Environmental Influences in the Development of Neural Crest Derivatives: Glucocorticoids, Growth Factors, and Chromaffin Cell Plasticity[1]

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Abstract

The neural crest gives rise to three major adrenergic cell types: sympathetic principal neurons, adrenal chromaffin cells, and small intensely fluorescent (SIF) cells. All of these derivatives synthesize and store catecholamines, but they differ in numerous other characteristics. SIF cells appear intermediate in phenotype between the other two. We have examined the role of several environmental factors in the differentiation of sympathetic principal neurons and adrenal chromaffin cells.

In previous studies of young rat adrenal chromaffin cells in dissociated cell culture, differentiated characteristics such as the presence of the enzyme phenylethanolamine N-methyltransferase (PNMT), epinephrine (E) synthesis, and large catecholamine storage vesicles were not well maintained. Here we describe long-term culture of chromaffin cells which, in the presence of micromolar glucocorticoid, maintained all of these characteristics. In addition, chromaffin cells of a variety of ages were found to be dependent on glucocorticoid for long-term survival in culture. In the absence of glucocorticoid, many adrenal chromaffin cells from neonatal rats could be rescued by nerve growth factor (NGF) administration. They extended neurites, as previously described by Unsicker and colleagues (Unsicker, K., B. Krisch, U. Otten, and H. Thoenen (1978) Proc. Natl. Acad. Sci. U. S. A. 75: 3498–3502). In contrast to previous studies, however, with long-term exposure to NGF these cells became indistinguishable from mature sympathetic neurons, as judged by the following morphological and biochemical criteria: (i) increased cell size and loss of intense CA fluorescence in their cell bodies; (ii) acquisition of characteristic neuronal ultrastructure, including morphologically specialized synapses; (iii) loss of chromaffin granules, PNMT, and E synthesis; and (iv) acquisition of neuronal markers, including tetanus toxin labeling and immunoreactivity to neurofilament protein. This conversion to neurons was markedly enhanced by addition of a non-neuronal cell conditioned medium (CM) containing a neurite-promoting factor, which acted by increasing the NGF responsiveness of the chromaffin cells. Even chromaffin cells from adult rats, which are known to grow few processes in response to NGF alone, became neuronal in the presence of this CM plus NGF. While converting to neurons, adrenal chromaffin cells transiently assumed an intermediate phenotype resembling type I SIF cells, which suggests particular developmental relationships between the different cell types of the sympathoadrenal lineage.

In response to a factor known to induce cholinergic function in adrenergic sympathetic neurons, the chromaffin cell-derived neurons were also capable of developing cholinergic properties, including acetylcholine synthesis and storage, choline acetyltransferase activity, and the presence of cholinergic terminals after permanganate fixation. The interconversions of these phenotypes in culture demonstrate the remarkable plasticity of rat adrenal chromaffin cells and provide further evidence for the importance of environmental factors in neural crest development.

Adrenergic neural crest derivatives are of three major types: sympathetic principal neurons, adrenal chromaffin cells, and small intensely fluorescent (SIF)[3] cells. These cells all synthesize and store catecholamines (CAs) but differ in a number of other respects which are summarized diagrammatically in Figure 1. Mature neurons have large cell bodies (40 to 50 μm) (Burnstock and Costa, 1975) and long processes containing microtubules and neurofilaments. These neurites have specializations for release of transmitter at localized sites adjacent to target cells (Grillo, 1966). Neuronal amine-storing vesicles are small (mean diameter, 50 nm) (Hokfelt, 1969; Gabella, 1976), are concentrated in the processes, and usually contain norepinephrine (NE) (Burnstock and Costa, 1975; Gabella, 1976). In contrast, adrenal chromaffin cells have small cell bodies, usually 20 μm in diameter, and have few or no processes (Wood, 1963; Coupland, 1965a). Their cells bodies are packed with large amine-

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[3] The abbreviations used are: ACh, acetylcholine; BCE, bovine corneal endothelial (cell); CA, catecholamine; CAT, choline acetyltransferase; CM, conditioned medium; DBH, dopamine β-hydroxylase; E, epinephrine; ECM, extracellular matrix; FCS, fetal calf serum; GOA, glyoxylic acid; GS, goat serum; HBSS, Hanks' balanced salt solution; HS, horse serum; kD kilodalton; NE, norepinephrine; NGF, nerve growth factor; PBS, phosphate-buffered saline; PNMT, phenylethanolamine N-methyltransferase; RS, rat serum; SIF, small intensely fluorescent (cell); TX-100, Triton X-100.

storing vesicles (diameters, 100 to 350 nm) and, therefore, exhibit intense formaldehyde-induced fluorescence (Eranko, 1955; Coupland, 1965b; Elfvin, 1967; Grynszpan-Winograd, 1975). They release their CA (epinephrine (E) or NE) directly into the circulation. SIF cells appear intermediate in properties between neurons and chromaffin cells (Grillo, 1966; Eranko, 1975; Taxi, 1979; Taxi et al., 1983).

How do these different adrenergic phenotypes arise during development? It is clear from a variety of experiments *in vitro* and *in vivo* that the local environment of neural crest derivatives during development can play an important part in determining their final fate. Most of this work has focused on the decision of neural crest cells to become either adrenergic or cholinergic (Patterson, 1978; Le Douarin, 1980). Another environmental cue, nerve growth factor (NGF), has been implicated in the development of crest cells into either neurons or chromaffin cells. NGF is required for the survival and differentiation of sympathetic neurons both *in vivo* (for reviews see Levi-Montalcini and Angeletti, 1968; Thoenen et al., 1979; Thoenen and Barde, 1980) and *in vitro* (Levi-Montalcini and Angeletti, 1963, 1968). It remains unclear whether adrenal chromaffin cells also require NGF during normal development (Aloe and Levi-Montalcini, 1979; Gorin and Johnson, 1979; Unsicker, Millar and Hoffman, 1982). There are many indications, however, that chromaffin cells can respond to NGF *in vivo* and *in vitro* (Olson, 1970; Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979; Tischler et al., 1980). Exposure of chromaffin cells to NGF results in the acquisition of certain neuronal characteristics, including neurites, small synaptic vesicles, and larger soma size. In particular, the experiments of Unsicker et al. (1978) with dissociated cell cultures showed that individual differentiated chromaffin cells can acquire these properties. In all of these studies, however, the development of neuronal characteristics was not accompanied by the complete loss of chromaffin cell properties: large CA granules and intense CA fluorescence in cell bodies as well as processes were maintained. It was therefore unclear whether adrenal chromaffin cells could acquire the normal phenotype of sympathetic neurons. The interpretation of these studies is further complicated by the observation that adrenal chromaffin cells from 1- to 2-week-old rats lose a number of differentiated characteristics in cell culture even without NGF addition. E content decreases markedly (Muller and Unsicker, 1981), and only cells with the ultrastructural appearance characteristic of NE storage are observed (Unsicker and Chamley, 1977; Unsicker et al., 1978). In contrast, rat and bovine chromaffin cells from adult animals maintain E synthesis and storage granules for 1 to 2 weeks in culture (Trifaro and Lee, 1980; Unsicker et al., 1980). However, these mature cells do not grow processes in response to NGF (Naujoks et al., 1982; Tischler et al., 1982).

In the present studies, conditions were established for long-term dissociated cell culture of adrenal chromaffin cells from both neonatal and adult rats. These cells remained well differentiated by a number of criteria. The cultures were then used to examine the effects of a variety of environmental factors on the differentiation of chromaffin cells and on their segregation from the other closely related cells of the sympathoadrenal lineage. The factors examined were: (*i*) glucocorticoid hormones, which are present in very high levels in the normal environment of the adrenal gland (Jones et al., 1977); (*ii*) NGF, with particular attention given to the age dependence of NGF effects and to the question of whether chromaffin cells can become fully neuronal; (*iii*) extracellular matrix-associated molecules from conditioned medium (CM), which are known to promote neurite outgrowth from neurons and PC12 cells (Collins, 1978; Fujii et al., 1982; Lander et al., 1983); and (*iv*) a protein factor produced by non-neuronal cells which causes adrenergic sympathetic neurons to acquire cholinergic characteristics (Patterson and Chun, 1977; Weber, 1980). Preliminary reports of this work have appeared previously (Doupe et al., 1982; Doupe and Patterson, 1983).

Materials and Methods

Cell preparation

Adrenal medullae from early postnatal (1 to 3 days), young (7 to 10 days), or adult (250 gm or more) rats (CD strain, Charles River Breeding Laboratories, Wilmington, MA) were dissected free of cortex, cut into small pieces, and dissociated according to the method of Unsicker et al. (1978), as follows. Cells were washed once in calcium/magnesium-free Hanks' balanced salt solution (HBSS) and then incubated at 37°C for 40 to 70 min in HBSS containing 0.1% elastase (Sigma Chemical Co., St. Louis, MO) plus 0.1% collagenase (Worthington Biochemical Corp., Freehold, NJ); the cell suspension was then centrifuged at 1000 rpm, resuspended, and incubated (with stirring) for 10 to 20 min in 0.125% trypsin in phosphate-buffered saline (PBS; M.A. Bioproducts, Walkersville, MD) supplemented with 2% glucose. The final cell suspension was centrifuged and washed three times in serum-containing medium, and was plated into culture dishes at a density of 400 to 3000 cells/well. Medullae from 10 rat pups yielded 20 dishes, each containing 500 to 1000 cells.

Culture dishes and substrates

Cells were grown on Aclar (Allied Chemical Corp., Morristown, NJ) or polystyrene (Lux, 25 mm) coverslips in the center well of 35-mm Falcon Petri dishes (Hawrot and Patterson, 1979). The coverslips were sterilized with UV irradiation and coated with one of several substrates: (*i*) sterile collagen (Bornstein, 1958); (*ii*) sterile collagen coated with extracellular matrix (ECM) produced by bovine corneal endothelial (BCE) cells (gift of Dr. Jeremy Brockes, Medical Research Council Biophysics Unit, London, England), by the method of Fujii et al. (1982) with minor modifications. Alternatively, the tissue culture-treated surface of polystyrene culture dishes (Falcon) was incubated with 1 mg/ml of poly-D-lysine (Sigma) for 24 to 48 hr, as described by Collins (1978). In experiments on substrate factors, polylysine-coated dishes were incubated with heart cell CM (undiluted or × 10 concentrate; see below) for 8 to 12 hr at 4°C, and washed three times with serum-containing medium.

Culture media

Cells were prepared in plating medium made with L15-Air and grown in L15-CO$_2$ growth medium, as described by Mains and Patterson (1973). Growth medium was supplemented with 5% adult rat serum (RS) and, in some cases, with 5% FCS as well. Methocel (0.6%; Dow Chemical USA, Midland, MI) was added to medium for culture when substrates were formed only of collagen. Depending on the particular experiments, other additives included the following.

i. Various concentrations of dexamethasone phosphate (Merck, Sharp & Dohme, Teterboro, NJ), hydrocortisone succinate, corticosterone, and progesterone (all from Sigma). Corticosterone and progesterone were prepared as 10^{-2} M solutions in absolute alcohol and then diluted in L15-Air.

ii. NGF (7 S) (Mains and Patterson, 1973), at a final (saturating) concentration of 1 μg/ml except in NGF concentration dependence experiments. In some experiments, an antiserum to 2.5 S NGF (Collaborative Research, Waltham, MA), at a concentration of 1/200 to 1/400 was used.

iii. Serum-free CM, prepared as described by Fukada (1980). The serum-free medium was collected and frozen at −20°C for later use. CM either was used unconcentrated and mixed with an equal volume of growth medium (containing double-strength additives including RS), or was concentrated by 100% ammonium sulfate precipitation as described by Fukada (1980). In the latter case, the pellet was resuspended in 1/10th the original volume, dialyzed against phosphate buffer, and used at 1 ml/10 ml of medium (equivalent to 100% CM).

After plating, cultures were allowed to stand for 2 days before the medium was changed. Subsequently, the medium was changed every 2 to 4 days. Rapidly proliferating cells were eliminated with cobalt-60 irradiation (5000 rads) at day 2 or 3, or by treating cultures with a mixture of 10^{-5} M arabinosylcytosine, 10^{-5} M fluorodeoxyuridine, and 10^{-5} M uridine (Sigma) during days 2 to 4 and days 6 to 8 *in vitro*.

Histochemistry

CA histofluorescence was visualized by the Falck-Hillarp method (see Falck et al., 1962; Corrodi and Jonsson, 1967; Eranko, 1967) or by a modification of the glyoxylic acid method of de la Torre and Surgeon (1980). In the first procedure, cultures were rinsed once with serum-free medium, drained completely, and dried over P$_2$O$_5$. The cultures were reacted over paraformaldehyde powder (equilibrated to 70% humidity) at 80°C for 60 to
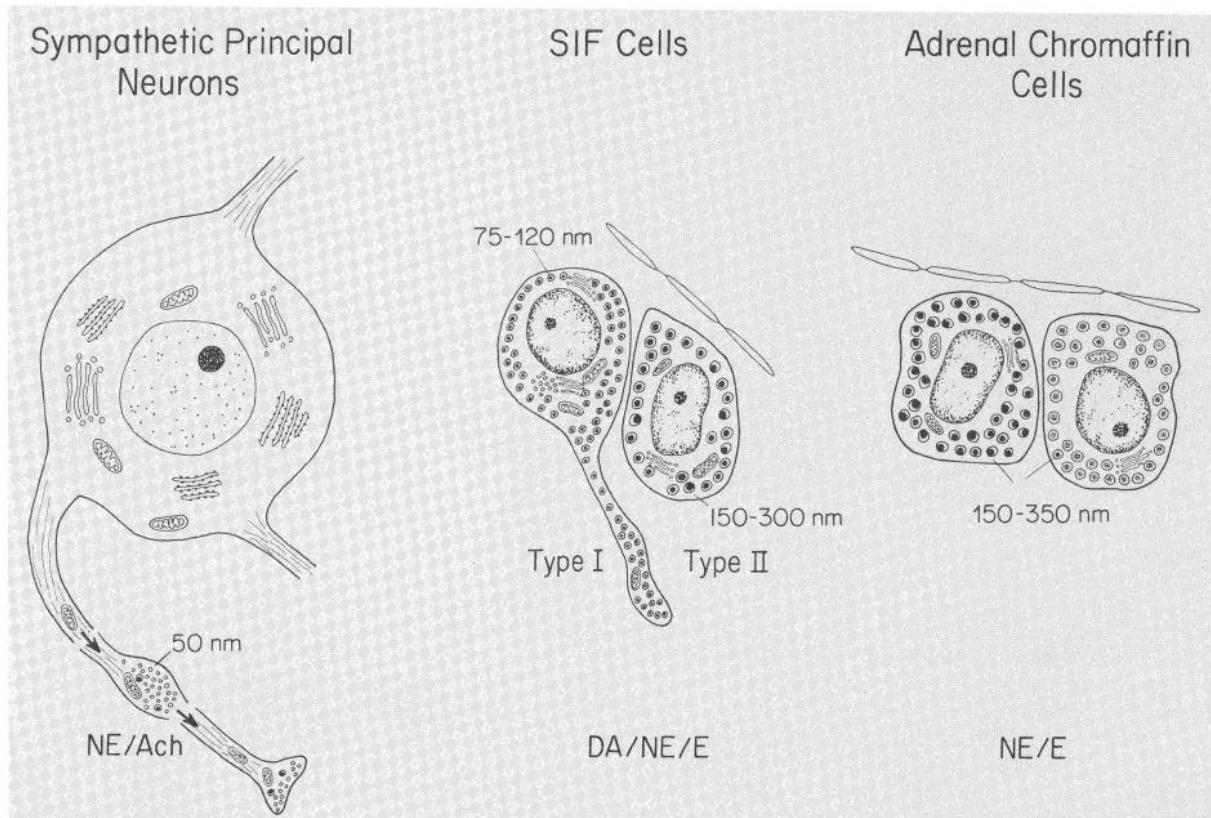
Figure 1. Schematic summary of the three adrenergic derivatives of the neural crest and their similarities and differences.

90 min in a closed container. The coverslips were mounted in glycerol and examined on a Zeiss inverted microscope with epi-illumination fluorescence and a CA filter set. For the glyoxylic acid (GOA) reaction, the cultures were rinsed once with PBS and washed for 3 sec with GOA solution made as described by de la Torre and Surgeon (1980), except that in some experiments it included 0.1% paraformaldehyde to improve the preservation of cell morphology. The cultures were then dried under a cool air stream for 30 min. After drying, several drops of light mineral oil were placed on each coverslip, and the cultures were reacted for 2.5 to 3 min in a 96°C oven, mounted in mineral oil, and examined.

