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Long-term effects of dexamethasone and nerve growth factor on adrenal medullary cells cultured from young adult rats

Arthur S. Tischler[1], Robert L. Perlman[2,3], Gretl Nunnemacher[1], Gardiner M. Morse[1], Ronald A. DeLellis[1], Hubert J. Wolfe[1], and Beth E. Sheard[2]

1 Department of Pathology, Tufts University School of Medicine, Boston, USA;
2 Department of Physiology, Harvard Medical School, Boston, USA

Summary. Normal postnatal rat chromaffin cells and rat pheochromocytoma cells are known to show extensive Nerve Growth Factor (NGF)-induced process outgrowth in culture, and this outgrowth from the postnatal chromaffin cells is abolished by the corticosteroid dexamethasone. To determine whether adult rat chromaffin cells respond to NGF and dexamethasone, dissociated adrenal medullary cells from 3-month-old rats were cultured for 30 days in the presence or absence of these agents. Such cultures contained typical chromaffin cells, chromaffin cells with processes, and neurons. Fewer than 2% of normal adult chromaffin cells formed processes under any of the conditions studied, and statistically significant changes in this proportion were not detectable in the presence of NGF or dexamethasone. Adrenal medullary neurons, however, were observed only in the presence of NGF, in cultures with or without dexamethasone, and thus appear to be previously unreported NGF targets which require NGF for survival or process outgrowth. Dexamethasone markedly increased total catecholamine content, total content of epinephrine, and tyrosine hydroxylase activity in cultures with or without NGF. In contrast, postnatal rat chromaffin and rat pheochromocytoma cells which have been studied in culture do not produce epinephrine under any of these conditions. It is concluded that rat adrenal chromaffin cells undergo age-related changes in both structural and functional plasticity. The in vitro characteristics of rat pheochromocytoma cells more closely resemble those of postnatal than of adult rat chromaffin cells, but may not entirely reflect the properties of the majority of chromaffin cells in either age group.

Key words: Adrenal chromaffin cell – Nerve growth factor – Nerve plasticity – Catecholamine – "Short" and "long" adrenergic neurons

Send offprint requests to: Dr. A. S. Tischler, Department of Pathology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Ma 02111, USA

3 Present Address: Department of Physiology and Biophysics, University of Illinois College of Medicine, Chicago IL, USA

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It has been previously reported that adrenal chromaffin cells from postnatal rats show extensive Nerve Growth Factor (NGF)-induced outgrowth of neurite-like processes in culture, and that this process outgrowth is inhibited by high concentrations of dexamethasone. The cultured cells have also been noted to contain chromaffin granules which are smaller than those in vivo, and are exclusively of the norepinephrine (NE)-type (Unsicker and Chamley 1977; Unsicker et al. 1978, 1980 b). These changes in granule morphology have been reported to occur in the presence or absence of dexamethasone (Unsicker and Chamley 1977; Unsicker et al. 1978, 1980 b), and to be accompanied by progressively reduced storage of epinephrine (E) in relation to NE and dopamine (DA) with increasing time in vitro (Müller and Unsicker 1981).

The present investigation was undertaken to compare the morphological and catecholaminergic characteristics of adrenal medullary cells cultured from young adult rats in the presence or absence of NGF and Dexamethasone with those reported for cells from postnatal animals, and to correlate changes in granule morphology with absolute changes in catecholamine content.

