 ± 3.7
N2	3	55 ± 2.1
N2 +dbcAMP +IBMX	3	60 ± 6.4
N2 + NGF	3	57 ± 5.2

NOTES. dbcAMP concentration was 1mM, IBMX concentration was 0.25mM.

NGF concentration was 250 ng/ml 7S NGF (equivalent to 50 ng/ ml 2.5S NGF).

* $p < 0.05$
*** $p < 0.001$

TABLE NUMBER 8.

THE EFFECTS OF FCS AND N2 MEDIA

ON M17 TYROSINE HYDROXYLASE ACTIVITY

MEDIUM	N	TOTAL CELL PROTEIN	TYROSINE HYDROXYLASE SPEC. ACT. ± S.E.M.
10% FCS	7	1.70 ± 0.16	34.97 ± 0.17
N2	8	1.14 ± 0.15*	28.40 ± 2.80**
N2 +10% FCS	3	1.65 ± 0.03	35.34 ± 4.90

NOTES. Total cell protein is expressed as mg protein per culture ± standard error of the mean.

Specific activity is expressed as nmoles tyrosine converted to DOPA per hour per mg total cell protein.

* $p < 0.05$
** $p < 0.01$

TABLE NUMBER 9.

THE EFFECTS OF CELL NUMBER

ON M17 TYROSINE HYDROXYLASE ACTIVITY

MEDIUM	TIME DAYS	N	TOTAL CELL PROTEIN	TYROSINE HYDROXYLASE SPEC. ACT. ± S.E.M.
10% FCS	3	3	0.36 ± 0.01	33.47 ± 4.42
	6	2	1.07 ± 0.01	43.46 ± 3.00
.	9	1	1.92	46.41
	13	2	2.23 ± 0.33	59.57 ± 4.75*
N2	2	2	0.32 ± 0.01	33.99 ± 4.65
	3	3	0.76 ± 0.07	24.93 ± 1.65
	5	3	0.90 ± 0.04	28.13 ± 1.20
	7	2	1.59 ± 0.38	20.73 ± 0.90
	9	3	2.21 ± 0.08	22.28 ± 0.75*

NOTES. Initial plating densities for FCS $1.8 \times 10^4 / cm^2$, for N2, $3.5 \times 10^4 / cm^2$.

Total cell protein is expressed as mg per culture.

Specific activity is expressed as nmoles tyrosine converted to DOPA per hour per mg total cell protein.

* $p < 0.05$

TABLE NUMBER 10.

THE EFFECTS OF dbcAMP

ON M17 PROTEIN AND TYROSINE HYDROXYLASE ACTIVITY

MEDIUM	N	TOTAL CELL PROTEIN	TYROSINE HYDROXYLASE SPEC. ACT. ± S.E.M.
10% FCS	7	1.70 ± 0.16	34.97 ± 1.7
FCS +dbcAMP +IBMX	6	1.75 ± 0.08	39.38 ± 2.7
10% FCS +butyrate	3	1.22 ± 0.11	42.45 ± 1.9*
10% FCS +N2	3	1.65 ± 0.03	35.34 ± 4.9
10% FCS +N2 +dbcAMP +IBMX	3	1.35 ± 0.02	45.87 ± 9.4
N2	8	1.14 ± 0.15	28.40 ± 2.8
N2 +dbcAMP +IBMX	5	1.49 ± 0.18	41.79 ± 4.7**
N2 +butyrate	2	0.40 ± 0.08	49.45 ± 11.8**
N2 +IBMX	3	1.44 ± 0.15	33.55 ± 1.8
N2 +dbcAMP	3	1.61 ± 0.03	51.16 ± 8.0***

NOTES. * $p < 0.05$
** $p < 0.01$
*** $p < 0.001$

TH activity is expressed as nmoles tyrosine converted to DOPA per hour per mg total cell protein.
Total cell protein expressed as mg per culture.

TABLE NUMBER 11.

THE EFFECTS OF NGF

ON M17 TYROSINE HYDROXYLASE ACTIVITY

MEDIUM	N	TOTAL CELL PROTEIN	TYROSINE HYDROXYLASE SPEC. ACT. ± S.E.M.
N2	8	1.14 ± 0.15	28.40 ± 2.8
N2 + NGF	3[a]	1.06 ± 0.05	26.29 ± 2.2
10% FCS	7	1.70 ± 0.16	34.97 ± 1.7
10% FCS+NGF	3	1.50 ± 0.02	37.96 ± 3.2
N2 +10%FCS	3	1.65 ± 0.03	34.34 ± 4.9
N2+10%FCS+ NGF	3	1.39 ± 0.09*	36.24 ± 1.5

NOTES. a. N for tyrosine hydroxylase assay = 2.
* $p < 0.05$

NGF supplied as 7S molecule at a final concentration of 500 ng/ml.

Specific activity of TH expressed as nmoles tyrosine converted to DOPA per hour per mg total cell protein.
Total cell protein expressed as mg per culture.

DISCUSSION AND CONCLUSIONS

GROWTH OF SY5Y AND M17 IN N2 MEDIUM

N2, serum-free hormone supplemented medium, supported the growth of both SY5Y and M17 human neuroblastomas. The cellular morphology was altered apparently due to changes in the interaction with the substrate. This change in adhesion needs to be investigated further, as it may be due to a deficiency in the culture system with respect to available attachment molecules or may represent alterations of cell surface molecules in the N2 medium. The underlying ultrastructure of both cell lines, however, was similar to that observed for cultures maintained in 10% FCS supplemented medium. While the expression of neurotransmitter synthesizing enzymes, TH and DBH, was similar in both media, some quantitative variation was observed.

The growth of SY5Y and M17 was slower than parallel cultures maintained in 10% FCS. Again, substrate interactions may be at least partially involved in this response. The cells in N2 established a more fragile attachment than cells in FCS, and the number of cells remaining attached to the substrate with medium withdrawl or addition was lower than in FCS cultures. For many cell types mitogenic factors in serum stimulate growth in a concentration dependent fashion, and this probably contributes to the differential growth rate with SY5Y and M17.

The apparent growth rate supported by N2 medium, while slower than with FCS supplementation, may prove an advantage in examining the potential of cells for the expression of differentiated neuronal functions in response to specific factors. While the initial expression of some differentiated features <u>in vivo</u> occurs prior to the cessation of cell division in peripheral adrenergic neurons (Rothman et al., 1978) the appearance of other features follows the cessation of

cell division. Outgrowth of neurites from sympathetic neurons, contact with peripheral targets and establishment of preganglionic synapses occur largely during the postmitotic period (Black, 1978). The preganglionic and target tissue influences which act to stabilize the neuronal phenotype are exerted during this period. Whether the cessation of cell division is causally related to any aspects of the mature expression has not been examined directly, but nonetheless is part of the maturation of the sympathetic neuron. The serum-free defined medium culture system provided an in vitro environment with a reduced content of proliferation stimulating factors which may mask the expression of developmental capabilities of the neuroblastoma in FCS medium.

EXPRESSION OF NEUROTRANSMITTER-SYNTHESIZING ENZYME ACTIVITY

The expression of neurotransmitter synthesizing enzymes in SY5Y and M17 was effected by N2 medium. SY5Y cultures exhibited higher specific activity for DBH than cells in FCS. The increased activity is present during log phase growth but was most pronounced at stationary phase when DBH levels decline in FCS medium. The increase in DBH was not accompanied by increase in either total cell protein per culture or TH specific activity. The increased DBH levels demonstrated that the cells were able to maintain neuron specific functions in N2. The decrease in population growth rate did not reflect overall cellular degeneration.

The levels of DBH in adrenergic cells are subject to multiple controls. The rate of synthesis and degradation determine the steady state levels of the enzyme. Increased synthesis or decreased catabolism both lead to observed increases in activity. In vivo, DBH degradation in the adrenal medulla is accelerated after hypophysectomy and causes a decline in enzyme activity which is observed prior to the reduction in the rate of synthesis. Enzyme

degradation may be an ongoing cellular process which can be regulated independently of synthesis. Radiolabelled amino acid incorporation studies show that adrencorticotropic hormone (ACTH) specifically acts to maintain steady state DBH levels by inhibition of degradation. Transsynaptic induction by neural stimulation increases DBH above steady state levels by increasing the synthesis of the enzyme without influencing the rate of degradation (Ciaranello et al., 1975).

Serum may influence the activity of either synthetic or degradative enzymes. If serum factors are required to maintain degradative processes, DBH activity would be predicted to increase with its removal. N2 medium may be inadequate to support the level of degradative enzyme activity found in serum medium. Alternatively, factors in the N2 formulation may specifically increase synthesis and thereby account for the observed elevation of DBH specific activity.