To detect the presence of immunoreactivity to phenylethanolamine-*N*-methyltransferase (PNMT) and dopamine, β-hydroxylase (DBH), immunohistochemistry was performed as follows. Cells were washed free of culture medium and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 40 min at room temperature. After several rinses in PBS and L15-Air, cells were preincubated at room temperature for 1 to 3 hr in dilution buffer containing 10 mM phosphate, 0.5 M NaCl, 10% horse serum (HS), 3% goat serum (GS), and 0.3% Triton X-100 (TX-100). For visualization of PNMT immunoreactivity cells were then incubated overnight at 4°C in rabbit anti-bovine PNMT antiserum (provided by Dr. S. Watson, University of Michigan, Ann Arbor, MI), diluted 1/1500 to 1/2000 in dilution buffer. The cultures were then washed in PBS and incubated for 30 min at 37°C in goat anti-rabbit IgG (Sternberger-Mayer Inc.), diluted 1/40 in dilution buffer. Cultures were again washed in PBS, incubated for 30 min at 37°C in peroxidase-antiperoxidase (Sternberger-Mayer, Inc.), and diluted 1/80 in PBS containing 10% HS, 3% GS, and 0.1% TX-100. The peroxidase was developed for 10 min in 0.05% 3,3'-diaminobenzidine hydrochloride (Sigma) in PBS activated with 0.002% H_2O_2. The coverslips were mounted in glycerol-PBS, 1:1, and cell counts were made at × 80 with brightfield optics. No positive cells were observed if normal rabbit serum was substituted for the primary antiserum. For visualization of DBH immunoreactivity, fixed and permeabilized cells were incubated overnight at 4°C in guinea pig anti-rat DBH antiserum (provided by Dr. R. Grzanna, Johns Hopkins University, Baltimore, MD; Grzanna et al., 1977). The primary antiserum was diluted 1/250 to 1/400 in dilution buffer. After washing with PBS, cultures were incubated for 30 min at 37°C in horseradish peroxidase-conjugated goat anti-guinea pig IgG (supplied by Dr. R. Grzanna) diluted 1/40 in PBS containing 10% HS, 3% GS, and 0.1% TX-100. Cells were then washed in PBS and reacted as described for PNMT. No staining was evident if normal guinea pig serum was substituted for the primary

antiserum. Cultures of a pancreatic cell line without DBH immunoreactivity (AR42J, provided by M. Womack and Dr. T. Jessell, Harvard University) also showed no staining when incubated with the primary antiserum and stained exactly as for experimental cultures.

To detect the presence of immunoreactivity to neurofilaments, cells were fixed with 4% paraformaldehyde for 20 min, washed with PBS and L15-Air, and permeabilized for 5 min in PBS with 0.2% TX-100. After washing in PBS, cells were preincubated for 15 min in PBS and 3% GS, then incubated at room temperature for 60 min in a rabbit antiserum to melanocyte-stimulating hormone (anti-MSH, Immunonuclear Corp., Stillwater, MN), which has been shown to detect the 140-kilodalton (kD) subunit of neurofilaments (Drager et al., 1983). The antiserum was diluted 1/50 in Tris-buffered saline, 2% bovine serum albumin, 0.1% TX-100, and 0.2% azide. Cells were washed in PBS containing 3% GS, incubated in fluorescein-conjugated goat anti-rabbit IgG (Cappel, Malvern, PA) diluted 1/125 in PBS containing 3% GS, and mounted in 0.2 M sodium carbonate, pH 9.0:glycerol (1:1), containing 2 mg/10 ml of *p*-phenylenediamine to prevent photobleaching of the fluorescein (Johnson and Nogueira Aranjo, 1981), and examined under epi-illumination fluorescence. No specific immunofluorescence was evident if normal rabbit serum (diluted 1/50) was substituted for the primary antiserum.

In order to visualize the cell surface antigen recognized by the mouse monoclonal antibody ASCS4 (Sweadner, 1983), immunohistochemistry was performed on living cells. Cultures were washed in L15-Air containing 10% HS and then incubated at room temperature for 30 min in ASCS4 diluted 1/4 in L15-Air containing HS. Cells were washed in L15-Air, incubated for 30 min at room temperature in rhodamine-labeled goat anti-mouse IgG (F(ab')$_2$ (Cappel Laboratories, Malvern, PA) diluted 1/50 in L15-Air plus 10% HS, and then examined. No staining was observed if cultures were incubated with a monoclonal antibody raised against sheep red blood cells (provided by Dr. J. Dodd, Harvard University) instead of ASCS4.

Tetanus toxin binding was also determined on living cells using the method of Mirsky et al. (1978) and Raff et al. (1979). The rabbit anti-tetanus toxoid antiserum was kindly provided by Dr. J. Brockes, Medical Research Council Biophysics Unit, (London, England) (Raff et al., 1979). If cultures were incubated with the equivalent concentration of rabbit serum instead of anti-tetanus toxin, or if the tetanus toxin incubation was omitted, no immunofluorescence was observed.
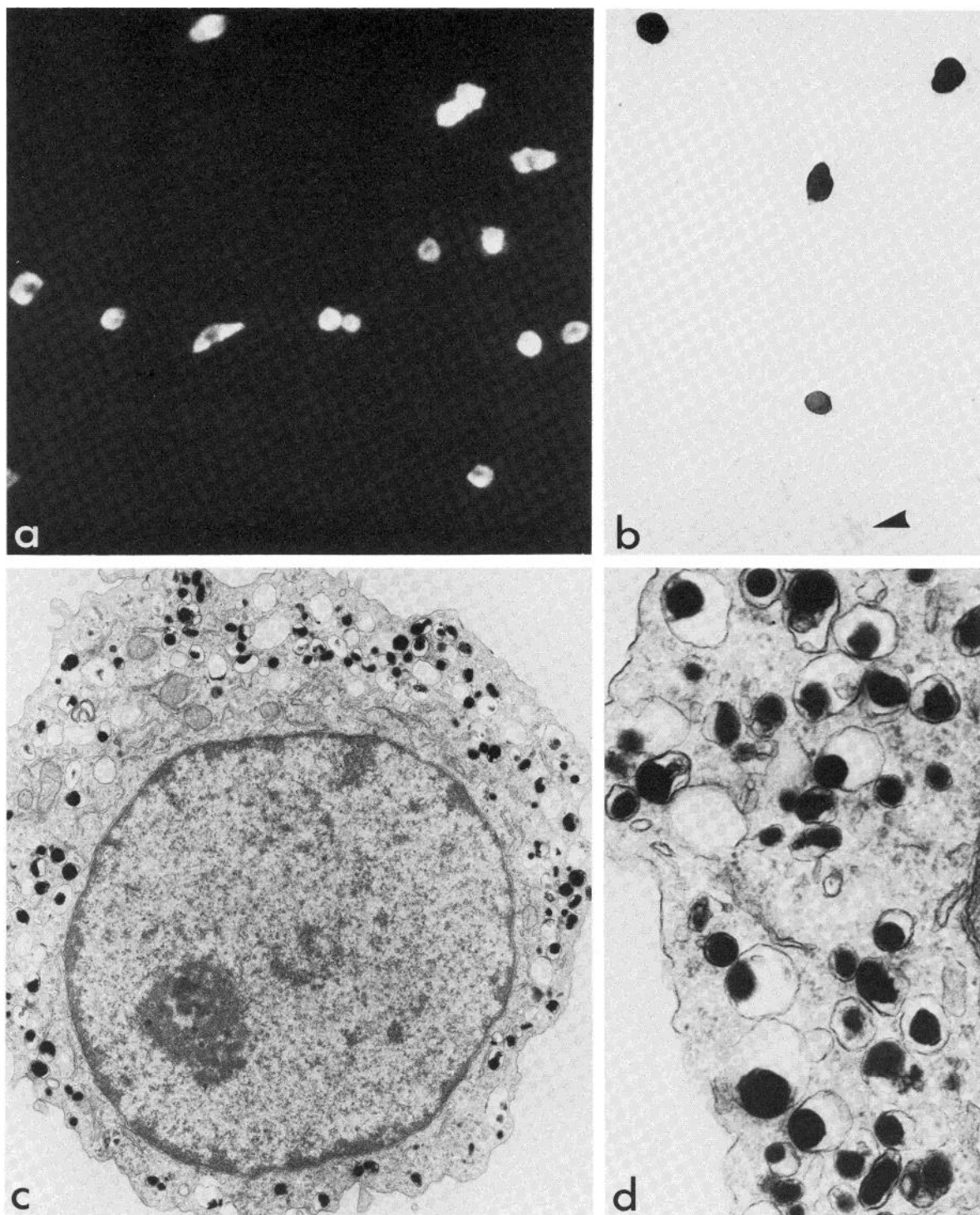
Figure 2. Differentiated properties displayed by newly plated adrenal chromaffin cells from neonatal (1- to 3-day-old) rats. *a,* GOA-induced CA fluorescence. Magnification × 420. *b,* Indirect immunoperoxidase staining with an antibody to the epinephrine-synthesizing enzyme, PNMT. Note that one cell in this field does not contain detectable PNMT (*arrowhead*). Magnification × 420. *c,* Ultrastructure of adrenal chromaffin cells 9 hr after plating. Note the high nuclear-cytoplasmic ratio, the clumped heterochromatin, and the presence of large chromaffin granules. Magnification × 11,800. *d,* Chromaffin granules of a cell from the same culture as in *c.* Magnification × 43,700.

Biochemical assays

High pressure liquid chromatography. Synthesis and accumulation of CAs were determined by incorporation of radioactive precursors (Mains and Patterson, 1973). After a 20-min incubation in tyrosine-deficient medium, living cultures were incubated for 48 hr with 50 μM L-[U-^{14}C]tyrosine (New England Nuclear, Boston, MA) in 0.8 ml of tyrosine-free L15-CO_2 supplemented with the usual additives, and 5×10^{-6} M or 10^{-8} M dexamethasone

or 1 μg/ml of 7 S NGF depending on the particular culture. The specific activity of the isotope was 495 to 510 mCi/mmol. Ascorbic acid (Sigma, 25 μg/ml in water, made immediately before addition) was added at 3- to 7-hr intervals throughout the incubation. At the end of the incubation, cultures were washed for 20 min at 37°C in plating medium and were scraped off the dish into microhomogenizers containing 200 μl of cold 0.17 M acetic acid and 0.1 mg/ml of unlabeled standards (tyrosine, tyramine, dopamine, NE, and E; Sigma). Cultures were homogenized and left on ice for 20 min. The

TABLE I

Differentiated characteristics of newly plated chromaffin cells versus chromaffin cells grown for 4 weeks with or without dexamethasone

Differentiated characteristics of newly plated chromaffin cells are shown on line 1; those of chromaffin cells grown for 4 weeks in the presence or absence of dexamethasone are shown on lines 2 and 3, respectively. Values are expressed as mean ± SEM.

Chromaffin Cells	Percentage PNMT+ Cells	CA Synthesis		Vesicle Diameter (nm)
		fmol CA/Cell	E/NE	
1. Newly plated	86.7 ± 0.7	ND[a]	ND	177 ± 11
	(n = 2)[b]			(n = 8)
2. +dex,[c] 4 weeks	87.8 ± 1.1	9.6 ± 0.9	2.10 ± 0.11	149 ± 6
	(n = 6)	(n = 18)	(n = 10)	(n = 13)
3. −dex, 4 weeks	0.0 ± 0.0	9.1 ± 1.9	0.02 ± 0.02	113 ± 6
	(n = 4)	(n = 11)	(n = 3)	(n = 6)

[a] ND, not done.

[b] n, number of cultures assayed or cells in which vesicles were measured.

[c] dex, dexamethasone.

radioactive CAs in the supernatants were separated using reverse phase high pressure liquid chromatography on a C_{18} μBondapak column (Waters Associates, Milford, MA) with a precolumn attachment. The mobile phase was a glycine-HCl buffer, pH 3 (0.08 M glycine, 0.08 M NaCl, 0.019 N HCl in distilled water, as described in the unpublished method of G. J. Glover and T. O. Fox, Harvard Medical School, Boston, MA; see Glover, 1981; Role et al., 1981). Detection was by UV absorption at 254 nm, in the order: NE, E, DOPA, dopamine, tyrosine, and tyramine. The separation was complete in 15 to 20 min at a flow rate of 0.8 to 1.0 ml/min. Seventy 0.25-ml fractions were collected for each sample and counted after addition of 4 ml of Ultrafluor liquid scintillation cocktail (VWR Scientific Inc., Boston, MA). With a Packard model 3320 scintillation counter (Packard Instruments Co., Inc., Downers Grove, IL), the counting efficiency for ^{14}C was 70%.

High voltage electrophoresis. Synthesis and accumulation of acetylcholine (ACh) and CA were determined simultaneously as described by Mains and Patterson (1973). Living cultures were incubated for 8 hr in 50 μl of medium containing 60 μM [3,5-^{3}H]tyrosine and 60 μM [*methyl*-^{3}H]choline (New England Nuclear). The final specific activities of the isotopes were 15 Ci/mmol of tyrosine and 40 Ci/mmol of choline. Cultures were harvested and the transmitter products were separated by electrophoresis as previously described (Mains and Patterson, 1973).

In some experiments, the identity of ACh spots was further confirmed by incubation of cultures with acetylcholinesterase (Worthington Biochemical Corp.) in 0.02 M sodium phosphate with 0.1 M NaCl, 0.02 M $MgCl_2$, and 0.1% TX-100 for 20 min at 37°C. Cultures were then acidified to bring them to pH 2 and were electrophoresed with unlabeled electrophoretic standards as described above.

Choline acetyltransferase assay. Choline acetyltransferase (CAT) activity was assayed in homogenates by measuring the conversion of [acetyl-^{3}H] coenzyme A to [acetyl-^{3}H]choline using electrophoresis at pH 1.9 to separate the precursor and product, as described by Patterson and Chun (1977).

Electron microscopy. Two different methods of fixation were used to study the ultrastructure of SIF and adrenal chromaffin cells. In order to examine general structure, cultures were fixed for 20 min in 3% glutaraldehyde in 0.12 M phosphate buffer, pH 7.3, at room temperature directly after the removal of growth medium. The cultures were rinsed with 0.12 M phosphate buffer (pH 7.3), postfixed with 1.3% osmium tetroxide in phosphate buffer for 20 min, stained en bloc with uranyl acetate, dehydrated with ethanol, and embedded in Epon 812. Thin sections were cut parallel to the collagen substrate and stained with lead citrate (Landis, 1980).

To examine endogenous CA stores, cultures were fixed in 4% potassium permanganate (Richardson, 1966) on ice for 15 to 20 min. They were then rinsed thoroughly with cold 0.1 M acetate buffer, stained en bloc with uranyl acetate, dehydrated, and embedded. Thin sections were cut parallel to the collagen substrate and examined without further staining.

The areas of the granular vesicles were measured from prints at a final magnification of × 152,000 using a Houston Instruments electronic digitizing tablet and stylus connected to an Apple IIe/Bioquant morphometry system (R & M Biometrics, Inc., Nashville, TN). Profile diameters were calculated from the measured area, assuming all vesicles to be circular, although a small percentage of the vesicles were elongated rather than circular. Because many of the measured profiles represent sections through vesicles away from the equator where the diameter is maximal, the mean vesicle profile diameter (d) is an underestimate of the true mean diameter. In some cases, the estimated mean true vesicle diameter, D, was calculated from the

measured mean profile diameter (d). This was done by completing the profile size distribution for the loss of small profiles, calculating the corrected mean profile diameter d', and using d' to calculate D, using the correction $D = 4d'/\pi$, as described by Weibel (1979). This relationship applies strictly to a single class size of spheres but also represents an approximate mean diameter for a population of spheres of varying diameter. Measurements were not corrected for section thickness. Eighty to 500 vesicles were measured per cell. In experiments in which vesicle diameters of cells were measured, the vesicle diameters in a large number of other cells were also assessed qualitatively and compared to those of the measured cells.

Results

Role of glucocorticoid. To investigate the role of glucocorticoids in chromaffin cell development, adrenal medullary cells from newborn rats were grown in dissociated cell culture in the presence or absence of dexamethasone, a synthetic glucocorticoid. Treatment of the cultures with antimitotic agents or gamma-irradiation at 2 days after plating virtually eliminated non-chromaffin cells. Chromaffin cells grown in the absence of antimitotic agents did not show significant mitosis as assessed by [^{3}H]thymidine incorporation or by sequential cell counts (data not shown), and the treatments to eliminate replicating cells did not decrease chromaffin cell survival in culture.

Chromaffin cells from neonatal (1- to 3-day-old) rats were well differentiated when examined 9 to 12 hr after plating in the presence or absence of glucocorticoid: essentially all of the cells had intense CA fluorescence (Fig. 2a), and 86.6 ± 0.7% of the cells stained with an antiserum to the enzyme for E synthesis, PNMT (Fig. 2b, Table I). When the cultures were examined ultrastructurally, the cells were small, with scant cytoplasm which contained little rough endoplasmic reticulum. Numerous large chromaffin granules with a mean profile diameter of 177 ± 11 nm (SEM) were present (Fig. 2, c and d, Table I). *In vivo,* NE- and E-storing cells have been distinguished on the basis of the ultrastructural features of their granules after glutaraldehyde fixation (Coupland, 1965b). Granules of NE-storing cells have electron-dense, eccentric cores whereas those of E-storing cells have centrally placed, relatively granular and electron-lucent cores. Many of the newly plated cells had chromaffin granules with electron-dense and eccentric cores, characteristic of NE storage (Fig. 2d), but the majority of the cores appeared E-like after several days *in vitro* (see below).