Materials and methods

Cell culture

Four three-month-old male Long-Evans hooded rats were killed rapidly by exposure to dry ice vapor. Under sterile conditions, the adrenal glands were removed, placed in calcium and magnesium-free Hank's balanced salt solution (HBSS, GIBCO), and bisected transversely, and the gray medullary tissue from each half gland was dissected away from the yellow and brown cortex as a single fragment. The fragments were then pooled, incubated at 37 °C for 40 min in calcium and magnesium-free HBSS containing 2 mg/ml of collagenase (Type II, Sigma) and for an additional 40 min in the same salt solution with 0.25 % trypsin (GIBCO) plus collagenase. At the end of the incubation period the softened tissue fragments were aspirated repeatedly into normal and then flame-constricted Pasteur pipettes until they were completely dissociated. The resulting cell suspensions were centrifuged at 600 × g for 5 min, the supernatants were discarded, and the cells were resuspended in RPMI 1640 medium (GIBCO) with 10 % heat-inactivated horse serum (KC Biologicals), 5 % fetal calf serum (KC Biologicals), 50 mg/ml streptomycin and 50 U/ml penicillin (GIBCO). Replicate aliquots of 3×10^4 cells were plated on 35 mm Falcon polystyrene culture dishes coated with air-dried rat-tail collagen (Bornstein 1958). Three additional aliquots were assayed for catecholamines and for tyrosine hydroxylase (TH) activity, and a fourth was fixed for electron microscopy (see below). Half of the cells in each aliquot were estimated to be chromaffin cells and the remainder degenerating cortical cells, from low-magnification electron micrographs of the cell pellets. Beginning 15 h after plating, the medium in all of the culture dishes was supplemented with 10^{-6} M cytosine arabinoside (Ara-C, Sigma) to retard fibroblast proliferation. Sets of four dishes each were also supplemented with 2.5 S mouse salivary gland NGF (50 ng/ml, courtesy of Dr. Lloyd Greene), Dexamethasone disodium phosphate (10^{-5} M, courtesy of Merck, Sharp and Dohme Research Laboratories), NGF plus dexamethasone, or no additives. NGF was prepared by the method of Bocchini and Angeletti (1969), and its biological activity established in the PC 12 cell bioassay (Greene 1977). Virtually all cells had attached to the dishes by the time the additions were made to the medium, so that selective effects of the various additives on cell attachment were eliminated. Cultures were maintained for 30 days at 37 °C in a water-saturated atmosphere of 93 % air and 7 % CO_2. Medium was changed every 2–3 days.

Live cells were observed and photographed on a Leitz Diavert inverted phase contrast microscope. Individual cells were identified by phase contrast as typical chromaffin cells, chromaffin cells with processes, or neurons, and their identity was confirmed by electron microscopy. Numbers of these cells (Varon and Raiborn 1972) and percentages with and without processes (Greene 1977; Tischler and Greene 1975) were quantitated by strip counts. In these determinations three diagonal strips at 60°

angles to each other were scanned at 320 × magnification for each set of growth conditions. Each strip was 0.32 mm wide, corresponding to the width of a rectangular viewing reticle, and 24 mm long, corresponding to the diameter of the dish minus a peripheral area of sub-optimal visibility. This method resulted in scanning of about 2.5 % of the surface area of each dish. Consecutively scanned processes in each strip were measured with an ocular micrometer. A process was defined as a slender projection at least 2 cell diameters in length.

Electron microscopy

For electron microscopy, cultured cells were fixed in situ for 45 min at room temperature in 3 % glutaraldehyde (Ladd Research Industries) in 0.1 M Na cacodylate buffer, pH 7.2, with 2 mM $CaCl_2$. They were then post-fixed for 45 min in 1 % OsO_4 (Ladd) in the same buffer, dehydrated in a graded ethanol series and embedded in Epon. After hardening, cells which were identified by phase-contrast as chromaffin cells or neurons were circled from below with a diamond-tipped marking objective (Leitz) and from above with a diamond pencil. Discs of Epon, which retained the cell monolayers, were then broken out of the culture dishes by immersion in liquid nitrogen. The circled cells were cut out with a saw and glued to the bottoms of Epon-filled BEEM capsules. The capsules were transilluminated from below and examined on a bright field microscope to ensure that the circled cells were not lost during trimming. Ultrathin sections (80 nm) were cut parallel to the growth surfaces and were stained with aqueous uranyl acetate and lead citrate. Dissociated cells in suspension from the first day of the experiment were centrifuged at 600 × g for 5 min, resuspended in fixative for 45 min, centrifuged again, washed, post-fixed, embedded, and sectioned identically to the cells in culture.

Secretory granules were measured from electron micrograph prints on a Bioquant II Biological Image Analyzer (R & M Biometrics, Nashville, TN), which utilizes a Houston Instruments electronic digitizing tablet and an Apple II microcomputer. Prints were placed on the digitizer tablet, overlayed with a transparent ruled sheet divided into 5 mm squares, and scanned in 5 mm rows from left to right and top to bottom. The maximum dimension of the electron-dense content of each granule-containing vesicle was measured in a single direction (Coupland 1971). All consecutively encountered granules up to the first 100 were measured and stored in separate computer files for each individual cell and individual process. One hundred granules were measured for each chromaffin cell, 24 to 100 for each neuronal cell body, and 6 to 38 for each process by this method.