The clustering of cells observed in N2 medium may increase cell-cell contacts and this could influence the enzyme activity. Such effects have been described in PC12 cell cultures and shown to be due to intimate cell contact and not diffusible factors (Lucas et al., 1979).

A large portion of DBH is associated with the vesicular membrane where is functions to catalyze the conversion of dopamine to noradrenaline (Potter & Axelrod, 1963; DePotter et al., 1977). Increased vesicle formation could lead to accumulation of enzyme activity due to enhanced stability of the enzyme or protection from degradation with vesicle association. However, the ultrastrucural data for SY5Y and M17 did not suggest a major change in the population of dense core vesicles though quantitative analysis might reveal such a change. Vesicle turnover, determined by vesicle formation and rate of exocytosis also influences the level of DBH. DBH is released into the extracellular space along with other vesicle contents upon exocytosis

(Viveros et al., 1969).

Clearly, more work needs to be done to evaluate the basis of the observed increase. The further characterization of this effect should determine the contributions of RNA and protein synthesis. The increase could represent synthesis of more molecules or increases activity of molecules at posttranslational levels. Immunotitration studies with antibodies to TH and DBH would determine whether more molecules are present.

The level of protein per cell and enzyme activity calculated per cell could help distinguish between increases related to generalized effects of agents or medium conditions from specific effects on neurotransmitter synthesizing enzymes. These remain to be determined.

The failure to stimulate TH activity in SY5Y was probably due to the cellular defect associated with low basal level of the enzyme. SY5Y cells may possess mutations in the structural gene for TH or in regulatory genes associated with its expression.

Alternatively, the low TH levels as determined by activity assay of SY5Y cell homogenates could be due to defects in activating mechanisms. Phosphorylation of TH, for example, has been postulated to be associated with production of catalytically active TH (Joh et al., 1978). If levels of TH were at the limits of detection by activity assay due to failure of activation alone, substantial amount of the inactive form might be present in the cytoplasm. As some enhancement of detectable activity was found with cyclic AMP addition to the medium, TH content was probed using immunocytochemical staining. The results obtained with antibodies specific to TH do not indicate that immunoreactive catalytically inactive TH was present in the SY5Y. Rather the results corroborate the low TH content indicated by the in vitro activity assay.

The concentration of antibody used was two-fold higher than that used to demonstrate TH in sections of human fetal tissue (Pickel et al., 1980) and five-fold higher than required to obtain intensive staining of the M17 cells. The results suggest that, despite the presence vesicles which morphologicily resemble catecholamine storage granules, SY5Y did not synthesize significant amounts of TH, the rate limiting enzyme for catecholamine biosynthesis. This indicates that amounts of TH, while at the limits of detection by both assays, may be catalytically active in SY5Y and sufficient to support some catecholamine synthesis. Alternatively, the dense core vesicles may contain little or no catecholamine and density of the cores result from osmophilic reaction of other molecules in the vesicle. Qualitative and quantitative determination of catecholamine content by permanganate or chromate-dichromate fixation and high pressure liquid chromatography, respectively, could provide information necessary to resolve this apparent discrepancy. Data on catecholamine content would also allow for clearer evaluation of TH activity in the SY5Y cells.

LACK OF COORDINATED EXPRESSION OF TH AND DBH IN SY5Y

The high levels of DBH activity measured in the absence of significant TH activity in SY5Y indicates a dissociation in the control mechanisms that determine enzyme levels and rates of synthesis for these two enzymes. Neuroblastoma tumors generally release high levels of DOPA and its metabolites. Despite the presence of detectable TH, DDC and DBH in most neuroblastomas, spectrofluorophotometric determination of catecholamine content reveals low norepinephrine content. The urinary excretion of this catecholamine or its metabolites is not typical of neuroblastoma (Itoh & Ohmori, 1973). Production of DOPA, dopamine or their metabolites from radioactive precursors is observed in cultured neuroblastomas, but norepinephrine is not detected (Imashuku et al., 1973; Beidler et al., 1978). The

level of DOPA and its metabolites is generally high and suggests an absence of intracelluar DBH activity. The low level of norepinephrine may act to stimulate high TH activity due to the absence of end product inhibitor. The absence of norepinephrine occurs despite the detection of variable levels of DBH in homogenates of tumors and neuroblastoma cultures. (Bohoun & Comay, 1973 ; Hortnagl et al., 1972; Itoh & Ohmori, 1973; Ross et al., 1980).

The dense core vesicle is the putative storage granule for catecholamine. Catecholamine free in the cytoplasm is labile due to the presence of degradative enzymes. Defects in storage of norepinephrine could lead to low levels in the tumor despite synthesis due to rapid catabolism (Bohoun & Comay, 1970). The observation of variable but relatively low numbers of dense core granules in neuroblastoma suggests this may be related to low norepinephrine levels (Romansky et al., 1978). Maturation of neuroblastoma to ganglioneuroma leads to a more mature neuronal morphology including increased numbers of dense core vesicles (Kadin & Bensch, 1974) and increased norepinephrine content perhaps due to improved storage (Bohoun & Comay, 1973).

The high rate of DOPA formation associated with the clinical manifestations of neuroblastoma, while primarily due to tumor mass, also may involve abnormal regulatory mechanisms in the cell (Imashuku et al., 1975). Low norepinephrine, due to inactive DBH, ineffective storage or excessive catabolism could release the feedback controls operative in normal adrenergic neurons (Ikeda et al., 1966). The kinetic parameters of neuroblastoma TH compare favorably to human adrenal TH with respect to cofactor and substrate affinity. Sensitivity of the enzyme to norepinephrine can be demonstrated in vitro (Imashuku et al., 1975). The enzyme does not appear to be defective but rather regulatory mechanisms may not be functional in neuroblastoma cells.

Levels of reduced pteridine cofactor are thought to be limiting in normal adenergic cells. If levels of the pteridine cofactor or the dihydropteridine reductase are elevated, TH activity could be elevated as a consequence of increased cofactor availability (Imashuku et al., 1975). The biopterin content measured in the SK-N lines, SH and SY5Y, were higher than those in BE(2) (Albrecht et al., 1978). The reductase activity was not measured directly. Interestingly, the SY5Y cells exhibit TH levels at the limits of detection by in vitro assay in the presence of excess cofactor while BE(2) expresses high levels of TH (Ross et al., 1980). The high biopterin levels in the low TH line indicate that the low activity observed was not due to limiting cofactor and suggests non-coordination between the production of enzyme and cofactor in SY5Y. The SH line exhibits a biopterin content twice that of BE(2) (Albrecht et al., 1978), though the TH activity measured in vitro is 2500-fold lower (Ross et al., 1980). The production of dopamine from radioactive precursor, though not determined directly for the SH, was determined for various clonal derivatives of the line. As compared to BE(2), the levels of dopamine were 10 to 100-fold lower in SH clones (Biedler et al., 1978).

Evidence from biochemical assay and immunocytochemical staining indicated that the SY5Y clone and the lines from which it derives possess low levels of TH enzyme in distinction to many other neuroblastoma lines. These findings are consistent with data from other labs (Biedler et al., 1978; Ross et al., 1980). These cells present a interesting situation with respect to expression of TH and DBH. The SY5Y may contain a mutation in the structural gene for TH or in regulatory proteins associated with its expression.

IMMUNCYTOCHEMICAL STAINING FOR DBH

To test whether the hydroxylating activity measured in cell

homogenates of SY5Y was due specifically to neuronal DBH and not other enzymes capable of mimicking the activity, immunocytochemical staining with anti-DBH antiserum was done. This immunocytochemical staining, however, did not provide conclusive evidence for neuron specific DBH in SY5Y. Heavy nuclear staining, in both SY5Y and M17, suggests some nonspecific reaction of the antibody with human tumor cells, an abnormal localization of the enzyme in the nuclear fraction or antigen migration during fixation. In mouse neuroblastoma cells DBH was found localized with the plasma membrane rather than the synaptic vesicle when fractions were isolated and reacted with specific DBH antibody or assayed for activity (DePotter et al., 1980). This ectopic localization is suggested to result from defects in membrane recycling in the neuroblastoma, specifically in reuptake of membrane following exocytosis. Such a failure to retrieve the membrane could leave DBH, an enzyme associated with the synaptic vesicle, at the plasma membrane and result in defective synthesis and storage of catecholamine.