When grown in the presence of 5 μM dexamethasone, chromaffin cells dissociated from neonatal rat medullae survived for many weeks in low density cell culture. They remained well differentiated by a number of morphological and biochemical criteria. The cells were increased in size compared to newly plated chromaffin cells but still had the characteristic phase microscopic morphology of chromaffin cells in culture: their phase-bright cell bodies were round or slightly flattened and fusiform, with a small nucleus and a single nucleolus (e.g., Figs. 6a and 8a). All of the chromaffin cells had intense CA histofluorescence (Fig. 3a). The ultrastructure of the cells was also typical of chromaffin cells: the small cell bodies had little
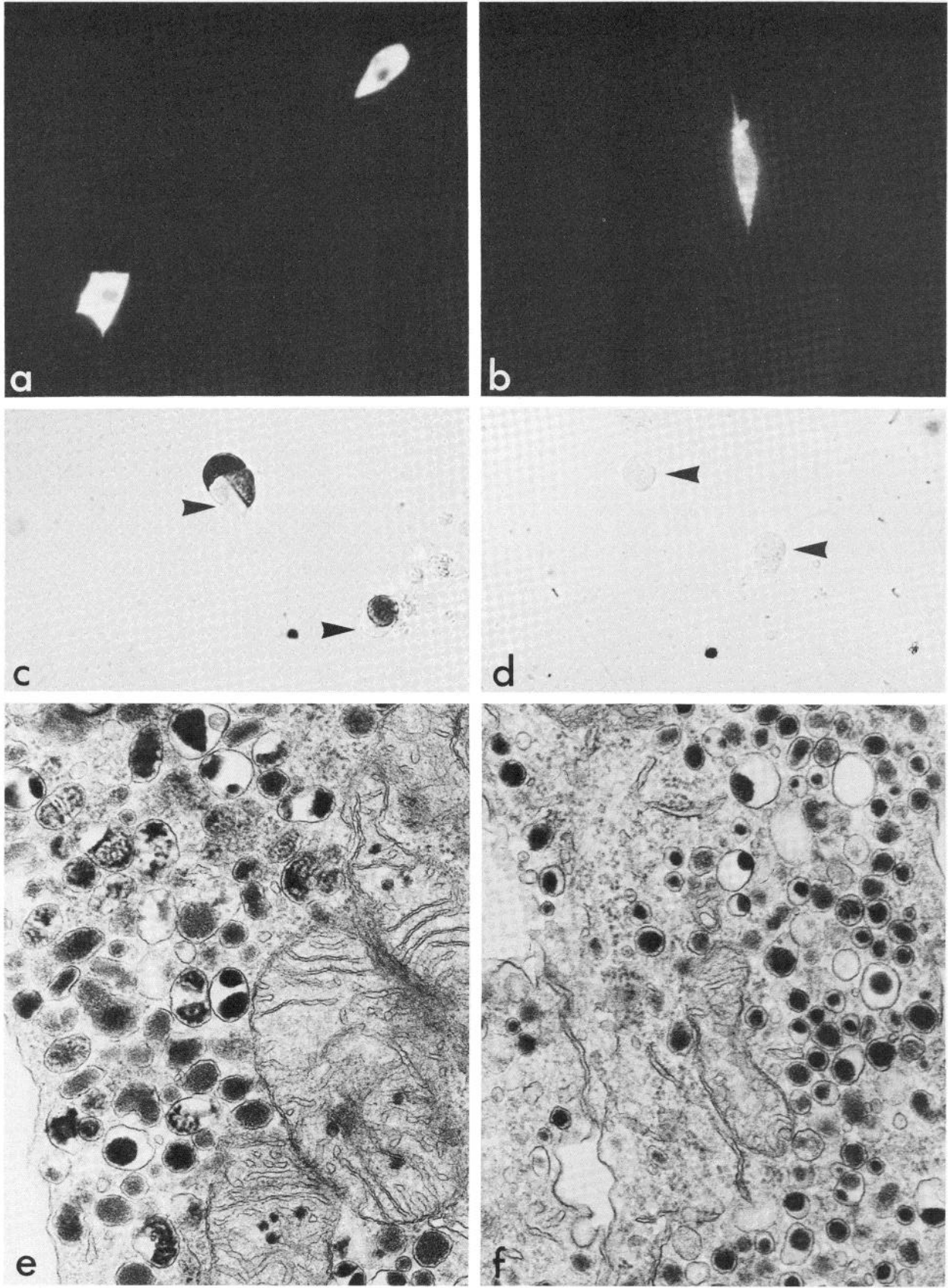
Figure 3. Comparison of adrenal chromaffin cells from neonatal rats grown for 4 to 6 weeks in the presence or absence of 5 µM dexamethasone. *a, c,* and *e,* With 5 µM dexamethasone. *b, d,* and *f,* without corticosteroid. Chromaffin cells grown both with (*a*) and without (*b*) corticosteroid exhibit intense GOA-induced CA fluorescence. Magnification × 420. Most cells grown with corticosteroid have immunohistochemically detectable levels of the enzyme PNMT (*c*), whereas chromaffin cells grown in the absence of the hormone (*d*) lose this immunoperoxidase staining. Magnification × 420. *Arrowheads* point to cells without immunoreactivity. Ultrastructurally, chromaffin cells grown with (*e*) or without (*f*) hormone appear well differentiated and contain numerous chromaffin granules, although the granules appear to be slightly smaller and to have denser cores in cells grown without corticosteroid (*f*). Magnification × 43,700.
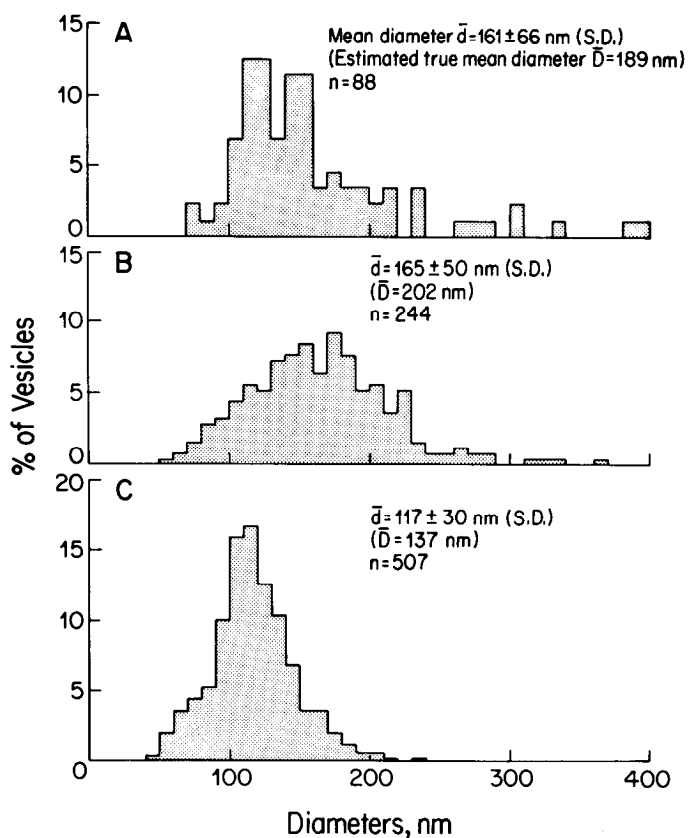
Figure 4. Histograms of vesicle diameters in representative cells from sister cultures at three different times *in vitro*: newly plated neonatal chromaffin cell cultures (*A*), chromaffin cells grown for 4 weeks with 5 μM dexamethasone (*B*), and cultures grown for 4 weeks without corticosteroid (*C*).
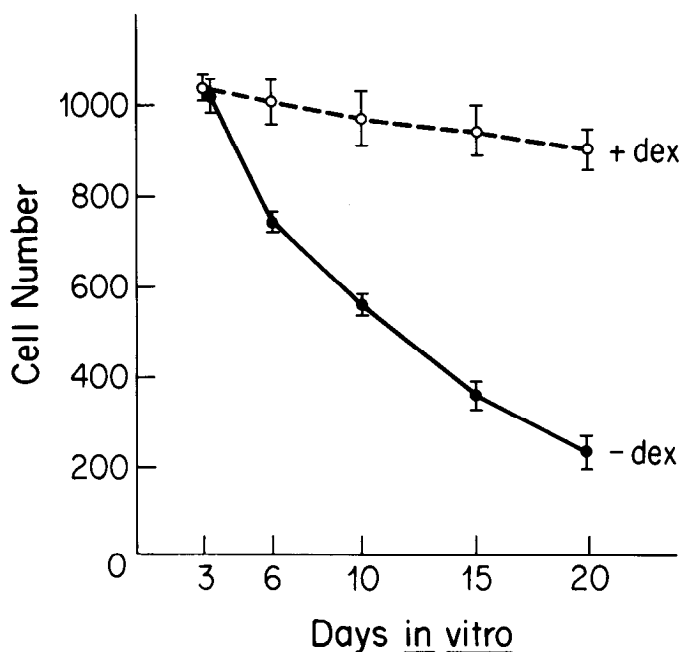

Figure 5. Long-term survival of chromaffin cells from neonatal rats in culture with 5 μM dexamethasone or without dexamethasone. For each condition all of the cells in each of three sister cultures were counted in the phase microscope at the indicated days after plating. Total cell number was expressed as the mean ± SEM.

rough endoplasmic reticulum but were filled with numerous large chromaffin granules (Fig. 3e). Most cells in the cultures grown in the presence of corticosteroid had E-like granules, although a smaller number of cells with NE-like granules was also observed. The chromaffin granules exhibited very large diameters, although there was variation from plating to plating (103 ± 22 nm to 190 ± 44 nm) (Table I). In some experiments, the mean diameter of chromaffin granules after 4 weeks *in vitro* was smaller than that of the newly plated cells.

The cells in these cultures synthesized and stored CAs, primarily E. The E/NE ratio was 2.1 ± 0.1 after 4 to 6 weeks in dexamethasone (Table I). Because trans-synaptic activity is known to increase the activity of CA-synthetic enzymes in the adrenal medulla (Molinoff et al., 1970; Ciaranello and Black, 1971; Otten and Thoenen, 1975) and can frequently be mimicked in culture by elevated levels of potassium (Walicke et al., 1977), 20 mM potassium was included in the corticosteroid-containing growth medium in some experiments. The inclusion of high levels of potassium did not significantly increase the total amount of CA synthesized and stored per cell in 48 hr but shifted the E/NE ratio to 5.9 ± 1.0 ($n = 5$), a value very similar to that observed for newly plated adult rat chromaffin cells (Tischler et al., 1982). All of the cells grown in dexamethasone alone displayed immunoreactivity to DBH, and 87.8 ± 1.1% of them were immunohistochemically labeled with an antiserum to PNMT (Table I, Fig. 3c). This value is similar to the proportion of E-synthesizing cells observed in rats *in vivo* (Hillarp and Hokfelt, 1953; Eranko, 1955; Coupland, 1965a). Similar results were seen in cultures grown with 5 μM hydrocortisone or with the normally circulating rat hormone, corticosterone. To test the specificity of the glucocorticoid effect, cells were grown for 3 to 4 weeks in the same concentration of a different steroid hormone, progesterone. No PNMT immunoreactivity or E synthesis were observed (data not shown).

When chromaffin cells from newborn rats were grown in the absence of corticosteroid, all of the cells displayed intense CA fluorescence (Fig. 3b). They also synthesized and stored CA as assayed biochemically (9.1 ± 1.9 fmol/cell, Table I). This amount is very similar to CA production in cultures containing corticosteroid (Table I). The CA production included little or no E (E/NE = 0.02 ± 0.02), as would be expected from the well documented dependence of PNMT on corticosteroid (for review, see Pohorecky and Wurtman, 1971). Growth in 20 mM potassium without glucocorticoid did not increase E synthesis (E/NE = 0.09 ± 0.04, $n = 3$). In 10^{-8} M corticosteroid, the E/NE ratio was intermediate between that in 10^{-6} M steroid and no hormone (0.91 ± 0.12, $n = 4$). None of the cells expressed immunohistochemically detectable levels of PNMT after growth in the absence of hormone (Table I, Fig. 3d). The cells still contained numerous large chromaffin granules, although the mean diameter was slightly smaller than in cells grown with dexamethasone (Table I, Figs. 3f and 4), and in many cells the granule cores were denser and more NE-like than those of cells grown in glucocorticoid (Fig. 3, e and f). Typical histograms of chromaffin cell granule diameters are shown in Figure 4. In the absence of corticosteroid a small number of chromaffin cells grew processes and became more neuronal in appearance. These cells remained brightly fluorescent, however, and contained chromaffin granules. Process outgrowth was frequently from cells in the vicinity of the rare fibroblast-like cells in the cultures, which might represent a source of NGF (Harper and Thoenen, 1980).

The most striking effect of the absence of corticosteroid was poor cell survival (Fig. 5). Chromaffin cell survival in cultures grown without dexamethasone for 4 to 6 weeks ranged from 3.4 to 37.8% (mean = 13.1 ± 3.5%, $n = 11$) of the survival of sister cultures grown with the steroid. This hormone dependence was not restricted to cells from 1- to 3-day-old rats: after 4 weeks growth without corticosteroid, the survival of chromaffin cells dissociated from 7-day-old rats was only 14% of that of sister cultures in dexamethasone. Survival in 10^{-8} M dexamethasone was intermediate between that in 0 and 5 μM corticosteroid. Glucocorticoids therefore appear to be required
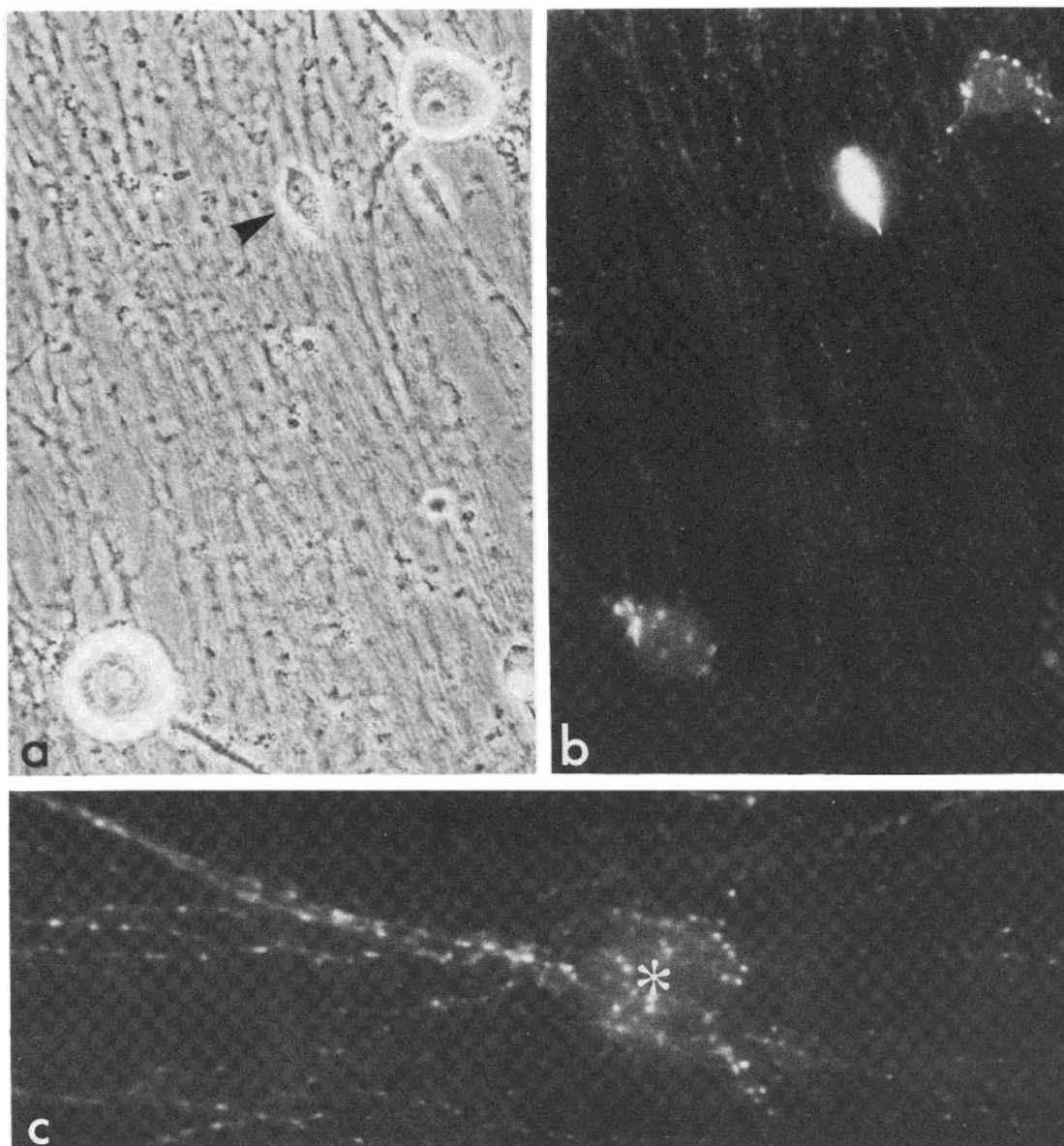
Figure 6. Adrenal medullary cells grown for 6 weeks in the presence of NGF. With NGF the cells extend neurites, and their cell bodies enlarge (*a*) and lose intense CA fluorescence (*b*). Note that the magnification in *a* and *b* is the same as for chromaffin cells in Figure 3. Because the camera is focused on the cell bodies, the dense layer of neurites underlying the cells is out of focus. The occasional chromaffin cells which do not respond to NGF (*arrowhead in a*) retain intense CA histofluorescence (*b*). Magnification × 420. *c*, At higher magnification, the chromaffin cell-derived neurons are seen to have brightly fluorescent neurites. Numerous brightly fluorescent varicosities which resemble synaptic boutons surround the pale cell bodies. ∗, pale cell soma. Magnification × 670.

for long-term survival of chromaffin cells as well as for maintenance of E synthesis.