Catecholamine and tyrosine hydroxylase assays

Catecholamine content and tyrosine hydroxylase (TH) activity were measured in cells cultured for 30 days and in dissociated cells in suspension from the first day of the experiment by a sensitive method previously applied to rat pheochromocytoma cell suspensions (Erny et al. 1981) and to cultured rat PC12 pheochromocytoma cells (Tischler et al., submitted for publication). For cell cultures, the growth medium was aspirated from the dishes, and the cells were washed three times with 1 ml of a Hepes-buffered Krebs-Ringer saline solution (KRH), prewarmed to 37 °C. This solution contained 125 mM NaCl, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 5.6 mM glucose, and 25 mM Hepes (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), adjusted to a final pH of 7.3 by the addition of approximately 8 mmol/liter of NaOH. The cells were then incubated for 60 min in 0.4 ml of an incubation medium consisting of the above solution supplemented with 100 μM L-tyrosine (Sigma), 100 μM brocresine (an inhibitor of aromatic L-amino acid decarboxylase, courtesy of Lederle Laboratories, Pearl River, NY) and 100 μM EDTA, in an air atmosphere at 37 °C. Experiments with pheochromocytoma cells have documented that cells incubated with brocresine produce 3,4-dihydroxyphenylalanine (dopa) at a constant rate, and release almost all of this dopa into the incubation medium (Vaccaro et al. 1980). Under the conditions of the experiments the rate of tyrosine uptake is greater than the rate of dopa production, and the intracellular tyrosine concentration is sufficient to support maximal rates of dopa production. Measured changes in rate of dopa production therefore directly reflect changes in TH activity, rather than in tyrosine transport (Vaccaro et al. 1980). At the end of the incubations, 70 % $HClO_4$ was added to the incubation medium in each dish, to a final concentration of 0.4 N. The cells were scraped from the dishes in this medium, rapidly frozen on dry ice, and then frozen and thawed two additional times. Preliminary experiments with pheochromocytoma cell cultures indicated that this procedure for disrupting cells yielded catecholamine contents equivalent to those obtained by

sonication, and slightly greater than those obtained by lysis of the cells in digitonin. After the final thawing the samples were centrifuged for 5 min at 12,000 × g in an Eppendorf microcentrifuge. Aliquots of the supernatant solutions were assayed for catecholamines and for dopa by liquid chromatography with electrochemical detection, as previously described (Erny et al. 1981). The mobile phase used for this chromatography was 100 mM H_3PO_4 containing 200 μM octyl sodium sulfate, 100 μM EDTA, and 9% methanol, and titrated to pH 2.6 with NaOH. This solvent was pumped at a flow rate of 1.2 ml/min over a 4.6 × 250 mm C-18 Ultrasphere-ODS column (5 μm particle size, Altex Scientific Inc., Berkeley, CA), which was maintained at 37 °C with an LC-22/23 temperature control system (Bioanalytical Systems, Inc., West Lafayette, IN). The effluent from the column was oxidized at a model TL-5 thin-layer electrochemical detector (Bioanalytical systems, Inc.). The glassy carbon working electrode in this detector was maintained at a potential of +0.6 V (vs. a Ag/AgCl reference electrode) with a model LC-2A amperometric controller (Bioanalytical Systems, Inc.). The output from the controller was recorded and integrated with a model 3390 A integrater (Hewlett-Packard, Palo Alto, CA). The amounts of catecholamines and dopa in the samples were estimated by integration of the areas under the peaks corresponding to each compound.

Dissociated cells in suspension were assayed comparably to cells in culture. After dissociation the cells were incubated in complete RPMI growth medium for 15 min at 37 °C, then centrifuged at 600 × g for 5 min. The growth medium was removed and the cells were washed twice with prewarmed KRH, resuspending them for each wash. After the second wash the cells were centrifuged again, then resuspended and incubated at 37° C for 1 h in the incubation medium described above. After the incubation period $HClO_4$ was added, extracts were prepared by freeze-thawing and centrifugation, and aliquots were assayed for catecholamines and dopa as described above.

Statistical analyses

Numbers of cells with processes under the various growth conditions were compared by Chi-square tests. Because of the small numbers of such cells, only differences with $p<0.01$ were considered statistically significant. This rejection limit was chosen to result in a 95% chance of detecting true differences of 40% or more (power = 0.95) with an average of 355 cells scored for each of the different growth conditions (Cohen 1970). Process lengths were compared by the Mann-Whitney U-test; and granule diameters, catecholamine content and dopa production by Student's *t*-test, with rejection limits of $p<0.05$.