The staining of sectioned human fetal tissue with this same antibody required the inclusion of Triton X-100 during the incubation with primary antibody to allow adequate antibody penetration. The localization of the antibody appeared to be thoroughout the cell under these conditions (Pickel et al., 1980). Even without this modification the antibody penetrated the cultured neuroblastoma cells. Dark reaction product was evident throughout the nucleoplasm when cultures were sectioned after staining. Nuclear staining was not evident with preimmune serum. Whether any of the cytoplasmic and/or nuclear staining represents DBH specifically was ambiguous. The problem of interspecies crossreactivity with DBH antibodies has been reported. Immunotitration of human DBH indicates that enzyme activity is more effectively reduced by homologous than heterologous antisera (Park et al., 1976). Heterospecific antibodies raised against bovine DBH reacted poorly with human DBH in radioimmunoassay while homospecific

antibodies demonstrated good reactivity (Fregon et al., 1981). This may contribute to the pattern of staining observed with the human neuroblastoma lines.

ADVANTAGES OF N2 MEDIUM

Defined media provide for the development of culture systems in which the participation of particular agents in the regulation of a cell function may be elucidated. The unknown and variable composition of serum complicates such analyses due to the possible presence of inhibitory or potentiating factors. The use of N2 medium for the growth of SY5Y and M17 revealed a morphological response of the SY5Y cells not observed with serum. The cells elaborated extensive neurites in N2 medium containing cyclic AMP but not in FCS medium containing cyclic AMP.

In addition, the participation of cyclic AMP dependent phosphorylation mechanisms in maintenance of TH specific activity was suggested from comparison of specific activity in N2 and FCS cultures. Cyclic AMP restores the level of TH activity to that observed in serum medium when added to SY5Y or M17 cultures in N2 medium. Addition of cyclic AMP to FCS medium did not lead to further increase in TH activity. In contrast, cyclic AMP increased DBH activity in SY5Y in both N2 and FCS medium.

N2 medium did not permit the morphological response of SY5Y cells to NGF that was observed with serum (Burmeister, 1982). This medium may be of use, however, in identifying the permissive factor(s) in serum which allow for responsiveness to NGF.

EFFECTS OF CYCLIC AMP

The addition of cyclic AMP to the culture medium increased the

specific activity of DBH in SY5Y with or without serum. That changes in the morphology and enzyme levels were observed with the dibutyryl or 8-bromo analogues suggests a critical role for the cyclic AMP moeity in mediating the effect in SY5Y. While the action of the 8-bromo analogue was identical to dibutyryl-cyclic AMP with respect to morphology and enzyme activity, the effect of it or its breakdown products on SY5Y growth rate have not been determined and therefore cannot be ruled out as contibuting factors to the observed effects in SY5Y. However, the growth rate of M17 cells in the presence of cyclic AMP was not significantly different from the controls. In mouse neuroblastoma, C1300, these two analogues are essentially the same in their effects with the notable exception that the 8-bromo-cyclic AMP does not inhibit growth rate (Waymire et al., 1978b).

Some effects of dibutyryl cyclic AMP can be due to butyrate formed when the molecule is degraded. Butyrate is inhibitory and at high concentration toxic to cells. As slowing or cessation of cell growth may secondarily influence cell morphology or enzyme expression, it is important to distinguish these effects from those mediated by cyclic AMP when dibutyryl analogue is used. Butyrate increased levels of TH specific activity in M17 cells. This increase, however, was accompanied by a drastic decrease in cell protein that was not found with cyclic AMP and IBMX treatment. Dibutyryl cyclic AMP and IBMX in the medium permitted growth rate comparable to the respective FCS or N2 control cultures of M17. In SY5Y, 1mM butyrate increased the specific activity of DBH, but also reduced total cell protein to levels below that observed with dibutyryl cyclic AMP. This is probably due to a combination of toxic and growth inhibitory effects. The morphology of the cells in N2 with 1mM butyrate was quite different from N2-dibutyryl cyclic AMP cultures. As butyrate also causes elevations in intracellular cyclic AMP (Prasad & Sinha, 1975), the enzyme increases may be due to this action. Butyrate at 1mM concentration did not account for the effects observed with dibutyryl cyclic AMP.

The addition of cyclic AMP to N2 medium provided for the consistent demonstration of TH activity in SY5Y, though it remained at the limits of detection by the in vitro assay. The levels were comparable to those found with FCS. The N2 medium may lack factor(s) which are present in serum and act to maintain higher levels of TH. Cyclic AMP may substitute for this factor and restore activity to the FCS level. The factor(s) may act via cyclic AMP dependent phosphorylation of TH. A similar effect on TH activity was observed in M17 where basal levels of the enzyme are high.

The cyclic AMP experiments with M17 also suggested that the levels of TH might be lower in N2 medium as a result of reduced cyclic AMP-dependent phosphorylation of the enzyme. N2 medium may not support the level of protein kinase activity comparable to that found in serum cultures. The activity of protein kinases can be stimulated by the addition of exogenous cyclic AMP or its analogues (Insel et al., 1975; Rosenfeld & Barrieux, 1978). As no further increases were observed when cyclic AMP and IBMX were added to FCS medium, it appeared that serum factors maintained sufficient kinase activity for the maximal expression of active TH.

The level of neurotransmitter synthesizing enzyme activity in SY5Y and M17 were modulated by cyclic AMP dependent processes. Whether the increases observed represent activation of preexisting molecules or de novo synthesis requires further work evaluating the effects of transcriptional and translational inhibitors on the expression of increased activity. Immunotitration studies would indicate if increased numbers of enzyme molecules are responsible for the observed elevation. The M17 cells in N2 medium may be a useful system in which to analyze further the actions of cyclic AMP on expression of TH activity.

The dramatic effect of cyclic AMP on the morphology of SY5Y cells in the presence of N2 but not FCS requires further investigation. The failure to find effects in the presence of serum indicates that intracellular or extracellular factors either degrade the cyclic AMP or antagonize its action on the cells. These factors are removed or fail to be produced without serum. Whether the increased process formation represents modified gene expression or results via the stimulation of tubulin polymerization or the activation of microtubule associated proteins remains to be determined. For example, phosphorylation of desmin and vimentin intermediate filaments by specific protein kinases may govern filament assembly by postranslational modification of the subunits (O'Connor et al., 1981).

Recent studies using a subclone of the mouse neuroblastoma c1300 suggested that specific increases in microtubule associated proteins (MAPS) are related to the morphological differentiation observed when cells are transferred from suspension culture to monolayer culture. Neurite formation resulted in a change in the distribution of total cell tubulin. The equilibrium between soluble and insoluble tubulin was shifted when cells were differentiated, probably related to increased polymerization. The failure to polymerize tubulin in the suspension cultures correlated with absence of MAPs (Olmstead & Lyons, 1981a).

A 215,000 dalton protein has been detected in extracts from differentiated cells but not undifferentiated ones. It was shown to participate in <u>in vitro</u> microtubule assembly and found to sediment with tubulin (Olmstead & Lyons, 1981b). The localization of MAP2 immunoreactivity specifically with microtubules of differentiating neuroblastoma line B104 when they were treated with dibutyryl cyclic AMP. The monoclonal antibody failed to react with microtubules in mitotic spindle, in nonneuronal cells or undifferentiated neuroblastoma. The MAP2 protein may function as a microtubule

polymerizing or stablizing agent promoting process formation in neuronal cells. It may prove to be a marker for neuron specific differentiation (Izant & McIntosh, 1981). The appearance of this protein in detectable amounts follows procedures known to stimulate neurite outgrowth, but whether this involves de novo synthesis has not been determined. Of interest will be further work which can evaluate the influence of cyclic AMP and NGF on the levels and activity of the MAPs.

CYCLIC AMP AND TYROSINE HYDROXYLASE ACTIVITY

The stimulation of protein kinase activity could have two consequences. The kinase could act at posttranslational level to phosphorylate preexisting enzyme molecules. The kinetic parameters of TH activity can be modified by incubation of tissue in vitro in the presence of a complete protein phosphorylating system. Full activation of TH requires Mg++, ATP, cyclic AMP and endogenous kinase. The activation is associated with an altered affinity of the enzyme for the pteridine cofactor if assayed at subsatruating cofactor concentrations. (Weiner et al., 1978). The increases in TH observed with phosphorylation resemble acute activation of enzyme following brief neural stimulation. In several different tissues studied including striatum (Lovenberg et al., 1975), caudate nucleus (Lloyd & Kaufman, 1975), hypogastric nerve-vas deferens (Weiner et al., 1978), TH activation subsequent to nerve stimulation is associated with an increased affinity of the enzyme for its reduced pterin cofactor while the Km for substrate remained unchanged. As the tyrosine concentration in the cell is thought to be saturating while cofactor may be limiting, the physiological relevance of such a mechanism can be apprecitated.