Chromaffin cells from adult rats were also grown in dissociated cell culture. In the presence of 5 μM dexamethasone they maintained the same differentiated characteristics as described for cells from neonates: all the cells had intense CA fluorescence, the ratio of stored E to NE was 4.1, the chromaffin granules had large diameters, and approximately 80% of the cells had immunohistochemically detectable PNMT. Without dexamethasone, these cultures showed no E synthesis or PNMT staining. The long-term survival of cells from adults was also dependent on corticosteroid, although cell number did not fall as rapidly as that of chromaffin cells from neonates: after 3 weeks, there were 50.2 ± 2.3% ($n = 2$) as many cells in cultures from adults without hormone as in cultures with dexamethasone, and after 6 weeks this decreased further to 36.8 ± 2.0% ($n = 4$). This is compared to an average survival of cells from neonates cultured in the absence of glucocorticoid of 26.2% after 3 weeks and 8.8% after 6 weeks.

NGF effects. To examine the effects of long-term NGF administration, 1.0 μg/ml of NGF was added to newly plated cultures of neonatal chromaffin cells in the absence of dexamethasone. This concentration of NGF is saturating for survival and differentiation of sympathetic neurons in dissociated cell culture (Chun and Patterson, 1977a). The adrenal medullary cells were examined after 4 to 6 weeks in NGF. Cell survival in the presence of NGF was generally higher than that in chromaffin cultures grown in the absence of steroid and without NGF: cell number with NGF was 164.1 ± 28.0% ($n = 9$) of that in cultures without NGF. Most of the chromaffin cells which did not respond to NGF died, such that more than 90% of the cells surviving after 4 to 6 weeks in NGF had neurites. Furthermore, these cells were indistinguishable from mature sympathetic neurons as judged by morphological criteria and by biochemical characteristics such as transmitter enzymes and immunological markers. Their cell bodies had increased markedly in size and had acquired the characteristic phase microscopic appearance of neu-
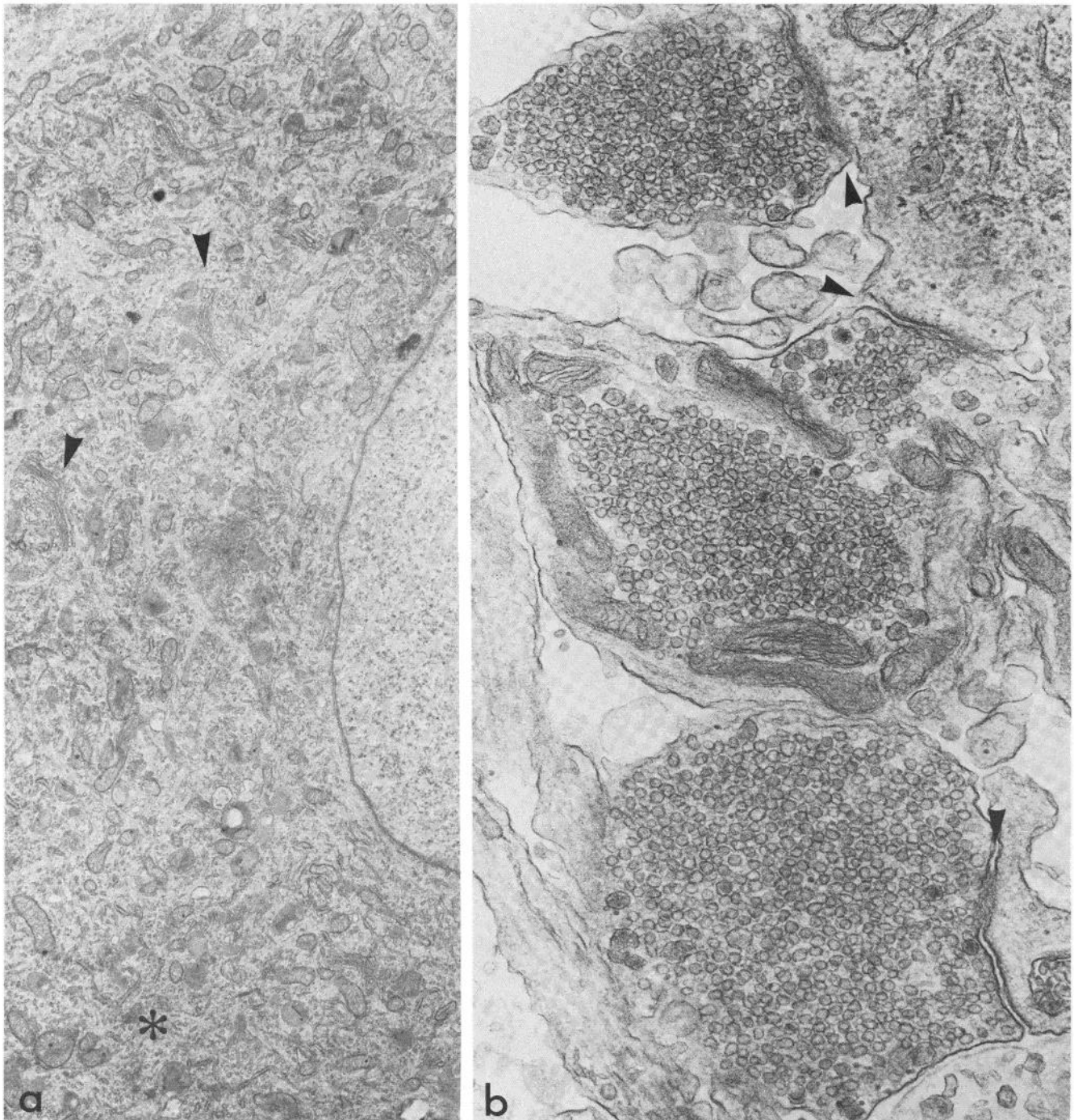
Figure 7. Ultrastructure of chromaffin cell-derived neurons. *a,* The cell somas have abundant rough endoplasmic reticulum (∗) and Golgi (*arrowheads*) and no longer contain catecholamine storage granules. *b,* The neurites have varicosities containing numerous small synaptic vesicles interspersed with occasional neuronal large granular vesicles, and they form morphological synapses with typical pre- and postsynaptic specializations (*arrowheads*). Magnification × 43,700.

rons (Fig. 6*a*). When reacted for GOA-induced fluorescence, these neuronal cell bodies were no longer intensely fluorescent (Fig. 6, *b* and *c*). In contrast, the neurites (Fig. 6, *b* and *c*) possessed numerous brightly CA-fluorescent varicosities, as do the neurites of cultured sympathetic neurons. Cultures with NGF also contained a small number of chromaffin cells and incompletely converted, process-bearing chromaffin cells that still displayed intense CA fluorescence in their cell bodies (Fig. 6, *a* and *b*).

After 4 to 5 weeks in culture with NGF, the chromaffin cell-derived neurons also displayed a characteristic neuronal ultrastructure (Fig. 7). Perikarya contained abundant rough endoplasmic reticulum and

numerous Golgi bodies but few or no chromaffin granules (Fig. 7*a*). The neurites contained microtubules and smooth endoplasmic reticulum typical of sympathetic axons. Varicosities filled with small, clear synaptic-size vesicles and occasional larger neuronal dense-cored vesicles were present (Fig. 7*b*). The chromaffin cell-derived neurons also formed numerous morphologically specialized synapses with each other. These synapses possessed prominent pre- and postsynaptic membrane thickenings typical for cultured sympathetic principal neurons (Fig. 7*b;* Rees and Bunge, 1974; Landis, 1976; Buckley and Landis, 1983).

Most of the cells with neuronal appearance showed no detectable
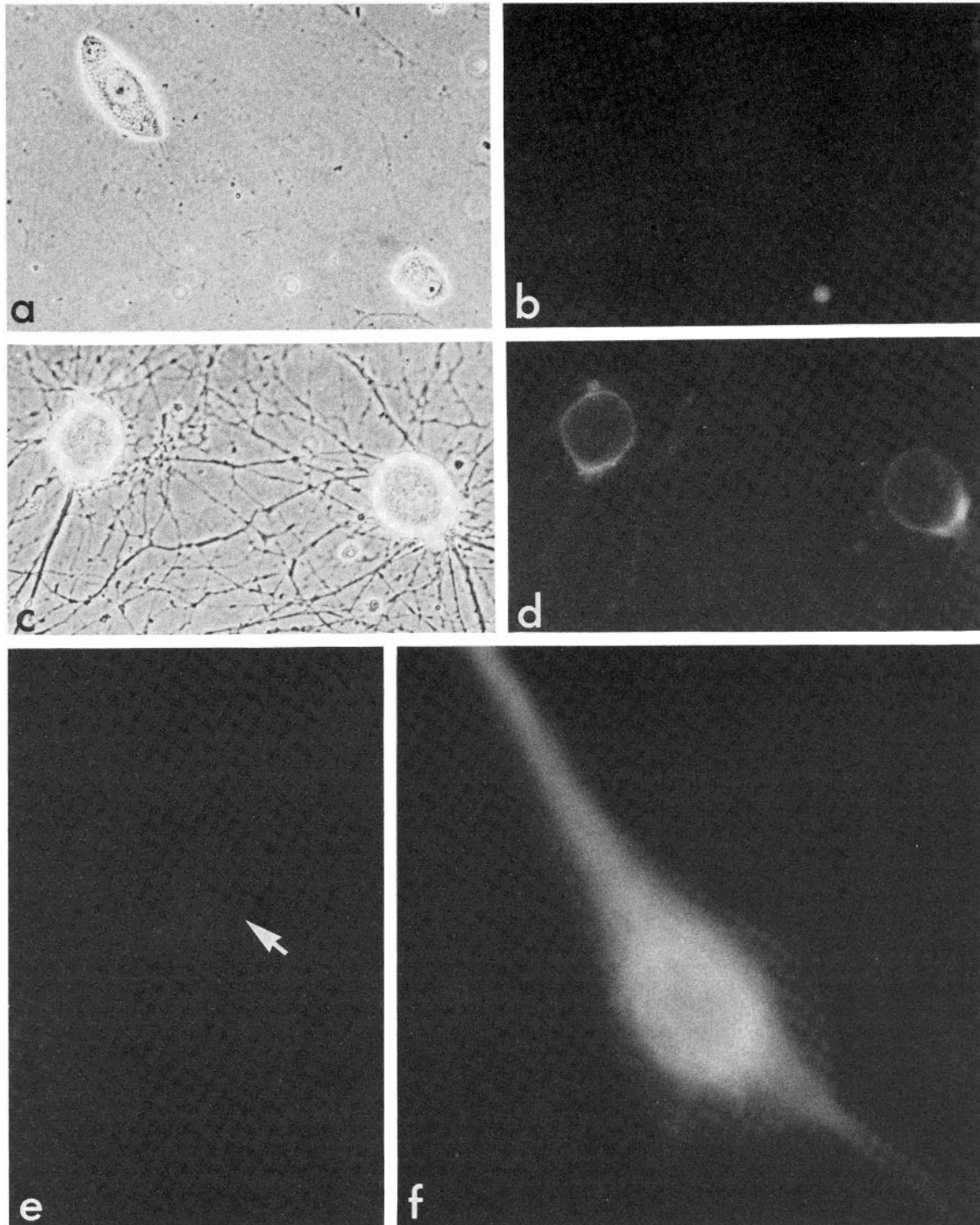
Figure 8. Neuronal markers acquired by adrenal chromaffin cells grown in the presence of NGF. Indirect immunofluorescence of live chromaffin cells (*b*) or chromaffin cell-derived neurons (*d*) stained with the monoclonal antibody ASCS4, and the corresponding fields with phase optics (*a* and *c*). Magnification × 420. Indirect immunofluorescent staining with an antibody to the 140-kD neurofilament subunit (*e, f*). *e,* Chromaffin cells grown for 6 weeks in dexamethasone. *f,* Chromaffin cell-derived neurons after 6 weeks in NGF. The cell in *e* was not visible at all if exposed as in *f*. In *e* exposure time during printing was therefore decreased four times to make the faint outline of the chromaffin cell apparent (*arrow*). Magnification × 1060.

PNMT immunoreactivity, even if dexamethasone was added to the culture medium with NGF for 2 days prior to the immunohistochemical staining to increase the level of enzyme (data not shown). In contrast, addition of dexamethasone did reveal PNMT immunoreactivity in incompletely transformed chromaffin cells with neurites. E synthesis and storage from [^{14}C]tyrosine were undetectable (<0.15 fmol/cell) in cultures of chromaffin cell-derived neurons.

The chromaffin cells that responded to NGF acquired other neuronal markers. The monoclonal antibody, ASCS 4 (Sweadner, 1983), which recognizes a determinant on NGF-inducible large external (NILE) glycoprotein (McGuire et al., 1978) and labels sympathetic neurons, did not label any chromaffin cells grown in dexamethasone (Fig. 8, *a* and *b*). In contrast, this antibody stained all chromaffin cell-derived neurons (Fig. 8, *c* and *d*). Similarly, tetanus toxin binding,
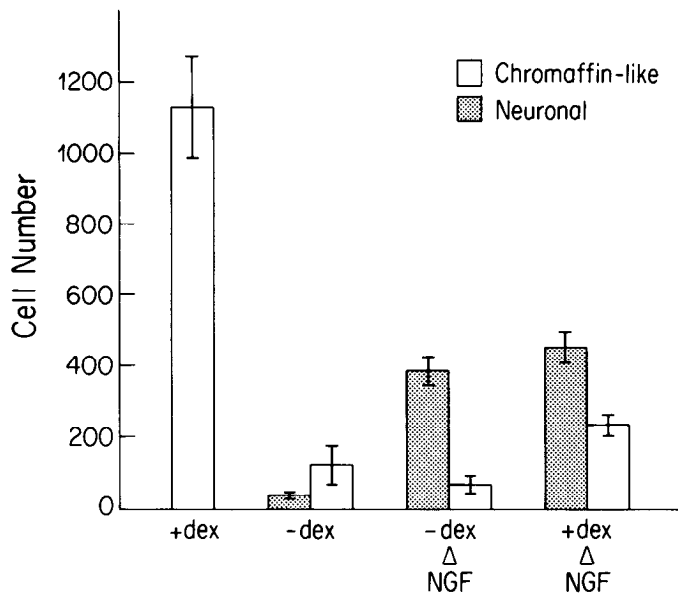
Figure 9. Conversion of chromaffin cells into neurons after growth for 1 week *in vitro* in the absence of NGF. Sister cultures initially containing the same number of cells were grown for 4 weeks in 5 μM dexamethasone (*+dex*) or without dexamethasone (*−dex*), or grown for 1 week in the presence or absence of steroid and then switched to medium containing NGF and no steroid (*−dex* Δ *NGF* and *+dex* Δ *NGF*). In this experiment medium with NGF also contained 20 mM potassium which enhanced survival in the presence of NGF but did not change the result qualitatively. Similar results were obtained in three other experiments in which cultures were switched to medium containing only NGF. *Hatched bars* represent cells with the phase microscopic appearance of neurons, and *open bars* represent small chromaffin-like cells. In this experiment, chromaffin cell cultures grown without steroid contained a small number of neuronal cells. This was occasionally observed; these cells generally had small chromaffin granules when examined ultrastructurally. *Numbers* represent counts of triplicate cultures ± SEM.

which was assayed with indirect immunofluorescence, is known to be a property of many neurons in culture (Raff et al., 1979). Ninety to 95% of chromaffin cells showed no immunofluorescent tetanus toxin labeling, either at the time of plating or after 4 weeks in steroid. Tetanus toxin binding was present, however, on all chromaffin cell-derived neuronal cell bodies and processes (data not shown; see also Lietzke and Unsicker, 1983). Finally, a rabbit antiserum which recognizes the 140-kD subunit of neurofilament protein (Drager et al., 1983), did not bind to chromaffin cells grown in dexamethasone. In contrast, all chromaffin cells that had responded to NGF displayed prominent immunofluorescence of a dense filamentous network in cell bodies and neurites (Fig. 8, *e* and *f*). Thus, by all criteria examined to date, these cells appear indistinguishable from mature sympathetic neurons.