Results

Light microscopy

For the first 48 h in vitro virtually all cells were rounded, refractile, and devoid of processes. Thereafter, it became possible to identify typical chromaffin cells, chromaffin cells with processes, and neurons by their appearance under phase contrast microscopy. Chromaffin cells with or without processes appeared as rounded or polygonal epithelial-like cells, 15 to 25 μm in diameter. Phase-dark cytoplasm and round or oval nuclei with one or two nucleoli of variable size could be observed in cells which flattened against the surfaces of the culture dishes, but the majority had a refractile appearance. Chromaffin cell processes were generally single and showed little or no arborization (Figs. 1, 2). Neurons were recognized as process-bearing cells 2–4 times the size of chromaffin cells, with 1 or 2 large nucleoli (Fig. 3). Processes of neurons branched extensively, often formed fascicles, and were markedly longer than processes of chromaffin cells.

After 30 days in vitro chromaffin cells with and without processes were present under each of the growth conditions studied. Approximately 75% of the cells in all of the dishes were estimated to be chromaffin cells and the remainder were

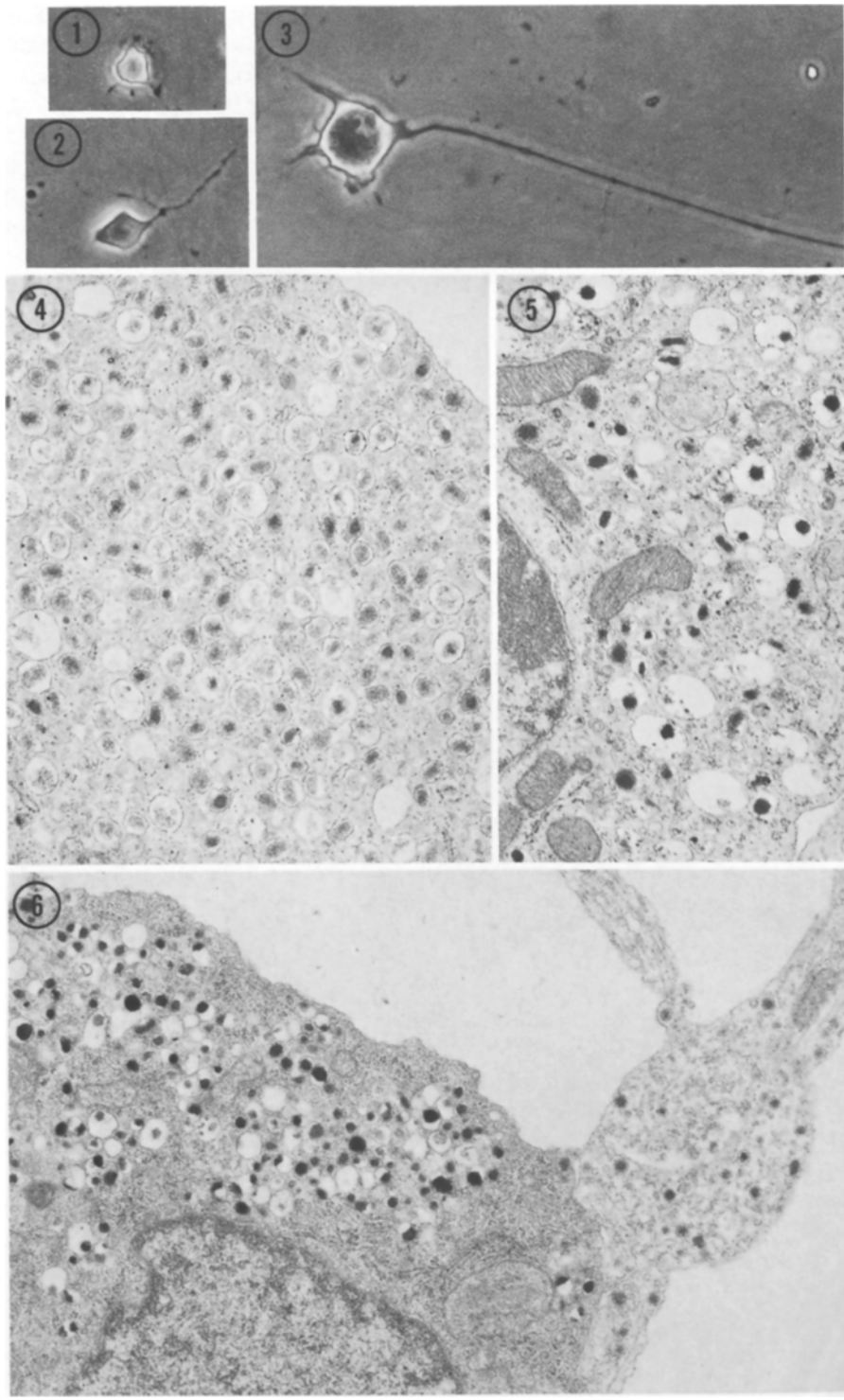
predominantly fibroblast-like. Rare vacuolated cells which may have been surviving cortical cells were also observed. Chromaffin cells were present mostly as single cells attached directly to the culture dishes. Occasional clusters of 2–4 chromaffin cells or of chromaffin cells attached to fibroblast-like cells were also present. Process outgrowth appeared to occur independently of contact with or proximity to other cells. Chromaffin cells with processes appeared to be slightly increased in number in cultures treated with NGF. Fewer than 2% of chromaffin cells formed processes in any of the cultures, however, and statistically significant differences in percentage of these cells with processes or in mean process length were not detected between the various growth conditions. NGF produced no statistically significant changes in chromaffin cell survival, and all of the dishes showed a 4- to 5-fold decrease in chromaffin cell number after 30 days in vitro ($3.7 \pm 0.2 \times 10^3$ surviving chromaffin cells/dish, mean ± SEM). This decrease appeared to result predominantly from loss of cells damaged by dissociation in the 15 h period after plating, rather than from slow attrition.