The presence of two kinetically distinct populations of enzyme is suggested by non-linear double reciprocal plots of 1/v vs. 1/[cofactor]. Analysis of homogenate from hypogastric nerve-vas

deferens preparation after electrical stimulation or following incubation with a complete phosphorylating systems reveals linear Lineweaver-Burk plots for TH. The combined effects of electrical and phosphorylation effects is equal to the maximal stimulation observed with either alone (Weiner et al., 1978).The effects of electrical stimulation and cyclic AMP may be exerted via a single common pathway and suggests that in vivo acute activation following neural stimulation involves cyclic AMP dependent protein kinase activity.

In vivo, acute stress stimulates TH activity by transsynaptically mediated mechanisms. Analysis of cofactor binding reveals non-linear Lineweaver-Burk plots in low stress animals sacrificed by halothane anaethesia. However, stresses caused by decapitation (Masserano & Weiner, 1979) or electroconvulsive shock (Masserano et al., 1981) increase adrenal and striatal TH and the enzyme exhibits Michaelis-Menten kinetics with respect to cofactor and substrate. Incubation of adrenals with a cyclic AMP-dependent protein phosphorylating system allows for the conversion of low affinity form of the enzyme to the high affinity form. A single homogeneous population of TH molecules with respect to Vmax and cofactor affinity is observed. (Masserano & Weiner, 1979).

Though phosphorylation was implicated as a mechanism for rapid activation, the demonstration of direct phosphorylation of the TH molecule and association with increased activity is more recent. The first report of phosphorylation of isolated TH suggested that phosphate was a constitutive part of the molecule and not related to the modulation of enzyme activity as the molecules isolated from experimental and control situations exhibited the same steady state levels of ^{32}P incorporation (Letendre et al., 1977). However, in vitro phosphorylation of purified enzymes from various tissues is correlated with changes in enzyme activity.

^{32}P from γ-^{32}P-ATP is incorporated into the 62kd subunit of highly purified rat caudate TH. The phosphorylated enzyme exhibits a two-fold increase in catalytic activity associated with an increased Vmax but no alteration in the affinity for cofactor or substrate. It is proposed that the increased catalytic activity represents recruitment of catalytically active molecules from an inactive pool. The changes in apparent Km found in crude enzyme preparations may represent summation of Km and Ki effects since inhibitors were present under these conditions (Joh et al., 1978).

Phosphorylation of TH purified from SK-N-BE(2) human neuroblastoma cells produces a two fold increase in activity. However, this increase is associated with a decrease in Km for cofactor but no change in Vmax. Whether these differences are related to species variation or to the neoplastic origin of the neuroblastoma cells remains to be determined (Joh et al., 1979).

Direct incorporation of phosphate into adrenal TH by a cyclic AMP mechanism has been demonstrated by coelution of the enzyme activity with the radioactive peak in gel filtration on Sephadex G-200. A 3-4 fold activation of enzyme activity follows the incubation of the purified enzyme under phosphorylating conditions (Yamauchi & Fujisawa, 1978).

Activation of purified PC12 TH by phosphorylating conditions is reported to involve change in cofactor affinity rather than change in Vmax. When assayed under limiting conditions for cofactor, the reduced Km is evident. Lineweaver-Burk plots exhibiting curvilinearity with repect to cofactor concentration in controls become linear with phosphorylation of the enzyme. Purified catalytic subunit of cyclic AMP dependent protein kinase added to purified TH resulted in enzyme activation which was proportional to amount of subunit added and highly correlated with ^{32}P incorporation into TCA precipitable material.

The activation appears to be specific for TH and proceeds at a rate 2 1/2 times that of histone H1 phosphorylation (Vuillet et al., 1981).

Partially purified bovine adrenal TH is also sequentially phosphorylated and dephosphorylated in vitro and concomitant increases and decreases were associated with the two processes. Endogenous kinases and phosphatases specifically modify the enzyme molecule and loss or gain of ^{32}P label is correlated with increased or decreased activity, respectively. The data suggest that a phosphorylation-dephosphorylation cycle controlled by cyclic AMP dependent protein kinase and phosphoprotein phosphatase mmay be related to modulation of enzyme activity in the adrenal medulla (Yamauchi & Fujisawa, 1979).

The kinetic basis for the phosphorylation associated alterations in TH activity remains controversial. A recent report suggests that phosphorylation may shift the pH optima for the enzyme. Depending on the conditions of the assay, apparent changes in several parameters can result. In addition the optimal pH ranges may vary with tissue type, state of enzyme activation, or assay conditions, e.g. cofactor concentration. This may provide a basis for some of the discrepancy in reports of effects of phosphorylation on TH activity (Acheson et al., 1981).

In summary, the modulation of enzyme activity in a variety of systems may share common mechanisms despite the diversity of stimulating signals. The changes in kinetic parameters favor increased catecholamine synthesis by increasing the sensitivity of the enzyme to cofactor while decreasing binding of end product inhibitor. The activation may involve direct phosphorylation of the enzyme molecule catalyzed by cyclic AMP-dependent kinase. The phosphorylated form may be the active TH or represent a TH molecule with modified affinity for cofactor or inhibitor or with different pH dependencies.

The changes in TH activity observed in M17 and SY5Y cultures with cyclic AMP treatment require further characterization. The action of cyclic AMP on enzyme activity in neuroblastoma cell lines may resemble regulatory events operative in mature adrenergic neurons. Alternatively, the changes could represent developmental changes associated with progressive differentiation. Comparison of the neuroblastoma data with that found in developing and mature neurons should be useful in elucidating the mechanisms underlying cyclic AMP action on enzyme activity.

Activation of protein kinase may also influence transcriptional events in cells (Jungman & Russell, 1977). The induction of TH and DBH follows chronic stimulation of sympathetic ganglia or adrenal medulla, as occurs during prolonged physiological stress. The increased levels of TH and DBH activity require transcription and de novo protein synthesis and result in increased numbers of enzyme molecules. This process is clearly distinct from short term activation (Otten & Thoenen, 1976). Some work suggests that elevated cyclic AMP may be a primary cellular response to increased neural stimulation and acts as a second messenger in transsynaptic induction of the enzymes (Guidotti & Costa, 1973). This, however remains controversial since increases in intracellular cyclic AMP preceding the inductive effects have been difficult to demonstrate directly (Otten & Thoenen, 1976). However, as the cyclic AMP signal is amplified in the cell, the physiologically active amounts may be very small. Detection of small transient increases in cyclic AMP in the neuronal population of the ganglion may be difficult due to the presence of other cell types.

Protein kinase consists of regulatory (R) and catalytic (C) subunits. The R subunits bind cyclic AMP and the C subunit dissociates from the R subunit upon cyclic AMP binding (Rall et al., 1969; Corbin et al., 1975; Granot et al., 1980). The C subunit catalyzes the

phosphorylation of endogenous cellular protein (Greengard, 1972; Rosenfeld & Barrieux, 1978). The protein kinase activity is compartmentalized, the cytosol containing cyclic AMP dependent kinases while the endogenous nuclear protein kinases are mainly cyclic AMP independent (Russell, 1978). The contribution of cyclic AMP to nuclear events was therefore dubious. However, the translocation of cytosol cyclic AMP dependent protein kinase or its catalytic subunit to the nucleus has been proposed (Jungman et al., 1975). The resultant phosphorylation of nuclear proteins is proposed to activate transcription of specific genes.

Reciprocal changes in cytoplasmic and nuclear cyclic AMP-dependent protein kinase activity are observed in adrenal medulla following nicotinic stimulation (Costa et al., 1977) or prolonged cold stress (Kurosawa et al., 1979). Activity of the free catalytic subunit in cytoplasm increases as cyclic AMP levels rise, but a subsequently declines simultaneously with a two-fold increase in nuclear phosphorylation and accumulation of protein kinase activity in the nuclear fraction (Kurosawa et al., 1979).

In primary cultures of adrenal chromaffin cells elevation of intracellular cyclic AMP by incubation with the 8-bromo cyclic AMP analogue for five hours leads to increased TH activity which peaks at 48-72 hours. The increase is specific and not related to generalized enhancement of protein synthesis. The increase is actinomycin D and cycloheximide sensitive and the Vmax for TH is elevated with no apparent change in cofactor affinity. The levels of cyclic AMP dependent protein kinase in the total cell pellet and in nuclei increase by an amount corresponding to a decrease in the cytosol activity. A similar sequence of changes was elicited by cholera toxin, which is known to increase intracellular cyclic AMP and induce TH. No such increases were seen with NGF or dexamethasone treatment. The effects of the above agents on chromaffin cells have not been related

to cyclic AMP. Colchicine blocks the TH induction stimulated by cyclic AMP as well as the phosphorylation of endogenous nuclear protein but does not affect the basal TH levels. Participation of microtubular system in translocation process was proposed (Kumakura et al., 1979).