We pursued three lines of experimentation to investigate whether the cells which became neurons in these cultures were originally chromaffin cells or instead represented a small, pre-existing population of medullary neurons which survived and differentiated in the presence of NGF.

i. Newly plated chromaffin cells (1 to 2 days *in vitro*) were examined for CA histofluorescence. More than 98% of these chromaffin cells showed intense CA fluorescence, in contrast to superior cervical ganglion neurons from young rats, which all lack bright CA fluorescence when assayed after 1 to 2 days in culture (see Doupe et al., 1985). In sister cultures of chromaffin cells grown for 4 to 6 weeks in the presence of NGF, however, virtually none of the cells had intense CA fluorescence, although almost half of the cells originally plated had survived.

ii. The medullary cell cultures were grown in the absence of NGF for 1 week, by which time it is likely that any NGF-dependent neurons

would have died (Chun and Patterson, 1977b). Nonetheless, when the cultures were then switched to NGF-containing medium, many of the cells grew neurites, lost their intense fluorescence, and became neuronal by the criteria described earlier (Fig. 9). This neuronal conversion occurred whether or not dexamethasone was included during the first week of culture, although cultures grown for 1 week with 5 μM dexamethasone before the switch to NGF subsequently contained more chromaffin cells than did cultures grown without dexamethasone for the first week (Fig. 9).

iii. One to 2 weeks after NGF addition, individual cells were observed which had both neuronal and chromaffin cell characteristics (see also Olson, 1970; Unsicker and Chamley, 1977; Unsicker et al., 1978). For instance, some cells with neurites had intense CA fluorescence in the cell body as well as in neurites and growth cones (Fig. 10). Moreover, at the ultrastructural level (Fig. 11), chromaffin cells with processes had abundant free polyribosomes, Golgi bodies, and rough endoplasmic reticulum. Their cell bodies still contained large chromaffin granules, intermingled with clusters of small synaptic vesicles. Many of the small vesicles had dense cores after glutaraldehyde fixation (Fig. 11*b*), in contrast to vesicles in older cultures and in mature sympathetic neurons (Rees and Bunge, 1974; Landis, 1976). We observed numerous lysosomes in cell bodies and varicosities, and many of the lysosomes contained granule-like inclusions. The neurites of chromaffin cells grown in NGF also contained both chromaffin granules and small synaptic vesicles, although there were few of the very large granules. The NGF-induced conversion was not completely synchronized: within cultures of the same age, some cells contained numerous large chromaffin granules whereas others had fewer vesicles and more synaptic vesicles than granules. Overall, however, the population of vesicles in individual cells shifted progressively toward smaller vesicle diameters (Fig. 11, *a* and *b*). The distribution of vesicle diameters in two representative cells from cultures at two different times after NGF addition is shown in Figure 12. Many cells appeared to pass through a stage in which they resembled type I SIF cells. They contained predominantly chromaffin-like vesicles of an intermediate size, similar in appearance to those of type I SIF cells *in vivo* (Siegrist et al., 1968; Lever et al., 1976), as well as occasional clusters of small synaptic vesicles (Figs. 11*b* and 12).

After 4 to 6 weeks with NGF, most cells had no chromaffin granules in their cell bodies and processes, and only small synaptic vesicles and occasional large neuronal-type granular vesicles in their varicosities. Morphologically specialized synapses were not observed until cells had been cultured for 3 to 4 weeks with NGF. The small number of partially transformed cells remaining in older cultures also contained predominantly vesicles of the type I SIF cell size as well as small synaptic vesicles.

To test whether the effect of NGF was reversible, NGF was withdrawn 1 or 3 weeks after plating and 5 μM corticosteroid was added back to the culture medium. The percentage of cells with neurites fell rapidly to zero. Similar results have been reported by Lillien and Claude (1983). In fact, in the cultures described here most of the neurite-bearing cells appeared to die after NGF withdrawal. Sequential cell counts showed that the number of cells which survived after NGF withdrawal and dexamethasone addition was equal to or less than the number of chromaffin cells without processes present in the cultures at the time of NGF withdrawal (Fig. 13). Thus, it is possible that the only cells that were still NGF-independent and/or responsive to glucocorticoid were the cells that had not yet responded to NGF with neurite outgrowth.

Effects of neurite-promoting factors. In our cultures of chromaffin cells from neonatal rats, the percentage of the cells originally plated that responded to NGF with neurite outgrowth and neuronal conversion varied from 10 to 40%. However, most of the chromaffin cells which did not respond to NGF died, such that only a small percentage (3 to 12%) of the cell population after 4 to 6 weeks in NGF consisted of chromaffin-like cells. It is not clear why some chromaffin cells failed to respond to NGF with neurite outgrowth. In order to
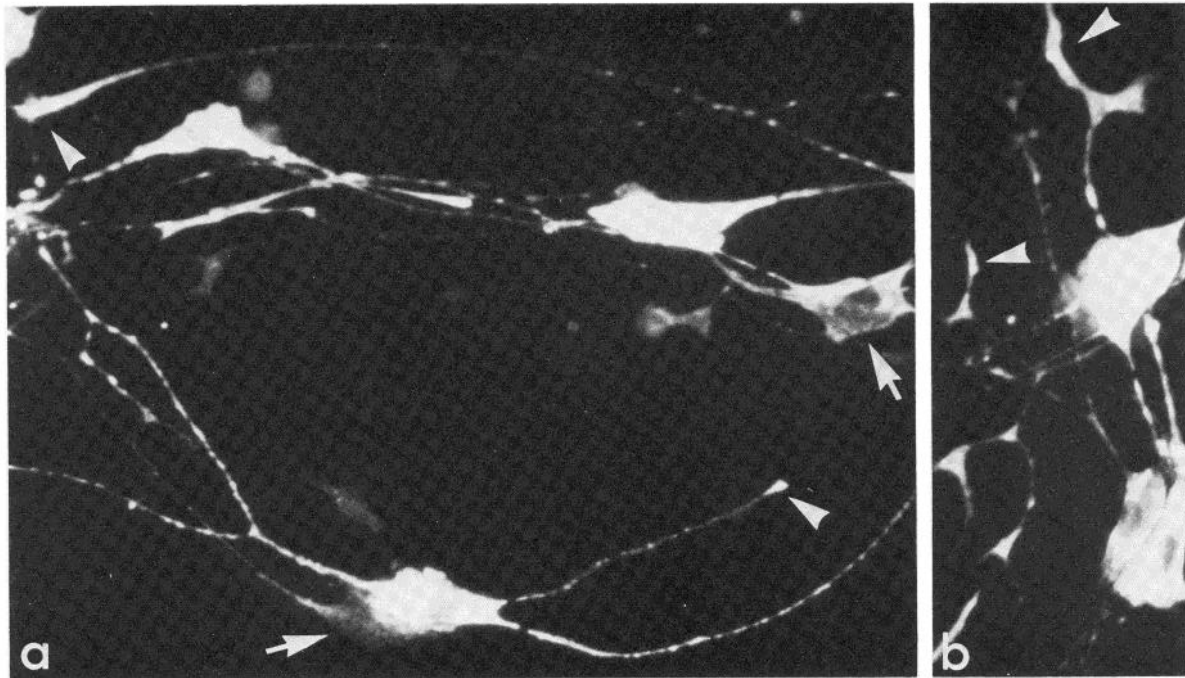
Figure 10. CA fluorescence of transitional chromaffin cells after 1 week in the presence of NGF. Note in *a* that some cells have intense CA histofluorescence in their cell bodies as well as their neurites, whereas other cells (*arrow*) have already lost this cell soma fluorescence but have very bright neurites or growth cones. In *a* and *b* fluorescent growth cones are indicated by *arrowheads*. Magnification × 420.

increase the percentage of cells responding to NGF, we added serum-free heart cell CM (Fukada, 1980) to the NGF-containing medium. Various CMs are known to contain factors which enhance neurite outgrowth of peripheral neurons (Collins, 1978; Coughlin et al., 1981; Edgar and Thoenen, 1982; Lander et al., 1982). In the presence of NGF, heart cell CM doubled the percentage of chromaffin cells that responded to NGF, compared to sister cultures without CM. Neurites also appeared 1 to 2 days earlier in the presence of CM (Fig. 14). When chromaffin cells were plated on a culture substrate coated with ECM from bovine corneal endothelial (BCE) cells and fed medium containing 50% CM as well as NGF, $86.1 \pm 1.2\%$ ($n = 2$) of the cells responded with neurite outgrowth.

This increase in conversion did not appear to be due to the presence of large amounts of NGF in the CM, as NGF levels in the × 10 concentrated CM were less than 2.6 ng/ml, as detected by a one-site radioimmunoassay (performed by Dr. R. Murphy, Harvard University). Furthermore, although newborn sympathetic neurons plated in the presence of the same CM without added NGF initially extended neurites very rapidly, these neurites retracted and the neurons died within several days (data not shown). This is reminiscent of the neurite-promoting effects observed with CM from BCE cells (Lander et al., 1982) and from PC12 cells (Matthew et al., 1982). These factors act by binding to the culture substrate. Similarly, the activity in heart cell CM which enhanced chromaffin cell conversion could be seen by precoating the culture substrate with heart cell CM. In some cases, however, the conversion-enhancing activity that we observed with substrate-attached heart CM was only one-half to one-third as potent as CM in solution in the medium (data not shown).

The factor in heart cell CM that enhanced the NGF-induced conversion of chromaffin cells did not act as an NGF-like molecule for chromaffin cells. In the absence of NGF and in the presence of CM and an antiserum to NGF, none of the chromaffin cells converted into neurons. In contrast to sympathetic neurons, chromaffin cells also exhibited no NGF-independent neurite outgrowth and subsequent retraction. This result was observed both with heart cell CM and with BCE cell ECM. Some chromaffin cells did grow and maintain processes in CM without NGF, but virtually all of these cells retained

their small size, intense CA fluorescence, and large chromaffin vesicles. This outgrowth of processes may reflect the presence of very small amounts of NGF-like activity in the CM or associated with the occasional fibroblast-like cells in the cultures, since the processes were frequently observed to be from chromaffin cells lying on or near such non-neuronal cells.

Although the CM factor did not act as a nerve growth factor itself, it appeared to increase the responsiveness of chromaffin cells to NGF. In the absence of CM, increasing the NGF level above the usual concentration (1.0 µg/ml) did not increase the percentage of chromaffin cells converting to neurons. In contrast, with CM there was a 60 to 100% increase in the number of cells responding at all NGF concentrations (Fig. 15).

Heart cell CM had several additional effects on chromaffin cell conversion into neurons. NGF-induced neurite outgrowth in chromaffin cells is age dependent. Bovine chromaffin cells lose the ability to grow processes early in fetal development, although they retain NGF receptors (Naujoks et al., 1982). Tischler et al. (1982) showed that few, if any, adult rat chromaffin cells in dissociated cell culture respond to NGF with process outgrowth. When we grew adult rat chromaffin cells in NGF plus heart cell CM, however, the number of cells that grew processes was markedly increased (Fig. 16). Many of these cells progressed to a well differentiated neuronal phenotype. Their cell bodies enlarged and they grew long neurites (Fig. 17). They also lost their intense cell body CA histofluorescence and many of their chromaffin granules (Fig. 18a) and made morphologically specialized synaptic junctions (Fig. 18, c and d). Therefore, adult rat cells can respond to NGF if heart cell CM is also added to the medium. Compared to cultures from neonatal rats, however, a higher proportion of cells in these adult-derived cultures still had chromaffin granules mixed with small synaptic vesicles in their cell bodies as well as in their varicosities (Fig. 18b). Most of the granular vesicles in these incompletely converted cells were intermediate in size, similar to those of type I SIF cells (80 to 120 nm).

Corticosteroids and NGF appear to act in competition on NGF-induced outgrowth. Administration of micromolar corticosteroid along with NGF markedly decreased NGF-induced neurite outgrowth in the cultures of neonatal chromaffin cells (Fig. 19, *left*). This was
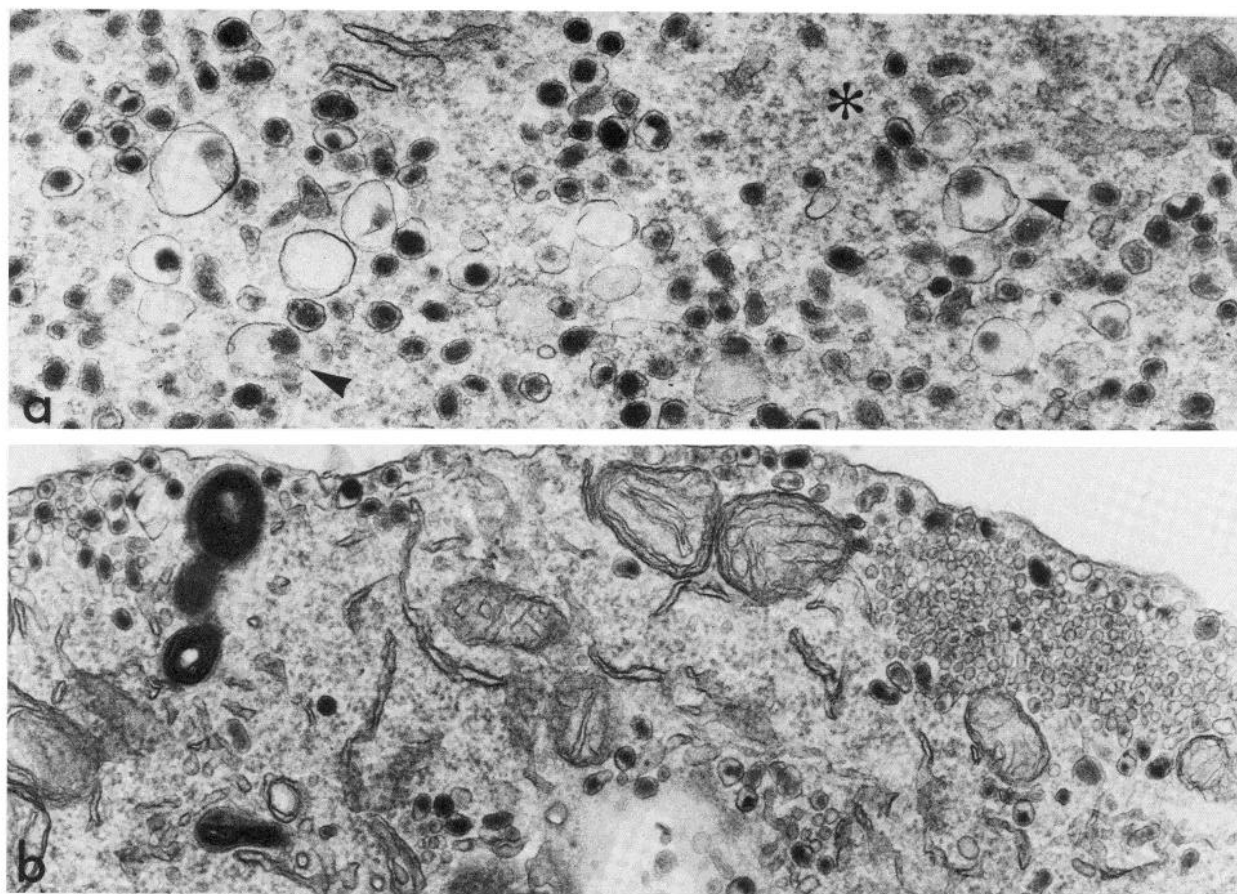
Figure 11. Ultrastructure of transitional chromaffin cells (glutaraldehyde-fixed), 5 days (*a*), or 9 days (*b*) after NGF addition. After 5 days in NGF, chromaffin cell bodies (*a*) have a mixed population of vesicles, which includes some large chromaffin granules (*arrowheads*) as well as intermediate sized ones. Cell bodies also contain abundant free polyribosomes (*). After 9 days in NGF, the number of CA vesicles in the cell bodies is decreased (*b*), and the vesicles present are largely small synaptic-sized vesicles or similar to the vesicles of type I SIF cells (80 to 120 nm). Many of the small synaptic vesicles have dense cores. Magnification × 43,700.

also reported by Unsicker et al. (1978) and by Lillien and Claude (1983). The absolute number of neurons in cultures grown with dexamethasone plus NGF was 3 to 4 times less than the number in cultures grown in NGF alone. The mechanism of the hormonal effect on neurite outgrowth is not known, although increasing concentrations of NGF seemed to increase its ability to compete with a fixed concentration of corticosteroid (Fig. 19, *left*). In agreement with the results of Lillien and Claude (1983), we frequently observed that some cells grew neurites after 4 to 6 weeks in NGF and steroid, although their neurite outgrowth had been markedly inhibited at 1 to 2 weeks in culture. Thus, part of the action of the glucocorticoid may be to delay neurite outgrowth, as suggested by Lillien and Claude (1983).