In contrast to chromaffin cells with processes, neurons were identified only in cultures treated with NGF. It was not determined whether this phenomenon reflected NGF requirements for survival or merely for process outgrowth, because only cells with processes were recognizable as neurons with certainty. Neurons were observed as single cells attached directly to the culture dishes, or to fibroblast-like cells, and comprised less than 2% of the cells in cultures with or without dexamethasone. After 30 days in vitro neuronal processes measured 1408 ± 215 μm in length, vs 550 ± 227 μm for processes of chromaffin cells (Mean ± SEM, $p < 0.01$). Mean process length was not significantly altered in cultures treated with dexamethasone.

Electron microscopy

Chromaffin cells in suspension from the first day of the experiment were identifiable as E-type or NE-type on the basis of chromaffin granule morphology (Coupland 1971). NE-type granules had rounded profiles and were homogeneously electron dense, while E-type granules were more pleomorphic and finely particulate, with an intermediate electron density. NE-type granules were almost invariably surrounded by a clear halo, as often observed in vivo. Many granules in E-type cells, however, appeared to have become distorted during the dissociation or fixation, and were also surrounded by halos (Figs. 4, 5). Occasional NE-type granules were observed in E-type cells, as also noted by Müller and Unsicker (1981) in the intact adult rat adrenal. Three of 13 chromaffin cells photographed on the first day of the experiment were of NE-type, and the remainder of E-type. Neurons were not identified in the cell suspensions.

After 30 days in vitro the number of NE-type granules was markedly increased and the number of E-type granules markedly decreased in chromaffin cells in all cultures. Many granules did not possess either typical E-type or NE-type morphology, however, and quantitative volume-density measurements were therefore not undertaken. Although granules in most cells exhibited a continuum of electron densities, granules of intermediate electron density were more numerous than NE-type granules in some cells treated with dexamethasone (Fig. 7), but not in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cells in control cultures. Some cells in dexamethasone-treated cultures also contained almost exclusively NE-type granules (Fig. 6). Treatment with NGF did not discernibly affect granule morphology. Only one chromaffin cell with a process was identified after ultrathin sectioning. This cell contained almost exclusively typical NE-type granules.

In addition to large granules, chromaffin cells in culture contained occasional clusters of predominantly agranular synaptic-like vesicles similar to those occasionally seen in vivo (Kobayashi 1977). These vesicles were very numerous in one cell treated with NGF and dexamethasone (Figs. 7, 8), but were too rare overall to permit assessment of differences in their prevalence under different culture conditions.

The ultrastructure of cultured adrenal medullary neurons was essentially the same as that described for rat sympathetic neurons from the superior cervical ganglion in vivo (Grillo 1966; Richard and Tranzer 1975) and in vitro (Bunge 1973). These neurons contained both "Large Granular Vesicles" (LGV) and predominantly agranular synaptic-like vesicles. The former were very sparsely distributed throughout the perikarya and also tended to accumulate at varicose regions of processes, while the latter were observed almost exclusively in processes (Fig. 9). Neuronal LGV exhibited a continuum of electron densities, and individually were indistinguishable from small chromaffin granules.