In NBD-2 mouse neuroblastoma cells, a 15 minute exposure to 8-bromo cyclic AMP results in a 100% increase in TH activity at 48 hours. The increase is associated with an increased Vmax probably due to increased numbers of enzyme molecules. The increases in cyclic AMP dependent protein kinase activity in the soluble and particulate cell fractions exhibit a reciprocal complementarity, protein kinase redistributing predominantly to the particulate fraction during the six hour period after cyclic AMP treatment. Nuclear extracts from the cyclic AMP treated cells preferentially phosphorylate non-histone proteins in vitro even without added cyclic AMP. This indicates that the catalytic subunit had been previously activated in the cytosol prior to translocation (Hollenback et al., 1979). Increases in TH and DBH with 8-bromocyclic AMP require transcription and translation. Immunotitration studies reveal that part of the increase is due to an increased number of enzyme molecules. TH from cyclic AMP treated cultures also exhibits a lower Km for cofactor (Waymire et al., 1979).

The level of neurotransmitter synthesizing enzyme activity in SY5Y and M17 can be modulated by cyclic AMP dependent processes. Whether the increases observed represent activation of preexisting molecules or de novo synthesis requires further work evaluating effects of transcriptional and translational inhibitors on expression of increased activity. Immunotitration studies would indicate if increased numbers of enzyme molecules are responsible for the observed increases. The use of serum free medium N2 revealed cyclic AMP effect on M17 TH activity not observed in FCS. The M17 cells in N2 medium may be a useful system in which to analyze further the actions of cyclic AMP on TH activity. Consideration of the processes operating in developing

and mature neurons with respect to cyclic AMP action is essential for evaluating the significance of the effects observed with neuroblastoma cell lines.

EFFECTS OF NGF

The effects of NGF on SY5Y at concentrations up to 100 ng/ml were limited to alterations in growth rate and morphology and no associated changes in neurotransmitter synthesizing enzyme activity were found under any conditions tested. The effects on neurite outgrowth were not correlated with changes in growth rate as the former occurs only in presence of FCS while the latter is observed only in N2 medium. Despite the increased neurite outgrowth with NGF the cells did not exhibit the overall increase in organelles, e.g. increases in rough endoplasmic reticulum, associated with the hypertrophic response in sensory and sympathetic ganglia (Levi-Montalcini et al., 1966) or PC12 (Luckenbill-Edds et al., 1979).

The M17 cells failed to exhibit morphological, enzymatic or growth rate changes with NGF. At the concentrations of NGF used, a slight but consistent decrease in total cell protein was observed and suggests that at higher concentrations NGF might produce more significant changes in M17 cell. Increased enzyme activity may require concentrations of the factor in μg/ml range. The absence of morphological and enzymatic effects at 100ng/ml does not exclude the possibility of effects at higher concentrations of factor. As the NGF receptor content of the BE(2) line or the M17 clone has not been determined, the failure to respond may indicate lack of receptors. Evaluation of NGF action with the M17 line requires more data especially from experiments performed at NGF concentrations shown to be optimal in other systems. Several possible explanations for the apparent uncoupling of effects in SY5Y and failure to elicit effects in M17 will be considered below.

Though high affinity binding of NGF to SY5Y cells has been demonstrated, the receptor content of the cells is low (Sonnenfeld & Ishii, 1979). The multiple effects of NGF on target tissues are mediated at several regulatory levels. Recent reports have identified cellular receptors with differing association kinetics for NGF (Sutter et al., 1979; Landreth & Shooter, 1980; Riopelle et al., 1980; Schechter & Bothwell, 1981). Receptor heterogeneity may be intrinsic to the population of receptors (Schechter & Bothwell, 1981) or conversion from low affinity to high affinity may be induced by ligand binding (Landreth & Shooter, 1980). The functional significance of different receptors remains speculative at present.

Fast and slow receptors are present on PC12 cells prior to ligand binding and exhibit different sensitivity to trypsin. A brief exposure of cells to trypsin destroys fast binding but does not prevent binding to the slow receptors. Treatment of the cells with Triton X-100 solubilizes the membranes leaving an insoluble cytoskeleton behind. While fast receptor binding is lost by such procedures, slow binding is preferentially retained. The cytoskeletally associated receptor may mediate specific morphological effects of NGF while transcription dependent events may require interactions of NGF at other sites e.g. the nucleus (Schechter & Bothwell, 1981).

The incubation of PC12 cells with NGF results in a portion of the bound ligand remaining associated with the cells even in NGF-free medium. Within this tightly bound fraction some of the binding is trypsin resistant while some is destroyed by trypsin treatment (Calissano & Shelanski, 1980). NGF binding has been followed to the nuclear compartment 24 hours after NGF treatment (Calissano & Shelanski, 1980; Yanker & Shooter, 1979). Translocation of surface receptors to the nuclear compartment is coincident with a transient reduction in surface binding. Internalization of the receptor,

associated with either degradation or transport to intracellular sites of action, reduces available receptors on the surface but the levels are subsequently restored to levels higher than present prior to binding. This modulation of available NGF receptors in response to NGF binding may involve de novo synthesis or unmasking of latent receptors. (Calissano et al., 1981).

Localization of surface binding sites and transport in the cell has been followed by immunofluorescent and autoradiographic techniques. In embryonic cells the initial binding is diffusely distributed over the surface but subsequently becomes clustered into immobile patches by a temperature sensitive process which precedes the eventual endocytosis of the molecules. More mature neurons exhibit in addition to mobile receptors, immobile sites localized at the tip of the axon and on the soma in which the receptors are aggregated prior to NGF binding (Levi et al., 1980; Marchisio et al., 1981).

NGF after binding to surface receptors in PC12, becomes internalized and is found in the cytoplasmic compartment and subsequently in the perinuclear region and in the nucleolar area of the nucleoplasm (Yanker & Shooter, 1979; Marchisio et al., 1980). In embryonic sympathetic neurons, however, the intracellular localization is restricted to the cytoplasmic compartment (Marchisio et al., 1981). The differences in surface binding and internalization may be related to developmental maturation of cell responsivity to NGF. These differences may be a reflection of the "priming" events observed with PC12 cells. Cells upon first exposure to NGF elaborate processes by a transcription-dependent mechanism. However, regeneration of processes occurs more rapidly and does not require RNA synthesis (Burstein & Greene, 1978).

The morphologic responses of SY5Y cells to NGF in the absence of effects on growth or enzyme activity could result from a reduced or

absent class of receptor(s) or alternatively to defects in cellular mechanisms following receptor binding, e.g. internalization, translocation or activation of a second messenger. The low surface receptor content may permit receptor occupancy below the threshold for enzyme inductive and growth effects but adequate for stimulation of neurite outgrowth. These cells might be useful in further analysis of NGF receptors aimed at correlating functional differences with various receptor types identified.

The concentration of B-NGF used in the experiments ranged from 10-100ng/ml. Morphological effects in SY5Y can be observed at concentrations in this range and appear to be maximal at 50 ng/ml (Sonnefeld, personal communication). However,these concentrations may not be sufficient for induction of enzyme activity. Morphological and enzymatic effects in sympathetic ganglia following in vivo NGF injections of neonatal animals can be distinguished. NGF induction of neurite outgrowth is temporally distinct from TH induction. In addition, the enzyme induction is actinommycin D sensitive while neurite outgrowth is not. NGF appears to act at level of tubule assembly as new synthesis of tubulin is not required for the increase of neurotubules observed by electron microscopy (Stoeckel et al., 1974).

In organ cultured adult rat superior cervical ganglia TH induction requires 5-10 µg/ml NGF while only 5 ng/ml is necessary for neurite outgrowth (Otten & Thoenen, 1976). In long term organ cultures of early postnatal rat superior cervical ganglia neurons display neurite outgrowth when maintained in a culture medium with 10 ng/ml NGF. However, low NGF could not support the detectable levels of TH. NGF at 1 µg/ml is required to restore TH activity following explantation and maintain it in culture. While the maximal effect on the density of neurites occurs at 10 ng/ml NGF, maximal neurite elongation, cell survival and enzyme induction requires 100 µg/ml of NGF. It is

postulated that the differential effects may be related to actions of NGF mediated at different cellular sites. The low concentration, similar to plasma levels, could stimulate axon formation via a mechanism involving cell surface receptors. The high concentrations, as would be encountered by nerve terminals at the target organ, might induce TH, survival and maximal neurite elongation by processes involving retrograde transport to the cell body following uptake in the terminal region (Hill & Hendry, 1976).

Human fetal ganglion cells in partially dissociated cell culture are supported by NGF at 10 ng/ml. Even when non-neuronal cell population is drastically reduced by the use of serum-free supplemented medium, N2, cells retain ultrastructural features characterisitc of sympathetic neurons. The cells exhibit dense core vesicles with permanganate or chromate-dichromate fixation indicating the presence of catecholmine stores and suggesting the presence of functional TH (Zeevalk et al., 1982, submitted for publication).