CM affects the interaction between corticosteroids and NGF. When chromaffin cells were grown with CM as well as corticosteroids and NGF, a larger number of neurons was present despite the presence of corticosteroid (Fig. 19, *right*). The extent of the decrease in dexamethasone inhibition of neurite outgrowth was somewhat variable. In some experiments, the corticosteroid did not inhibit the NGF-induced conversion at all in the presence of CM (data not shown). On average, cultures grown for 4 weeks in 5 μM dexamethasone as well as 1.0 μg/ml of NGF had 37.2 ± 10.2% (SEM; $n = 5$) as many neurons as sister cultures without glucocorticoid. In the presence of CM as well as NGF, cultures in steroid had 76.2 ± 13.6% ($n = 4$) as many neurons as cultures without the hormone. Here again, as with the percentage of neurite outgrowth from neonatal and adult cells, the CM may act to accentuate the NGF response.

Cholinergic induction. Adrenergic sympathetic neurons can be converted into cholinergic neurons by adding a factor from heart cell CM (Patterson and Chun, 1977; Weber, 1980). Chromaffin cells, although clearly able to convert into neurons, might nonetheless be committed to the adrenergic phenotype shared by chromaffin cells, SIF cells, and most sympathetic neurons. To test this, chromaffin cells from newborn rats were grown in the presence of cholinergic CM from cultured heart cells as well as NGF. In addition to the neurite-promoting activities described above, this serum-free CM is well known to contain a cholinergic factor (Fukada, 1980). The chromaffin cell-derived neurons did acquire cholinergic properties in these conditions. The cultures synthesized and stored labeled ACh from [³H]choline (Table II). The radioactivity co-migrating with cold ACh was further assessed in some experiments by acetylcholinesterase treatment of TX-100-permeabilized cholinergic cultures; this procedure eliminated 95% of the radioactivity in the ACh spot. CAT activity was present in homogenates (Table II) and was completely inhibited by the specific enzyme inhibitor naphthylvinylpyridine (data not shown). After cultures were fixed with potassium permanganate, which demonstrates endogenous CA stores as dense vesicular precipitate, samples of 100 or more terminals from each of three different areas of cholinergic cultures were counted. This revealed that more than a third of the terminals in these cultures contained only small clear vesicles and no small granular vesicles (Fig. 20*d*). Similar morphological evidence of cholinergic function in cultures of adrenal chromaffin cells has recently been reported by Ogawa et al. (1984), who also observed synaptic potentials with cholinergic pharmacology.

The cholinergic induction in chromaffin cell-derived neurons required NGF as well as CM, since little or no cholinergic function was seen in chromaffin cells grown with CM but without NGF. However, cell numbers in the absence of corticosteroid were low, so that a small amount of ACh synthesis might have gone undetected. Consistent with observations on cholinergic induction in sympathetic neurons (Walicke et al., 1977), depolarization made chromaffin cells resistant to cholinergic conversion (Table II, Experiment A). Furthermore, for sympathetic neurons as described by Wolinsky and Patterson (1984), rat serum in the absence of CM induced a small amount of cholinergic function in chromaffin cell-derived neurons. If

Figure 12. Distribution of vesicle diameters at two different times after NGF addition shows quantitatively the change illustrated in Figure 11. The *solid line* represents 5 days, and the *dashed line* represents 9 days. A representative cell was chosen from each time point for illustration. *n*, number of vesicles measured per cell.

chromaffin cells were switched to NGF and CM after 1 week in culture, they could still acquire cholinergic properties (Table II, Experiment B). Even adult rat chromaffin cells grown with NGF and CM for 6 weeks synthesized ACh in amounts similar to those in cholinergic neurons derived from newborn rat chromaffin cells (Table II, Experiment C).

The transmitter phenotype of transitional cells that had grown for only 1 to 2 weeks in cholinergic CM plus NGF was assayed morphologically using permanganate fixation. Many of these cells had small synaptic vesicles with the dense cores characteristic of CA storage (Fig. 20*b*). This observation suggests that the chromaffin cells may go through an adrenergic neuronal stage before becoming cholinergic. In older cultures, we also observed a number of terminals with a small percentage of small granular vesicles after permanganate fixation (Fig. 20*c*). These terminals are similar to those in single-neuron microcultures known to have both adrenergic and cholinergic properties (dual function neurons; Landis, 1976).

Discussion

These results demonstrate that glucocorticoid is required for the survival and maintenance of cultured adrenal chromaffin cells from both neonatal and adult rats in their differentiated state. In the absence of the steroid, many of the cells from young rats can respond to NGF by converting into neurons. This conversion is enhanced by the addition of a neurite-promoting factor, such that a higher percentage of chromaffin cells from neonates and even chromaffin cells from adult animals can become neuronal. When a cholinergic factor from heart cell CM is included as well as NGF, the cells can progress all the way from E-storing chromaffin cells to cholinergic neurons.

Newly plated chromaffin cells were well differentiated, as assessed by CA histofluorescence, PNMT immunoreactivity, and ultrastructure. PNMT immunoreactivity is known to be present in chromaffin cells *in vivo* at birth (Bohn et al., 1981), and although the CA content of the rat medulla increases postnatally, the ratio of E to NE storage is constant from newborn to adult animals (Eranko and Raisanen, 1957). The proportion of PNMT-immunoreactive cells in our newly plated cultures (approximately 85%) was also similar to the proportion of E-storing cells in adult rat adrenal medullae *in vivo* (Hillarp and Hokfelt, 1953; Eranko, 1955; Coupland, 1965a). The newly plated chromaffin cells from 1 to 3 day-old rats were smaller and had fewer CA storage vesicles than did adult rat cells, but the mean diameter of these vesicles (177 nm) was characteristically
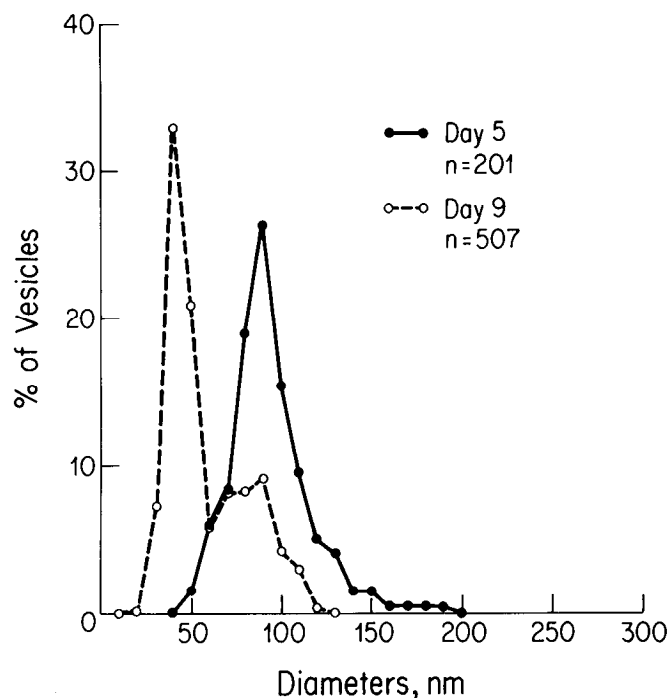

Figure 13. Effects of NGF withdrawal and corticosteroid replacement on total cell number *solid* (●) and number of neurons (O) 10 days after NGF addition (*B*) and 24 days after NGF addition (*C*). The *arrow* points to the day of NGF withdrawal. The progression of total cell number and of neuronal number in control cultures kept in NGF throughout the experiment is shown in *A*. In all conditions, all of the cells in duplicate cultures were counted and cell number was expressed as mean ± SEM.

Figure 14. Effect of non-neuronal CM addition on extent and rate of NGF-induced neurite outgrowth from neonatal chromaffin cells. Numbers represent the mean ± SEM of cells with neurites in each of three cultures counted at × 250 in the phase microscope on the indicated days after plating.

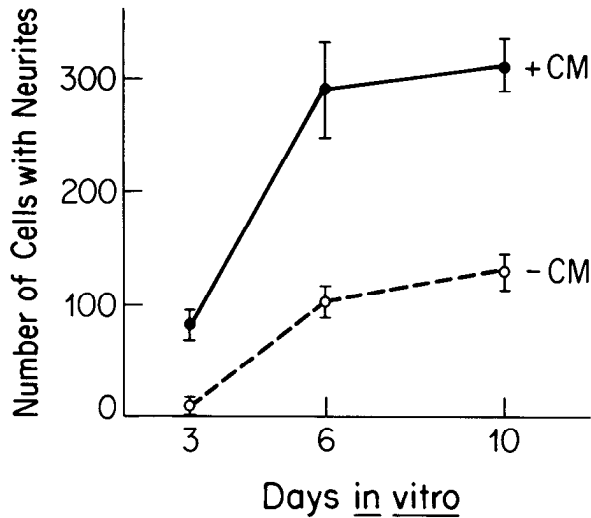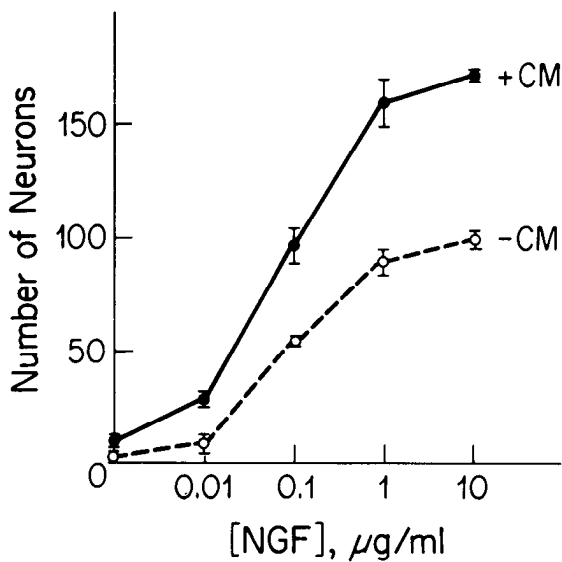Figure 15. The concentration dependence of NGF-induced neuronal conversion of chromaffin cells in the presence or absence of CM. Cells with neuronal phase morphology were counted in each of three 4-week-old sister cultures with or without CM, for each NGF concentration indicated. Numbers represent mean ± SEM.

large and similar to values reported for adult rat chromaffin cells *in vivo* (Coupland, 1965b) and in freshly dissociated cultures (Tischler et al., 1982). There was considerable variation among cells and among different platings, however. The means for individual cells ranged from 125 to 226 nm. This is also consistent with *in vivo* observations. The rat adrenal medulla matures morphologically during the first week of postnatal life. Many of the cells in newborn (immediately postnatal) rat medullae are immature: they contain intermediate-sized chromaffin storage granules (approximately 100 nm diameter) characteristic of immature chromaffin cells or SIF type I cells, and even in large vesicles many cores have the dense appearance associated with NE storage (Elfvin, 1967; El-Maghraby and Lever, 1980; Millar and Unsicker, 1981). By postnatal day 4 most of the cells have chromaffin granules of the same size as those of adult cells, and by postnatal day 8 the majority of cells also have vesicles with the ultrastructural appearance of E storage (Elfvin,
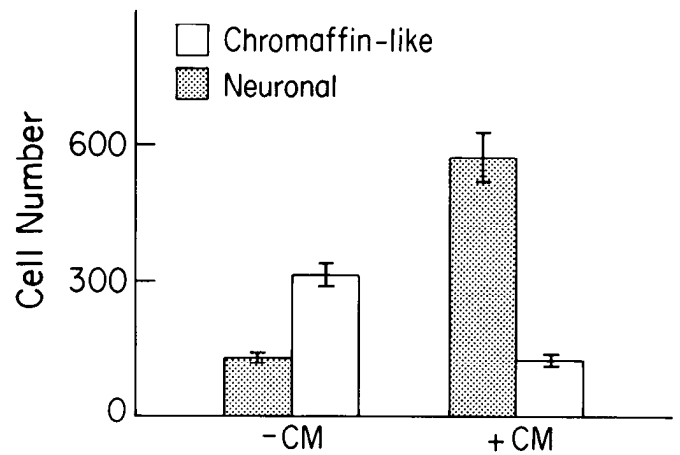

Figure 16. The effect of CM on NGF-induced neurite outgrowth of adrenal chromaffin cells from adult rats. Bars represent mean number of neuronal or chromaffin-like cells as assessed by phase morphology in each of three sister cultures with NGF and with or without heart cell CM.

1967; Millar and Unsicker, 1981). The variation between cells in our cultures from 1- to 3-day-old rats probably reflects the mixture of mature and immature morphological types in animals of these ages. As seen *in vivo* by Elfvin (1967), many of the granules in our newly plated cultures had the dense eccentric cores characteristic of NE storage (Coupland, 1965b). This is surprising, however, given that almost 90% of the cells were immunoreactive for PNMT, the enzyme for E synthesis. It is possible that PNMT, although present and increasing in amount (Bohn et al., 1981; Sabban et al., 1982), produced insufficient E accumulation to generate the characteristic granular and less electron-dense cores. By 2 days after plating many of the neonatal chromaffin cells grown in dexamethasone did possess E-like granules. It is also possible that the correlation between ultrastructural appearance and CA content does not always strictly hold (see also Doupe et al., 1985).

The chromaffin cell properties displayed in adrenal medullary cells grown with glucocorticoid for several weeks included intense CA fluorescence, large chromaffin granules, immunoreactivity for PNMT, and the synthesis and storage of CA, particularly E, as assayed biochemically. Previous workers reported that adrenal chromaffin cells from young rats were not able to maintain *in vivo* levels of PNMT or E synthesis for longer than 1 week in culture (Unsicker and Chamley, 1977; Unsicker et al., 1980; Muller and Unsicker, 1981). The cultures described here are different from those of previous studies in a number of ways. The cells were grown at relatively low density (500 to 1000 cells/cm^2), rat serum was included in the medium as well as fetal calf serum, and non-neuronal and non-chromaffin cells were virtually absent. Moreover, the synthetic corticosteroid dexamethasone was added to the culture medium. The amount of steroid added (5 μM) was similar to the level estimated to be in the adrenal medulla *in vivo*, which receives the corticosteroid-rich venous outflow from the surrounding adrenal cortex (Jones et al., 1977).