The sizes of individual granules in chromaffin cells varied between approximately 30 and 360 nm both on the first day of the experiment and after 30 days in vitro. The mean size of granules in the cultured cells, however, was markedly diminished under all of the growth conditions tested ($p < 0.001$). Cells cultured with dexamethasone showed small but highly significant increases in mean granule size compared to cells cultured with no additives ($p < 0.001$), and this increase was slightly augmented in the presence of NGF ($p < 0.001$). NGF alone did not

Fig. 1. Phase contrast photomicrograph of typical chromaffin cell in culture. The cell has a refractile appearance and an inconspicuous nucleus. × 350

Fig. 2. Phase contrast photomicrograph of cultured chromaffin cell with process. The process has a varicose appearance similar to that of neuronal processes, but is short and shows only minimal branching. × 350

Fig. 3. Phase contrast photomicrograph of cultured adrenal medullary neuron. The cell body is almost three times the size of chromaffin cell bodies, and has a prominent nucleus with two large nucleoli. The process extends out of the photograph at right. × 350

Fig. 4. Typical epinephrine-type cell in suspension of freshly dissociated adrenal medulla. The granules are comparable to E-type granules in vivo but have a less compact appearance as also noted by Unsicker et al. (1980) in suspensions of bovine chromaffin cells. ×16,126

Fig. 5. Typical norepinephrine-type cell in suspension of freshly dissociated adrenal medulla. The granules have characteristic electron-dense appearance and tend to be surrounded by clear halos. × 16,126

Fig. 6. Norepinephrine-type cell in culture maintained for 4 weeks in the presence of 10^{-5} M dexamethasone and NGF. The granules are similar in appearance to norepinephrine-type granules in vivo, but many are smaller. Varicose portion of neuronal process contacts the chromaffin cell at right, but there are no synaptic membrane densities. × 16,126