In PC12 cells, neurite outgrowth is maximally stimulated at 0.4 nM while induction of acetylcholinesterase requires 1.0-1.5 nM (Greene & Rickenstein, 1981). Induction of TH in the PC G2 cell line requires 1-10 μg/ ml while neurite outgrowth is not stimulated at any NGF concentration (Goodman & Herschman, 1978). The negative results presented for SY5Y do not exclude the possibility of NGF effects on neurotransmitter synthesizing enzymes at higher concentrations of the factor.

The role of other factors in potentiating the NGF action on cells must also be considered. The culture environment may lack molecules which are permissive for NGF action or modulate the cell response to NGF. Several studies indicate that glucocorticoids may have such a function in NGF responsivity of sympathetic cells. If animals are injected with NGF and the superior cervical ganglia

subsequently explanted into organ culture in a serum-free medium an increased level of TH activty is measured at 48 hours. In vivo administration of actinomycin D prior to NGF injection prevents this increase. Addition of cycloheximide but not actinomycin D prevents the increase. The initial action of NGF requiring transcriptional events is completed in vivo but continued protein synthesis is required for full expression of the effect in culture. Immunoprecipitation experiments of the increased activity demonstrated de novo synthesis of TH molecules (MacDonnell et al., 1977a).

The maximal stimulation of TH activity in response to a single NGF injection requires dexamethasone in the culture medium. However, injections of NGF administered on three consecutive days results in maximal induction regardless of dexamethasone in the medium (Nagaiah et al., 1977). It appears that some change is completed in the ganglion cells in vivo which overcomes the requirement for glucocorticoid in vitro. Recently it has been shown that NGF stimulates the pituitary-adrenocortical axis, thereby increasing plasma glucocorticoid (Otten & Towbin, 1980). The in vivo increase in glucocorticoid may allow for changes to be completed in the cells prior to culture and thereby relieve the in vitro requirement for dexamethasone.

The action of the glucocorticoids increases responsivity of superior cervical ganglia to NGF as evidenced by a shift in the dose response curve for NGF to the left. In the presence of glucocorticoid, 5-10 ng/ml NGF is suffucient for induction of TH as compared to the concentration of ug/ml without dexamethasone. The maximal inductive effect is greater than without glucocorticoid and the time of exposure to NGF necessary for induction is drastically reduced. These experiments suggest that glucocorticoids increase the responsiveness to NGF by a process which requires protein synthesis (Otten & Thoenen, 1977).

In PC-G2 cells, dexamethasone potentiates NGF and EGF effects on TH activity (Goodman et al., 1978). Enzyme levels in PC12 are not affected by NGF alone. However, in the presence of 10^{-6} M glucocorticoid, 40 ng/ml NGF enhances the level of TH (Schubert et al., 1980). Choline acetyltransferase activity, however, is stimulated by NGF while glucocorticoid inhibits the NGF induced increase (Edgar & Thoenen, 1978).

That dexamethasone encourages adrenergic differentiation while antagonizing expression of cholinergic enzymes in PC12 suggests that the steroid may be involved in the maintenance of the endocrine phenotype in the adrenal medulla, the normal counterpart of the pheochromocytoma. The PC12 cells may not provide an appropriate model system with which to study events related to neuronal differentiation, especially with respect to the expression and regulation of neurotransmitter synthesizing enzymes. Work with normal adrenal chromaffin cells in culture (Unsicker et al., 1980) and in vivo (Aloe & Levi-Montalcini, 1979) indicates that these endocrine cells also have specific responses to NGF which are different from NGF effects in sympathetic neurons. As the adrenal medulla and sympathetic neurons share a common embryological origin and synthesize catecholamine via same enzymatic pathway, there may be some regulatory processes common to both. However, the two systems are functionally distinct and regulation specific to each must develop as the phenotype becomes determined. Comparison of effects observed in neuronal and endocrine cell types will be of interest in understanding the development of the regulatory processes during differentiation. However, caution should be taken when proposing use of a cell line as a "model" for neuronal differentiation.

A recently isolated clone of PC12 exhibits an NGF sensitive TH induction not requiring the permissive action of glucocorticoid. Half maximal stimulation of TH occurs at low NGF concentrations (1.7 ng/ml)

and maximal induction is achieved at 10 ng/ml. Dexamethasone and NGF increased TH activity in an additive fashion suggesting the actions of the two agents are exerted via separate pathways (Hatanaka, 1981). The effective dose for half maximal response is comparable in magnitude to that reported for effects on neurite regeneration (Greene, 1977), CAT induction (Schubert et al., 1977) and TH induction in the presence of glucocorticoids (Schubert et al., 1980). The NGF required for response in PC12h is an order of magnitude lower than required for TH induction in PC-G2 (Goodman & Herschman, 1978).

Morphological and enzymatic responses to NGF are not necessarily correlated in the various PC12 clones isolated (Hatanaka, 1981). The loss or aquisition of NGF responsivity due to clonal variation in PC12 has also been noted in mutagenized cultures (Bothwell et al., 1980). Some of the discrepancies reported for PC12 responses, as well as those for other cell lines, may be due to variation even within clonal populations.

The SY5Y cells offer another system in which to study NGF action. Further work on this line should include characterization of the morphological response (Burmeister, 1982; Perez-Polo et al., 1979) with respect to the role of RNA and protein synthesis.

It would also be of interest to add glucocorticoids or other possible potentiating factors along with NGF and assess whether morphological and enzymatic parameters can be effected. The defined N2 medium may provide a system in which to reconstitute condtition required for NGF stimulated neurite outgrowth. The failure to observe a similar response in N2 may be due to deficient substrate. Attempts to modify the substrate by addition of fibronectin, collagen, polyornithine or polylysine would be a start to investigate this possibility. Alternatively,, some hormonal factor(s) might "prime" the cells and increase responsivity to NGF.

No single cell line studied thusfar has proven a conclusive model in which to study NGF action on it target cells. More information from alternative systems, such as the human neuroblastoma cell lines in particular SY5Y, may help in elucidating the mechanism of action for NGF.

COMPARISON OF SY5Y AND M17 TO EARLY SYMPATHETIC CELLS

The neuroblastoma lines SY5Y and M17 resemble early neuroblasts with respect to ultrastructural features. A heterogenous distribution of cells with respect to ultrastructural specializations populates the developing sympathetic ganglion. The most primitive neuroblasts possess large nuclei with variable amounts of condensed chromatin underlying the nuclear envelope and a cytoplasm with poorly developed membraneous organelles. Young sympathetic neurons contain more rounded or oval nuclei with evenly dispersed chromatin, well developed cytoplasmic organelles, dense core vesicles. Processes characterized by presence of microtubules, intermediate filaments, smooth endoplasmic reticulum and dense core vesicles indicate more advanced development (Pick et al., 1964; Hervonen et al., 1978).

The SY5Y cells contained a moderate number of large dense core granules of size comparable to those found in glutaraldehyde-osmium fixed fetal ganglia. The M17 cells possessed a richer population of dense core granules, whose size and shape were more variable than those of SY5Y cells. The mean diameter of the SY5Y vesicles was 100 nm as compared to a mean diameter of 130 nm for the M17. Groups of twenty or more vesicle were found in FCS and N2 cultures of M17. This was rarely encountered in an extensive survey of SY5Y under a number of medium conditions (see also Burmeister, 1982). Neither SY5Y nor M17 contained significant numbers of small (50nm) dense core vesicles.

The irregular shapes of vesicles in M17 may result from section

through endoplasmic reticulum which contained dense material rather than through vesicles. Several studies suggest that perikaryal and axonal endoplasmic reticulum may be a site for vesicle formation (Teichberg & Holtzman, 1973; Holtzman et al., 1973), and chromaffim positive material has been demonstrated in the cisternae of tubular endoplasmic reticulum, smooth endoplasmic reticulum and Golgi appartatus of superior cervical ganglion cells (Richards & DaPrada, 1980). Alternatively, the irregular shaped vesicle profiles resemble some of the vesicle types found in early sympathicoblasts, which are not as uniform in size and shape as at later stages of cellular maturation. The large granular vesicles are prominent at earlier developmental stages prior to appearance of small dense core vesicles (50nm) which are associated with synaptic function (Pick et al., 1963; Hervonen et al., 1978).