The factor(s) responsible for the development of separate E- and NE-storing populations in the rat adrenal medulla *in vivo* are not clear. A variety of early studies suggested a role of glucocorticoid (Shepherd and West, 1951; Margolis et al., 1966; reviewed in Doupe and Patterson, 1980). Recent studies of PNMT induction have demonstrated, however, that high levels of glucocorticoid are not required for the initial induction of PNMT during development (Bohn et al., 1981; Teitelman et al., 1982). There is as yet no candidate for another factor important in the earliest expression of PNMT, although the enzyme first appears *in vivo* as neural crest cells come in contact with the adrenal cortex (Teitelman et al., 1979; Verhofstad et al., 1979). After 4 to 6 weeks *in vitro* with 5 μM glucocorticoid, the percentage of chromaffin cells with immunohistochemically detect-
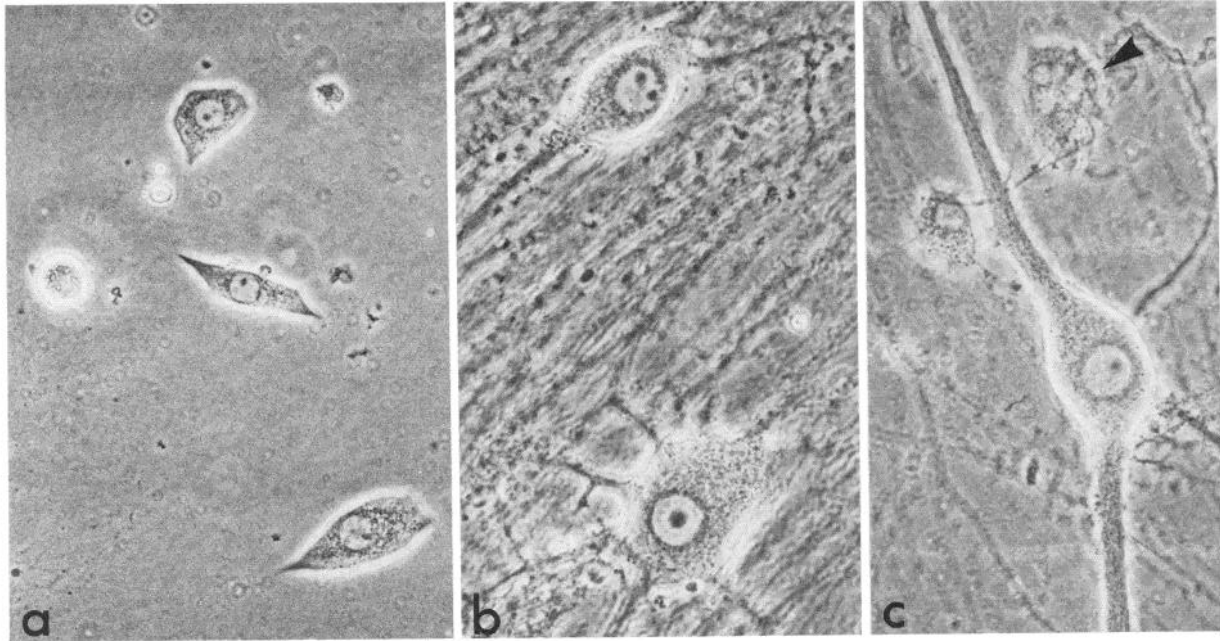
Figure 17. Phase appearance of adult rat chromaffin cells grown in the presence of dexamethasone (*a*) or in the presence of heart cell CM as well as NGF (*b*) for 6 weeks. Magnification × 420. As in neonatal cultures, occasional cells do not respond to NGF and remain small and chromaffin-like (*c*, *arrowhead*). Because the cell bodies in *b* and *c* are in focus, the dense layer of neurites underneath is slightly out of focus.

able PNMT in our cultures was approximately 85%, the same as that in newly plated medullary cells from neonates. Thus NE-containing chromaffin cells were apparently not induced to store E by these culture conditions. It is possible that the environment in the present studies did not contain the putative factor. Alternatively, approximately 15% of the chromaffin cells might have already become committed to the noradrenergic phenotype at birth and hence resistant to PNMT induction.

The ratio of E to NE storage in cultures from neonatal rats grown for 4 to 6 weeks with corticosteroid was similar to the ratios observed by Muller and Unsicker (1981) in freshly dissociated chromaffin cells from 10-day-old rats and by Tischler et al. (1982) in adult chromaffin cells grown *in vitro* with dexamethasone for 30 days. For comparisons between culture conditions and to other experiments, the ratio of E to NE synthesized by the cultures is the most reliable measure, since it is not influenced by variable cell yield, as is CA content. Inclusion of 20 mM potassium as well as glucocorticoid did not greatly increase the total amount of CA per cell but increased the ratio of E to NE to almost 5.0. This is very similar to the E/NE ratio observed in our cultures of adult chromaffin cells and to the ratio observed in freshly dissociated adult rat chromaffin cells (Tischler et al., 1982). In some culture situations, 20 mM K^+ can mimic the effect of depolarizing potentials (Walicke et al., 1977). Trans-synaptic activity has well documented stimulatory effects on CA-synthetic enzymes in the adrenal medulla, including PNMT (Molinoff et al., 1970; Ciaranello and Black, 1971; Otten and Thoenen, 1975). The effect of high potassium on E synthesis in culture suggests that it mimics the role of trans-synaptic activity in the maintenance of PNMT.

The mean diameters of chromaffin granules in adrenal medullary cells from neonatal rats cultured for 4 to 6 weeks with glucocorticoid was large (149 nm; range, 105 to 190 nm) and was similar to that of chromaffin cells *in vivo* (Coupland, 1965b). The majority of the granules had the granular cores characteristic of E storage. In some platings, however, the chromaffin cells had granule diameters that were smaller than those of mature cells *in vivo* and smaller than many of those of newly plated cells. This may reflect some variability in the initial population plated, the persistence of the intermediate-sized vesicles in the small number of cells plated that had immature

granules, or a decrease in vesicle size *in vitro*. Chromaffin granule diameter has been observed to decrease in numerous other studies using a variety of culture conditions (Unsicker and Chamley, 1977; Unsicker et al., 1978; Tischler et al., 1982). Some factor missing *in vitro* may be required for full maturation of all newly synthesized granules. Nonetheless, the majority of cells in our cultures displayed mature granule diameters after 4 to 6 weeks in culture. Therefore, these young rat chromaffin cells cultured in glucocorticoid maintain a range of differentiated characteristics which has not been observed in previous culture studies.

Chromaffin cells cultured without steroid had intense CA fluorescence and synthesized CA in amounts similar to those of sister cultures grown in dexamethasone. However, immunohistochemically detectable PNMT and the synthesis of E were not maintained in these cells. This is consistent with the *in vivo* dependence of adult PNMT on glucocorticoid for its maintenance (Wurtman et al., 1972; Ciaranello et al., 1978). Moreover, cells grown in the absence of glucocorticoid still possessed large chromaffin granules, but their mean diameter (113 nm) was slightly smaller than that of chromaffin cells grown in dexamethasone. Glucocorticoid thus appears to be required for maintenance of large chromaffin granules as well as for E synthesis and storage. These hormonal effects were specific to the glucocorticoids: similar maintenance of chromaffin cell differentiation was seen with hydrocortisone and with the normally circulating rat hormone, corticosterone, but not with progesterone.

The chromaffin cells also demonstrated a striking dependence on glucocorticoid for long-term survival in culture. Chromaffin cells from both adult and neonatal animals required the hormone, but cell loss occurred more rapidly in cultures of cells from neonates. Glucocorticoid levels undergo a number of changes in developing rats. The level of adrenocortical function starts to rise around embryonic day 17 (Margolis et al., 1966), at which time neural crest precursors are also beginning to invade the adrenal cortex. Corticosteroid levels then increase dramatically at term; serum corticoid levels are estimated to be in the micromolar range perinatally, and induce a variety of maturational changes (Liggins, 1976). Postnatally, hormone levels drop again and remain low until maturation of the hypothalamic-pituitary-adrenal axis (about postnatal day 19 in the rat). Lempinen (1964) showed that the normal disappearance of a variety of fetal
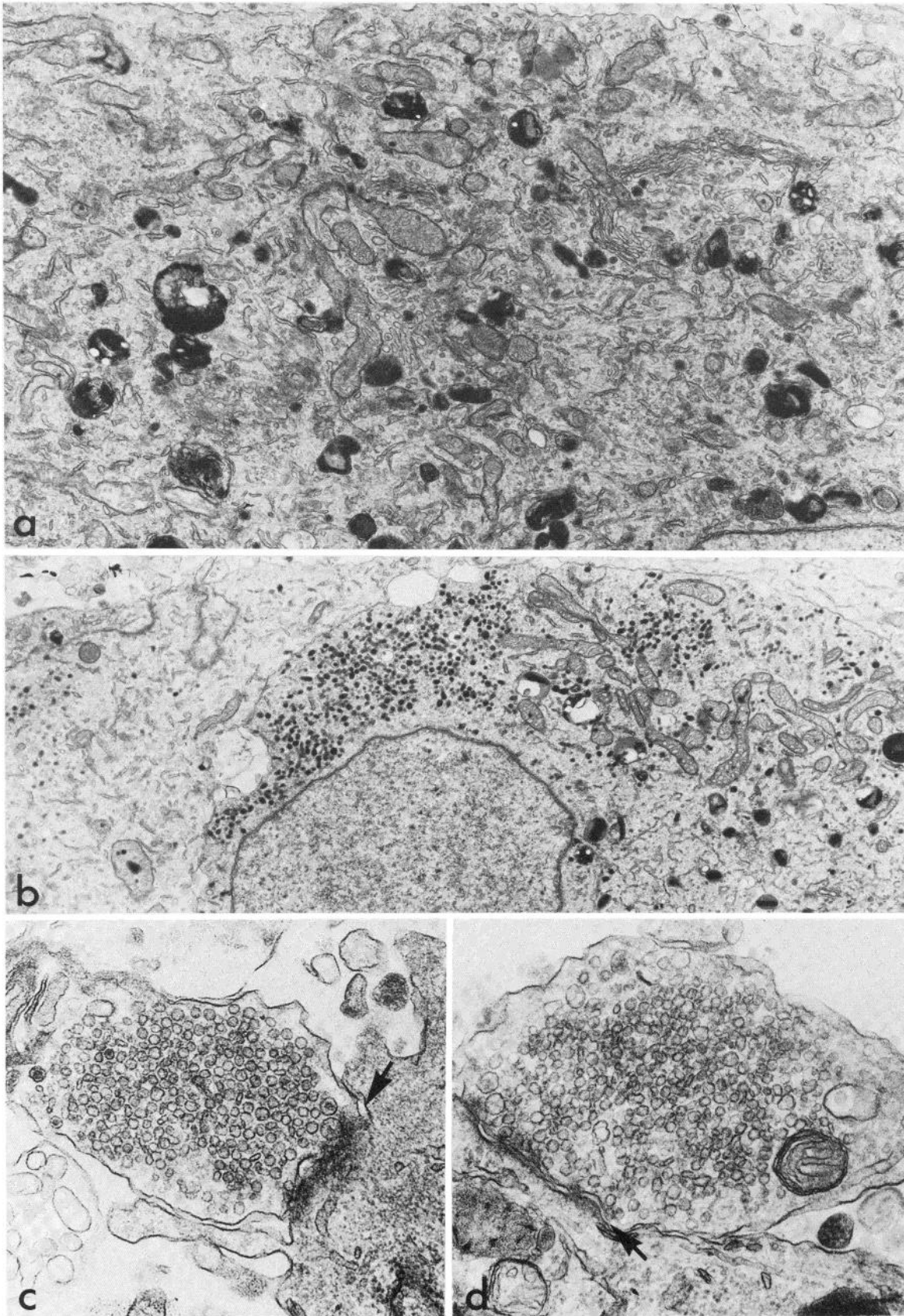
Figure 18. Ultrastructural appearance of adult rat chromaffin cells grown for 6 weeks in the presence of NGF and heart cell CM. Some cells are indistinguishable from neurons (*a*) whereas others remain in an intermediate stage similar in appearance to type I SIF cells (*b*). *c* and *d* show synapses formed by adult rat chromaffin cell-derived neurons, with typical synaptic membrane thickenings (*arrows*). Magnification × 43,700.

extra-adrenal chromaffin tissues, such as the organ of Zuckerkandl, correlated well with the postnatal drop in glucocorticoid, and that this disappearance could be prevented by hormone administration. Chromaffin cells of the adrenal gland, however, reside in an environment where corticosteroid levels are up to 100-fold higher than in the general circulation (Jones et al., 1977). Thus, even during the postnatal decrease, glucocorticoid levels in the adrenal circulation may still be relatively high. In culture, corticosteroid can be virtually eliminated. Our experiments thus revealed a marked dependence of chromaffin cells on glucocorticoid for survival. Other factors may also be missing in the cultures and enhance the observed dependence on glucocorticoid. Nonetheless, corticosteroid alone was able to maintain cell number at greater than 90% of the cells initially plated for many weeks.

When NGF was administered to adrenal chromaffin cells in the absence of corticosteroid, many cells that would probably have died
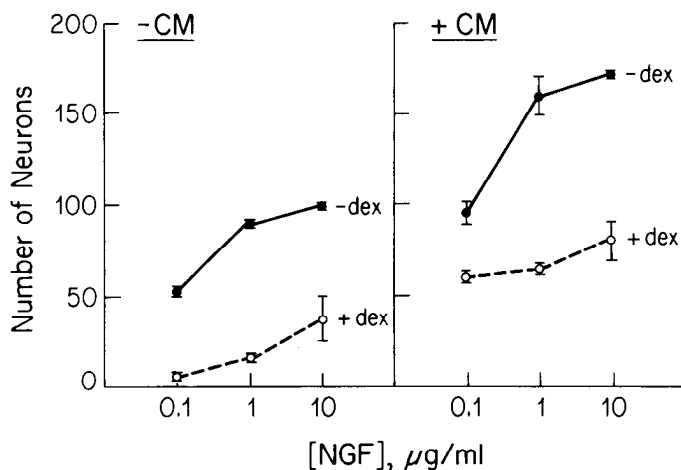

Figure 19. Effect of dexamethasone on NGF-induced neuronal conversion of chromaffin cells and effect of heart cell CM on the interaction of NGF and corticosteroid. The *left panel* shows the number of neurons (as assessed by phase morphology) in each of three sister cultures grown with the indicated concentration of NGF in the presence (O) or absence (●) of 5 μM corticosteroid. The *right panel* shows the same experimental conditions except that cultures contained 50% heart cell CM as well as NGF.

were rescued, grew neurites, and eventually became indistinguishable from mature sympathetic neurons. The criteria used for assessing neuronal conversion included: (*i*) the complete loss of intense cell body CA fluorescence, chromaffin granules, and E synthesis; (*ii*) the appearance of morphologically mature axons and synapses; and (*iii*) the acquisition of neuronal markers. These markers were tetanus toxin binding (Mirsky et al., 1978), ASCS4 labeling (Sweadner, 1983), and immunoreactivity to the 140-kD subunit of neurofilaments (Drager et al., 1983). The observation here of essentially complete neuronal conversion contrasts with earlier work in which rat chromaffin cells acquired some neuronal characteristics in response to NGF but maintained a number of characteristics of chromaffin cells, in particular, intense CA fluorescence and large chromaffin granules (Olson, 1970; Unsicker et al., 1978; Aloe and Levi-Montalcini, 1979). Our culture studies differ from those of Unsicker et al. (1978) in that, here, adrenal tissue was completely dissociated and single cells were grown at low density. Moreover, we grew our chromaffin cell cultures with NGF for several weeks longer than did other workers, and it is clear that the neuronal conversion is a slow process: at 1 to 2 weeks (the time studied by Unsicker et al., 1978) many cells still have characteristics of chromaffin cells, and synapses are not formed until cells have been exposed to NGF for 3 to 4 weeks.

Several lines of evidence show that these neurons are derived from chromaffin cells and not from the small population of preexisting neurons in the medulla (Aloe and Levi-Montalcini, 1980). Less than 2% of the cells in newly plated cultures lacked intense CA fluorescence, whereas a much higher percentage became neuronal. Moreover, if cultures were grown without NGF for a week to eliminate NGF-dependent neurons before adding NGF, many cells still became neurons. Finally, individual cells with characteristics of both neuronal and chromaffin cell phenotypes were observed at short times after NGF addition. Many of these transitional cells contained not only small, 50-nm vesicles and large chromaffin granules but also a population of intermediate diameter vesicles similar to those found in SIF cells. In explant cultures of postnatal rat medulla, Unsicker and Chamley (1977) also noted some similarities of neurite-bearing chromaffin cells to SIF cells. The mean vesicle size in the cells studied here gradually decreased with time in NGF, and vesicle diameters showed an intermediate stage (at 1 to 2 weeks after NGF addition) in which the majority of granules resem-

TABLE II

Cholinergic function in adrenal chromaffin cells

A, B, and C are three different experiments in which cholinergic function was assessed by measuring synthesis of ACh and CA from [³H]tyrosine and [³] choline, and by measuring CAT activity. Experiment A shows the cholinergic inducing effect of CM (−dex + CM + NGF) and the inhibitory effect of 20 mм potassium (−dex + CM + NGF + high K⁺). Experiment B shows cholinergic induction in chromaffin cells when CM is added immediately (−dex + CM + NGF) or when cells are grown with or without steroid for 1 week before switching to NGF plus CM (±dex Δ − dex + CM + NGF). Experiment C shows cholinergic function in chromaffin cells from adult rats. Values represent means of triplicate cultures ± SEM.