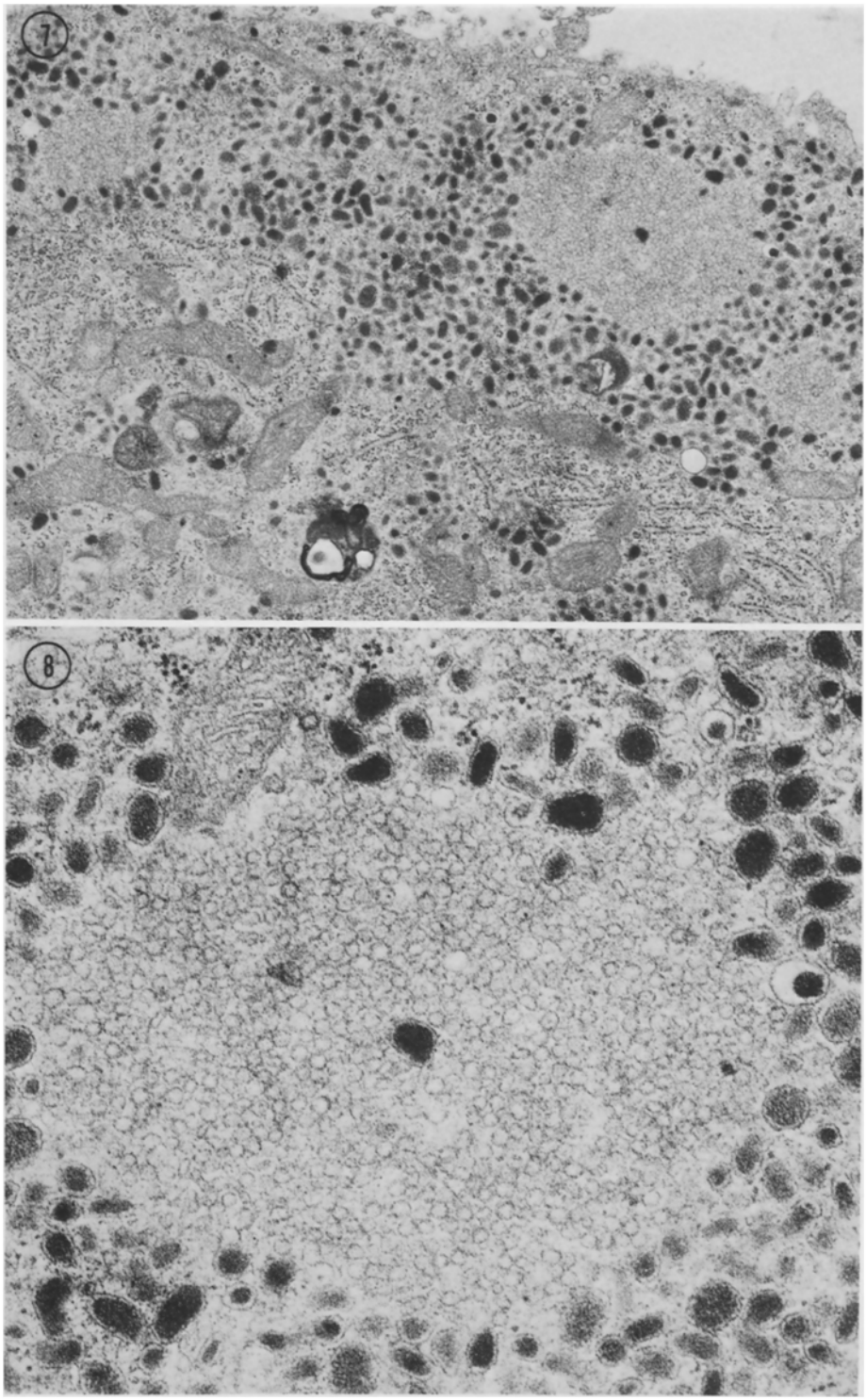

Fig. 7. Cell with a mixture of epinephrine-, norepinephrine- and indeterminate-type granules cultured for 4 weeks with 10^{-5} M dexamethasone and NGF. The granules are aggregated near the periphery of the cell. Clusters of numerous, small, synaptic-like vesicles are also present. × 16,126

Fig. 8. Higher magnification of synaptic-like vesicles from Fig. 7. The vesicles are predominantly agranular. × 50,000