In more mature neurons large granular vesicles are found in the cell body, along processes and in the terminals while the small granular vesicles (50nm) are almost exclusively localized in the terminals. Local formation of small granular vesicles in the terminals has been proposed (Hokfelt, 1973). The small vesicles may be formed by the fragmentation of the larger vesicles or by a partial exocytosis followed by endocytic retrieval of a smaller vesicle (Geffen & Livett, 1971). Though the mechanisms for the generation of vesicles and their specific functions remains controversial, it is generally agreed that the small vesicles are associated with synaptic function.

In the absence of synaptic interaction cells may not produce small dense core vesicles. The SY5Y and M17 cells rarely exhibited vesicles of 60-70 nm. The absence of this class of vesicle may be due to the early developmental stage of the cells or associated with the lack of synpase formation by these cells in culture.

Dense core vesicles are suggestive of catecholamine storage but

are not a sufficient demonstration of its presence. Vesicles with dense cores have also been observed in cholinergic terminals (Peters et al., 1976). Further, the dense core granule is a more complex organelle than the secretory granule and it has been suggested that it may also be the site for local synthesis of catecholamine (Pelegrino de Iraldi, 1980). Other molecules are associated with the vesicle, e.g., protein molecules which complex with catecholamine prevent diffusion of the transmitter out of the vesicle. Enzymes involved in the synthetic pathway have been localized in the vesicle, a large proportion of DBH in neuron is associated with the vesicle membrane (DePotter & Chubb, 1977). With standard glutaraldehyde-osmium fixation the density could be due to any of these other intravesicular molecules. In the absence of data from histochemical techniques specific for catecholamine, e.g., permanganate fixation (Kanerva et al., 1980,) fixation in presence of chromate-dichromate buffer (Tranzer & Richards, 1976), the content of the vesicle cannot be identified definitively.

While the morphology and size of the vesicles resembles that found in sympathetic ganglion cells, other neural crest derivatives exhibit similar storage granules. In the human fetus, granules in the paraganglia and adrenal medulla during early stages are of diameters in range of 110-150 nm. Adrenal chromaffin cells at eight weeks in the human fetus exhibit vesicle in the 60-140 nm range and dense core material is present by nine weeks. While the initial size is small, by 16 weeks larger vesicles of 240-430 nm diameters predominate in the endocrine cells. The vesicle size increases in the endocrine cells and some of the paraganglia between weeks 8-19 in the fetus, in distinction to the principle neurons of the sympathetic ganglion where the trend is toward smaller vesicle size. Large and small granular vesicles of 100 nm and 60 nm are present at this stage in the principle neurons of the sympathetic ganglion (Hervonen, 1971). The mature chromaffin cell of the adrenal medulla and some paraneurons exhibit vesicle diameters in the range of 200-400 nm (Seigrist et al., 1968; Taxi, 1975).

The vesicles of the M17 and SY5Y more closely resembled those found in the sympathetic neurons than in other cell types. The cores filled 50% or more of the vesicle, as is found for large granular vesicle type of the sympathetic neuron. The size of the vesicles falls within the range observed in sympathetic cells during development and in the mature sympathetic cell. The numbers of vesicles found and their distribution suggests closer similarity to sympathetic cell than chromaffin cell. The effects of dexamethasone and other adrenal glucocorticoids would be of interest to determine with respect to vesicle morphology and content in the light of the plasticity which appears to be retained in vivo in neural crest derivatives (see Backround section).

The immunoreactivity of the SY5Y and M17 cells with respect to TH and DBH also suggests a resemblance to early developmental stages. Immunoreactivity for TH in the sympathetic anlage has been demonstrated in human fetuses as early as five weeks (Pearson, 1980; Pickel et al., 1980; Hervonen et al., 1981). The staining in the 5 1/2 week fetus is observed only in sympathetic neuroblasts which are aggregating to form ganglion but not in the adrenal or the central nervous system. By 9-10 weeks immunoreactivity can been found in sympathetic terminals as well as in adrenal medulla, carotid body, locus ceruleus, substantia nigra and basal ganglia (Pearson, 1980). In the 14-22 week fetus the most intense TH immunoreactivity is associated with the larger more mature neuroblasts as compared to earlier stage neurons present at this time. The development of the neurons of the ganglion is asynchronous. DBH immunoreactivity is moderate during this period. Phenylethanolamine-N-methyltransferase (PNMT) immunoreactivity has not been observed in sympathetic ganglia of the human fetus (Hervonen et al., 1981).

The cytological features and distribution of immunoreactivity

correspond to the patterns observed by formaldehyde-induced flourescence (FIF), a histochemical technique for detection of catecholamine. Detection of catecholamine by histofluorescene is possible by about 7 weeks in the human fetus. Dense core vesicles are not observed until 10-15 weeks. At 10 weeks, weakly fluorescent cells are evident in the sympathetic ganglia but ultrastructurally the cells are predominantly of the primitive type. By 15 weeks larger neuroblasts with mature characteristics are evident in a mixed population. Outgrowth to the periphery begins during this period and precedes the arrival of cholinergic preganglionic fibers from the spinal cord. Synapse development ensues between 11-13 weeks in the superior cervical ganglion (Kanerva et al., 1974). PNMT is detected in human fetal adrenal at 13-14 weeks (Pearson, 1980; Hervonen unpub., cited in Hervonen et al., 1981). On the basis of immunocytochemical and ultrastructural characterization, the SY5Y and M17 most closely resemble early sympathicoblasts of the developing ganglion at a stage beyond initial expression of neurotransmitter synthesizing enzymes. The clones exhibit morphology which appears more advanced than previously reported for the parental lines, SH and BE(2) (Barnes et al., 1981). The observation of microtubule containing processes and presence dense core vesicles was much more frequent than with the parental lines. This may be due to selection a cell type of more mature morphology or to the richer medium supplied to cells in this study. The F12 formulation provides a richer basal medium than Modified Eagle's Medium which was used in previous studies. However, the removal of serum and replacement with N2 factors did not prevent the expression of these differentiated features.

PROCESS FORMATION IN M17 AND SY5Y

The fine structure of the processes observed in both SY5Y and M17 are immature in appearance. Even under conditions where the length of processes is significantly increased (SY5Y in presence of N2-cyclic

AMP), the ultrastructure remains essentially the same. The ultrastructure of the processes resembles the early outgrowth observed in developing dorsal root ganglion cells in vivo (Tennyson, 1970) and sympathetic (Bunge, 1973; Landis, 1978) and sensory neurons in culture (Yamada et al., 1971). Early neuronal processes contain smooth endoplasmic reticulum, mitochondria, vesicles, microtubules, and neurofilaments but also have substantial ribosomal content in distinction to mature axons. This may allow for some protein synthesis at a distance from the cell body during development in a region of very active growth (Tennyson, 1970). Ribosomes are absent from mature axons though an occasional cluster can be observed, the protein synthetic apparatus is localized in the cell body. Mature axons contain microtubules and neurofilaments in parallel orientation, with the former taking a more peripheral location and the latter forming a central bundle. The arrangenment is highly organized as indicated by the regular spacing between the elements (Yamada et al., 1971). This arrangement is probably associated with the transport systems carrying materials in orthograde and retrograde directions critical to the maintenance and function of the neuron.

The processes in SY5Y and M17 were rich in microtubules and some profiles also contained neurofilaments though this was not a common feature of all processes observed. Areas of expansion at the ends of processes resembled growth cones of elongating nerve fibers observed in vivo (Tennyson, 1970; Skoff & Hamburger, 1974) and in vitro (Yamada, 1971; Bunge, 1973; Landis, 1978). These areas are the presumed sites for membrane addition, neurite elongation and interaction with the environment.

The growth cone is a highly mobile structure and specific to neurons. Its morphology was described in the studies of Ramon y Cajal (1890) and its amoeboid movements described in early culture of neuronal cells by Harrison (1907). The cell body of the neuron becomes

established in a fixed position but axonal projections extend for great distances to establish connections with the periphery. Mobility becomes restricted to the growth cone region as the neuron matures. Elongation of the axon appears to occur at the growing tip and not in the more proximal regions of the axon (Bray, 1970). Filipodia and microspikes are extended from the tip of the growth cone and sequentially attach and detach from substrate during the course of its exploration. The network underlying the membrane of the filopodia consists mainly of a microfilamentous network similar to that found in the ruffled edges of fibroblasts. These ruffled edges have been suggested to represent the leading edges for cell migration and locomotion (Wessells et al., 1973). The surface extension in these regions suggests that the plasma membrane and lattice maintain a fluid association that allows for both extension and contraction associated with mobility (Luduefia & Wessells, 1973).