Experiment	ACh (fmol/cell)	ACh/CA	CAT (fmol/cell/20 min)
A. Neonates			
+ dex	0.07 ± 0.02	0.01 ± 0.01	0.09
−dex − CM + NGF	1.64 ± 0.60	0.07 ± 0.01	2.36
−dex + CM + NGF	62.12 ± 12.73	6.58 ± 0.57	22.71
−dex + CM + NGF + high K⁺	4.71 ± 0.53	0.23 ± 0.05	
B. Neonates			
+dex	0.0 ± 0.0	0.0 ± 0.0	
−dex	0.20 ± 0.21	0.02 ± 0.02	
−dex + CM + NGF	30.21 ± 3.93	3.62 ± 1.10	
+dex Δ − dex + CM + NGF	22.34 ± 14.12	3.39 ± 1.87	
−dex Δ − dex + CM + NGF	24.52 ± 6.35	3.15 ± 0.42	
C. Adults			
+dex	0.01 ± 0.01	0.01 ± 0.01	
−dex	0.02 ± 0.01	0.01 ± 0.01	
−dex − CM + NGF	0.85 ± 0.13	0.74 ± 0.18	
−dex + CM + NGF	91.23 ± 0.64	7.39 ± 1.83	
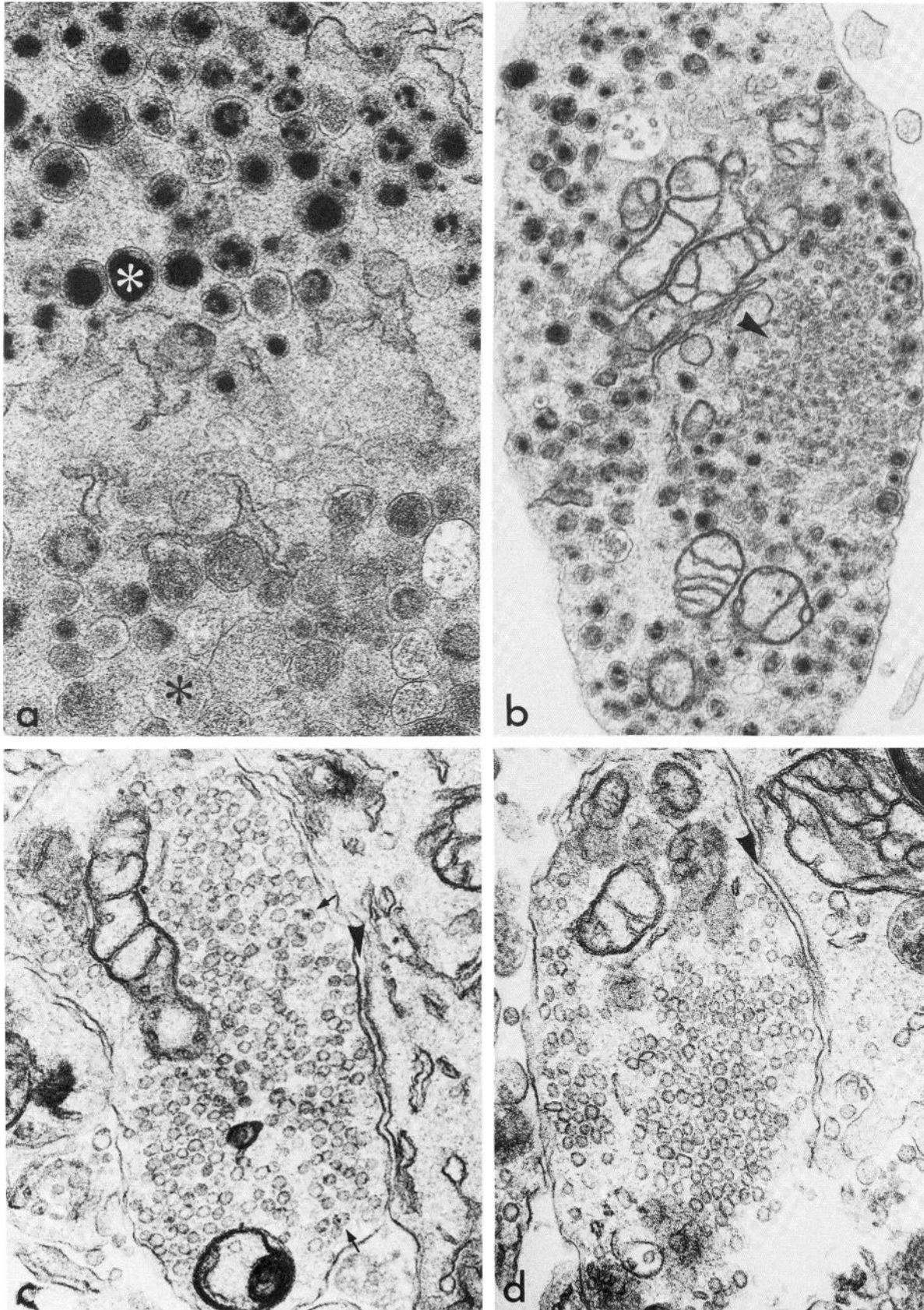
Figure 20. Ultrastructure of chromaffin cells and chromaffin cell-derived neurons after fixation in potassium permanganate. *a,* Characteristic appearance of chromaffin cells grown in dexamethasone; NE-storing cores (*white asterisk*) are dense, wherease E-storing cores (*black asterisk*) are pale and granular. *b,* Transitional cells grown for 1 to 2 weeks in CM and NGF have vesicles of a variety of sizes in their varicosities; note that small vesicles (*arrowhead*) as well as larger vesicles have dense cores. *c* and *d,* Synapses of chromaffin cell-derived neurons after 6 weeks in NGF plus CM. Some synapses (*c*) appear dual function (*arrows* point to occasional cores) and some (*d*) have only small clear vesicles and appear cholinergic. *Arrowheads* point to synaptic specializations. Magnification × 43,700.

bled those of Type I SIF cells in the superior cervical ganglion or those of the SIF-like small granule-containing cells of the adrenal medulla (Coupland et al., 1977, 1978). Vesicle diameter is one of a number of SIF cell properties which are intermediate between those of chromaffin cells and neurons. These observations on transitional phenotypes suggest that, rather than converting directly into sympathetic neurons, chromaffin cells may initially go through an SIF-like stage (see also Doupe et al., 1985). During development the immature chromaffin cells which initially populate the adrenal medulla (and also resemble SIF cells) gradually mature into chromaffin cells with large granules (Elfvin, 1967; Millar and Unsicker, 1981). Thus, the conversion of chromaffin cells into SIF-like transitional cells in the presence of NGF and the absence of corticosteroid may reflect the reversal of the normal developmental sequence undergone earlier by these cells.

Many but not all of the chromaffin cells from newborn rats can be converted into neurons by NGF alone. In contrast, essentially all of them will survive in the presence of corticosteroid. Unsicker et al. (1982) compared survival of chromaffin cells taken from rats of different ages grown with or without NGF. They proposed that NGF is an essential survival factor for chromaffin cells from rats less than 1 week old. Our results suggest a different interpretation: unlike glucocorticoid, NGF is not a chromaffin cell survival factor, but in the absence of corticosteroid, cells which can respond to NGF with neurite outgrowth are converted from corticosteroid dependence to NGF dependence instead, and thus survive. In vivo the effects of injection of an antiserum to NGF suggested that the developing adrenal medulla might go through an NGF-dependent phase prenatally (Aloe and Levi-Montalcini, 1979). In contrast, lowering fetal NGF levels by immunizing pregnant rats and guinea pigs with NGF did not noticeably affect the adrenal medulla (Gorin and Johnson, 1979, 1980). It is possible that the procedures used to manipulate NGF levels in vivo also influence endogenous glucocorticoid levels, and that this explains some of the differences between these studies.

In culture, when NGF was withdrawn and replaced by corticosteroid, the chromaffin cells with neurites did not readily revert to corticosteroid dependence, but died instead. In contrast, PC12 cells, which are derived from an adrenal medullary tumor and also grow processes in response to NGF, retract these processes when NGF is withdrawn (Greene and Tischler, 1976). Because of the apparent dependence of the chromaffin cell-derived neurons on NGF, it is difficult to assess independently of survival whether they can revert to a chromaffin phenotype. Nonetheless, one interpretation of the consequences of NGF withdrawal and corticosteroid replacement that we observed in vitro is that becoming a neuron is a terminally differentiated step in the sympathoadrenal lineage. In this context, one important difference between neurons and chromaffin cells is that differentiated chromaffin cells retain the ability to undergo mitosis in vivo (Jurecka et al., 1978), whereas mature sympathetic neurons are postmitotic (Hendry, 1977). In a number of developmental systems, loss of the ability to replicate occurs concomitantly with commitment to terminal differentiation (Sidman et al., 1959; Altman, 1969; Chi et al., 1975). One effect of NGF on chromaffin cells might be to eliminate their potential for mitosis. By analogy to other systems, chromaffin cell conversion into neurons could then be viewed as an irreversible commitment which occurs in conjunction with the loss of the capacity to divide.

Many cells which did not respond with neurite outgrowth to NGF alone did so when heart cell CM was added as well. The CM did not induce neuronal conversion in the absence of NGF but appeared to increase the responsiveness of chromaffin cells to NGF. A similar phenomenon has been observed by Edgar and Thoenen (1982), who showed that both total survival and NGF responsiveness of chick sympathetic neurons was increased by substrate-attached chick heart CM. In the present experiments, however, a marked shift in the half-maximal concentration of NGF was not observed. Unsicker et al. (1984) have recently described a neurite-promoting activity for chromaffin cells in C6-glioma CM, which was not blocked by an antiserum to NGF, but these workers did not assess the effects of CM and NGF in combination.

Some of the conversion-enhancing activity in CM could be preserved by precoating the culture substrate with CM. A variety of neurite-promoting factors found in other CMs share this property (Collins, 1978; Dribin and Barrett, 1982; Lander et al., 1982). The incomplete reconstitution of the activity of soluble CM seen here when it was bound to the substrate has several possible explanations. Binding of the CM to the substrate may not be complete; the factor may partially unbind from the substrate before initial neurite outgrowth from chromaffin cells, as this requires 2 to 3 days, in comparison to the 6- to 8-hr interval before process outgrowth from neurons (e.g., Lander et al., 1982); the CM factor may act in solution as well as on the substrate and thus may be present at a greater overall concentration when presented in solution; or CM may contain additional conversion-enhancing factors which are able to act in solution but are unable to bind to polylysine. Quantitative studies on neurite outgrowth have provided evidence for several components in CM (Henderson et al., 1981; Collins and Dawson, 1982; Dribin and Barrett, 1982).

The substrate-attached neurite-promoting factors described by Lander et al. (1982) and Matthew et al. (1982) are associated with a heparan sulfate proteoglycan fraction. The neurite-promoting factor in bovine corneal CM may exist as a complex of the proteoglycan and several other proteins (Lander et al., 1983). A monoclonal antibody to PC12 cell CM has been produced, which directly blocks the neurite outgrowth-promoting activity of that CM (Matthew and Patterson, 1983). Preliminary evidence (Doupe and Patterson, 1983) suggests that this antibody also blocks the substrate-attached activity in heart cell CM which enhances chromaffin cell conversion. In contrast, other monoclonal antibodies which recognize PC12 cell CM but do not block neuronal outgrowth (Matthew et al., 1982), as well as an antibody to Thy 1.1, do not block the enhancement of chromaffin cell conversion. Thus, it is likely that the substrate-bound molecule that acts on the phenotypic conversion of chromaffin cells is similar to a factor known to promote outgrowth from neurons. It is also unlikely that the conversion-enhancing activity is the same molecule as the cholinergic factor known to be present in heart cell CM, because the properties of the neurite-promoting factors as they have been described thus far (Coughlin et al., 1981; Lander et al., 1982, 1983) differ from those of the cholinergic molecule (Fukada, 1980; Weber, 1980).

Sympathetic neurons initiate NGF-independent neurite outgrowth in the presence of bovine ECM, PC12 cell CM (Lander et al., 1982; Matthew et al., 1982), and the heart cell CM described here. In contrast, chromaffin cells on bovine ECM or in the presence of heart cell CM did not spontaneously grow processes in the absence of NGF and then retract them. One difference between chromaffin cells and neurons that might be relevant is that sympathetic neurons form newborn rats have presumably been exposed to and "primed" by NGF in vivo. That is, they have already undergone some NGF-dependent RNA transcription and protein synthesis required for neurite outgrowth. NGF-induced neurite outgrowth by primed PC12 cells and sympathetic neurons is rapid and independent of RNA synthesis (Partlow and Larrabee, 1971; Burstein and Greene, 1978; Greene et al., 1982). Adrenal chromaffin cells, on the other hand, may not have been exposed to significant levels of NGF in vivo. Alternatively, the high concentrations of glucocorticoid in their normal environment may have prevented their response to local NGF. They would therefore not be primed for neuronal differentiation and hence might require the initial action of NGF on transcription as well as the neurite-promoting activity of CM for initial neurite outgrowth. In support of this idea is recent evidence that spontaneous neurite outgrowth of PC12 cells in the presence of bovine ECM does depend on NGF priming (Tomaselli and Reichardt, 1984; but see also Fujii et al., 1982).

Adult rat chromaffin cells are much less NGF responsive than are chromaffin cells from young rats (Tischler et al., 1982). Bovine

chromaffin cells also lose the ability to respond to NGF early in fetal life (Naujoks et al., 1982). The administration of heart cell CM, however, greatly increased the NGF responsiveness of adult rat adrenal medullary cells. Thus, these cells have not been irreversibly committed to the chromaffin phenotype, although they require a combination of factors in order to convert into neurons. The idea that the substrate factor interacts with NGF receptors (Edgar and Thoenen, 1982) suggests one possible explanation for the CM effect. The NGF receptors of adult chromaffin cells might have become unresponsive due to continued development and chronic exposure to high glucocorticoid. These receptors, then, require the extracellular matrix molecule(s) to reactivate the NGF response. A role for extracellular factors might also explain why NGF induces tyrosine hydroxylase in the adult adrenal medulla *in vivo* (Otten et al., 1977) but not in dissociated cell culture (Naujoks et al., 1982; Tischler et al., 1982).

Glucocorticoid in micromolar concentration markedly inhibited and delayed NGF-induced neurite outgrowth from chromaffin cells. This has also been reported by Unsicker et al. (1978) and by Lillien and Claude (1983). However, in contrast to Unsicker et al. (1978), and in agreement with others (Fukada, 1980), we observed no effect of corticosteroid on neurite outgrowth of sympathetic neurons. It is not clear how corticosteroids and NGF interact. Corticosteroid is known to increase the affinity of epidermal growth factor receptors (Baker et al., 1978). However, corticosteroids also have direct effects on gene expression and, thus, might interact with the NGF response at that level. In these experiments, inhibition by dexamethasone became somewhat less effective as the NGF concentration was increased, suggesting a competitive interaction. Thus, glucocorticoid not only promotes chromaffin cell survival and differentiation but also can compete with and even override the NGF signal promoting neuronal differentiation.

Inclusion of heart cell CM in the medium greatly complicated the inhibitory action of corticosteroid on the NGF response. In some cases, dexamethasone was completely ineffective in the presence of CM. In other cases there were more neurons present in CM than in its absence, but dexamethasone still reduced the number seen compared to CM plus NGF alone. There are many possible explanations for this result, including the presence of factors in the crude CM which directly complex with the hormone or inhibit its binding to the cells. Alternatively, because CM may act on NGF receptors, its action might be to increase the ability of a given amount of NGF to compete with glucocorticoid. Regardless of the mechanism, it is clear that the complexity of the interactions between NGF, glucocorticoids, and CMs must be considered in the interpretation of experiments on these factors.

Adrenergic sympathetic neurons can be induced to become cholinergic in culture by addition of a protein from heart cell CM. This transition was originally described *in vitro*, but recent evidence strongly suggests that a similar transition occurs during the normal development of cholinergic sympathetic neurons *in vivo* (Landis and Keefe, 1983; Yodlowski et al., 1984). The addition of the cholinergic factor as well as NGF to adrenal chromaffin cells from neonatal or adult rats resulted in the acquisition of cholinergic characteristics. These included CAT activity, the synthesis and storage of ACh, and the presence of numerous terminals containing exclusively small clear vesicles after fixation with potassium permanganate. Similar results have been reported by Ogawa et al. (1984), who further presented evidence for cholinergic synaptic potentials in cultures of adrenal chromaffin cells.

The cholinergic induction described here required NGF as well as CM. Another similarity to cholinergic induction in sympathetic neurons (Walicke et al., 1977) is that growth in depolarizing agents stabilized the adrenergic phenotype in chromaffin cell-derived neurons and prevented the cholinergic effect of CM. In terms of the relationships between the different phenotypes of the sympathoadrenal lineage, it is important to note that transitional cells grown for 1 to 2 weeks in CM and NGF had numerous small dense-core vesicles after permanganate fixation. This suggests that chromaffin

cells go through an adrenergic neuronal and/or dual function stage before becoming cholinergic (see also Doupe et al., 1985). The fact that even chromaffin cells from adult rats acquired cholinergic properties raises the possibility that adrenal chromaffin cells used as long-term neuronal grafts in the central nervous system (Freed et al., 1981) might also lose their catecholaminergic phenotype in certain locations in the brain.

Adrenal chromaffin cells show a remarkable plasticity in response to environmental factors. In the presence of NGF, extracellular matrix factors, and cholinergic CM, many of them can convert into cholinergic sympathetic neurons, apparently passing through intermediate stages in which they resemble other cell types of the sympathoadrenal system such as SIF type I cells and noradrenergic neurons. The analysis of these phenotypic interconversions *in vitro* shows that the transformation involves induction of individual cells and not selection for a population of previously committed cells. These studies also allow some of the controlling factors to be defined. Presumably, chromaffin cells have the same plasticity *in vivo*, and similar factors could operate during normal development. Indirectly, the culture experiments reported here further support the concept of neural crest cells as pluripotent cells which are responsive to environmental signals, and which can still respond to new cues by changing their fate, even after they have embarked upon a particular pathway of differentiation.

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