The growth cone region is characterized in the most distal region by the presence of mainly filamentous elements in the filopodia and membranous areas in the large expanse. This region lacks the organized arrays of microtubules and intermediate filaments found in the axon and more proximal growth cone region. The intermediate area contains numerous organelles including mitochondria, polysomes, scattered neurofilaments and microtubules. Membraneous mounds, plasmalemmal blebs filled with clear vesicles, are present and are the presumed site for new membrane addition (Bunge, 1973). In cultured sympathetic neurons the growth cone region contains permanganate positive vesicles indicating the presence of catecholamine storage. The vesicles may have functional role in the interaction with target organs though it has not been determined as yet whether there is release of transmitter from the growth cone. Only small clear vesicles were found in growth cones of dorsal root ganglion cells fixed with permanganate (Landis, 1980).

The growth cone region may exhibit localized responses to peripheral factors and have an important role in establishing initial contacts with the target organ and relaying information to the cell body. In platelets the microfilamentous lattice undergoes dynamic transitions from soluble monomers to filamentous polymers in response to localized control factors. Such transitions may influence platelet migration and aggregation (Behnke, 1971). The tip of the growth cone in sympatheitic cells grown in tissue culture also responds to environmental factors.

Local concentrations of NGF act to maintain the structural integrity of the elongating axon. Using chambers which separate the medium environment of the cell body from that of the growing tip, the action of agents at both locations can be evaluated. If NGF is present in the somal chamber but not in chamber with the growth cone, neurite elongation ceases. The elongation of the growing tip is sensitive to the concentration of NGF in that local environment and NGF interacting with the soma is not sufficient to support the continued elongation (Campenot, 1977). Whether the NGF acts in the terminal with cytoskeletal or other elements or whether its action is mediated in the soma following retrograde transport is not known. The direction of growth at the tip can be influenced by iontophoretic application of pulses of NGF. The predominant movement is in the direction of the NGF pulse. The response appeared to be mediated at the tip directly as the time course of the effect was more rapid than could be accounted for by retrograde transport to the cell body (Gundersen & Barrett, 1980).

Elongation of neurites continues in the presence of protein synthesis inhibitors (Spooner et al., 1971) and continues independently of actions of the cell body as demonstrated in transected axons (Hughes, 1953). Enucleated cells also respond to neurite promoting factors such as cyclic AMP (Miller & Ruddle,1974). The membrane composition of the growth cone region may be different from the somal

or axonal membrane. Frequently observed in growth cone regions are plasmalemmal protrusions containing vesicular elements suggesting these are areas of membrane addition or membrane retrieval associated with the functioning of the growth cone (Pfenninger & Bunge, 1974). The membrane of the mounds differs in lectin binding and in the numbers of intramembranous particles as compared to other sites along the axon or at the cell body (Pfenninger & Maylie-Pfenninger, 1975). Though the mound-like structures have been demonstrated to arise as an artifact of glutaraldehyde fixation in fibroblasts (Hasty & Hay, 1978) and are not seen in freeze substituted growth cones of neurons (Rees & Reese, 1981), they may indicate that differences in structure in that region render the membrane more susceptible to generation of this artifact.

Cell surface molecules of neuroblastoma B104 associated with adhesion sites formed by the cell body with the substrate differ from those found at growth cone attachment sites with the substrate. Remnants left following EDTA treatment of neurite bearing and non-neurite bearing cells differ biochemically and morphologically. Scanning electron microscopy reveals different distribution of neurofilaments in the attachment plaques. A specific high molecular weight polysaccharide is found only in neurite bearing cells but not in other cell types (Donan & Culp, 1981).

The presence of growth cone regions at the ends of processes in the neuroblastoma lines (see also Burmeister, 1982) suggests that these processes resulted from outgrowth rather than as a retraction fiber as the cell moved away from an attachment site. Such "process formation" has been observed in cinematographic recording of neuroblastoma cell movements following serum withdrawl (Booher et al.,1970). Processes thus formed are not appropriate to the study of neuronal axon formation. Cyclic AMP induced process outgrowth in the C1300 line, NBP2, resembles neuronal outgrowth as demonstrated cinematographically and suggested from the ultrastructure. The distal growth cones exhibit

filopodia whose content consists mainly of microfilamentous material, the intermediate area is rich in membraneous cisternae and "mound-like structures, and the more proximal regions contain a variety of organelles (Kataoka et al., 1980).

While neurites emerging from a cell may arise via several mechanisms, the ultrastructure may reveal distinguishing details from which to discern the origins of the outgrowth. The ultrastructure, most notably the presence of growth cones in the SY5Y processes, both spontaneous ones and those stimulated by NGF or cyclic AMP, suggests that these were formed in manner analogous to outgrowth from normal neurons. Preliminary cinematographic studies of neurite formation in SY5Y suggest it results from outgrowth rather than retraction (Ishii & Sonnefeld, personal communication). SY5Y cells may be useful model for probing mechanisms involved in neurite elongation.

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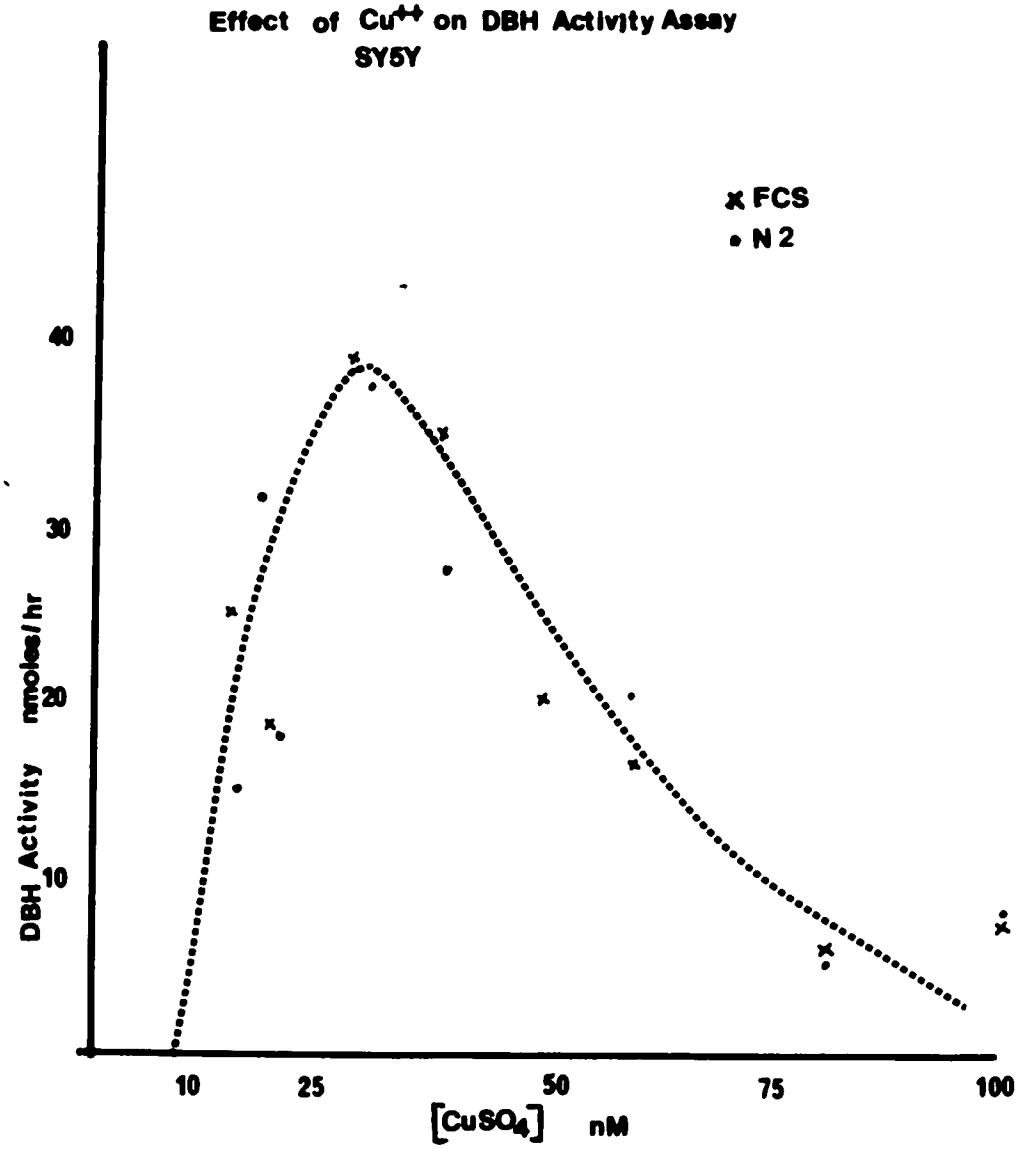
Fig. a


Effect of Cu++ on DBH Activity Assay
SY5Y
FCS
N 2
DBH Activity nmoles/hr
40
30
20
10
10
25
50
75
100
[CuSO4] nM