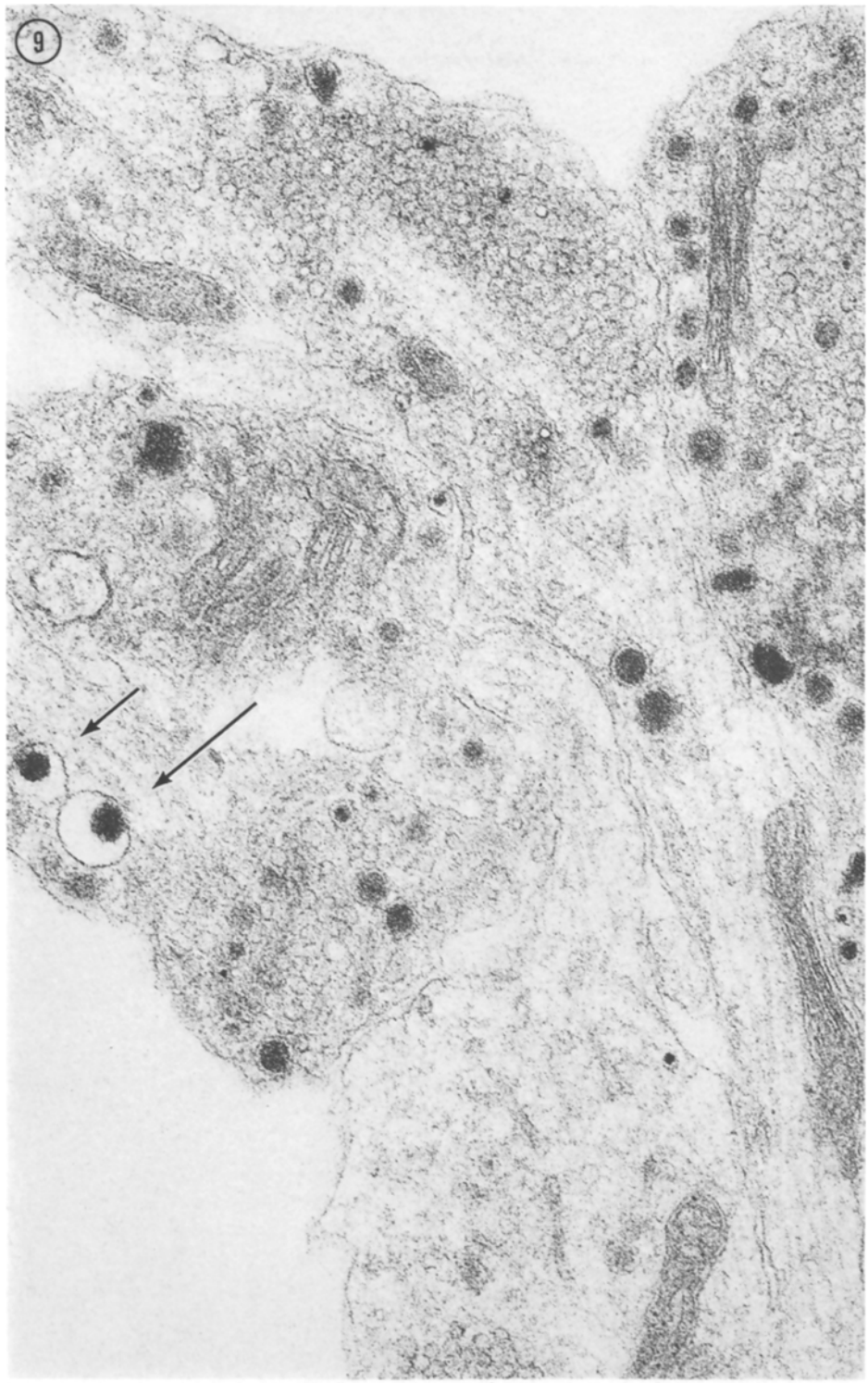

Fig. 9. Interwoven processes from cultured adrenal medullary neurons. The processes contain granules which individually are indistinguishable from those in chromaffin cells, including two "classic" norepinephrine-type granules at left (*arrows*). There are also numerous, predominantly agranular synaptic-like vesicles similar to those in chromaffin cell body in Fig. 8. × 50,000

Table 1. Mean granule size[a] in chromaffin cells

Days in vitro	Medium supplements	Granule size (nm) Range of means in individual cells, ± S.D.	Granule size (nm) Cumulative mean, ± S.D.	Number of granules measured
0	None	115 ± 39 – 212 ± 69	185 ± 63	1000
30	None	83 ± 24 – 116 ± 58	101 ± 34	1000
30	Dexamethasone (10^{-5} M)	85 ± 29 – 130 ± 36	112 ± 37	1000
30	NGF (50 ng/ml)	90 ± 29 – 132 ± 38	104 ± 32	1000
30	Dexamethasone + NGF	100 ± 29 – 159 ± 48	119 ± 41	1000

[a] Maximum granule dimension in a single direction, mean ± Standard Deviation, not corrected for section thickness. All measurements are of granules in perikarya. One hundred granules were measured in each of 10 cells for each set of conditions

Table 2. Mean granule size[a] in neuronal perikarya and processes, 30 days in vitro

Medium supplements	Granule location	Granule size (nm) Range of means in individual cells, ± S.D.	Granule size (nm) Cumulative mean ± S.D.	Number of granules measured
NGF (50 ng/ml)	Perikarya	91 ± 21 – 98 ± 19	94 ± 22	198
	Processes	66 ± 16 – 92 ± 21	80 ± 23	100
	combined		89 ± 23	298
NGF (50 ng/ml) + Dexamethasone (10^{-5} M)	Perikarya	92 ± 24 – 102 ± 28	97 ± 26	213
	Processes	86 ± 21 – 110 ± 29	93 ± 26	71
	combined		96 ± 26	284

[a] Maximum granule dimension in a single direction, mean ± Standard Deviation, not corrected for section thickness. All of the granules in electron micrographs of three perikarya and five processes were measured for each set of conditions. Perikarya and processes were discontinuous in most sections, and measurements may therefore not represent sampling of different parts of the same cells

significantly alter granule size. Highly significant differences ($p < 0.001$) were observed between mean granule sizes in individual cells both at the start of the experiment and in cultures maintained under each of the various growth conditions (Table 1).

The sizes of the dense cores of individual LGV in neurons varied between approximately 20 and 180 nm in perikarya and in processes. Mean sizes tended to be larger in perikarya than in processes, but the difference was statistically significant ($p < 0.001$) only in cultures without dexamethasone. In the limited number of cells sampled, combined mean sizes of LGV cores were significantly larger in cultures treated with dexamethasone + NGF than with NGF alone ($p < 0.001$). Individual neuronal perikarya and processes, however, exhibited differences in mean sizes of LGV cores which were of comparable magnitude to differences in mean granule size occurring from cell to cell in chromaffin cell cultures (Table 2).

Table 3. Effects of dexamethasone and NGF on absolute and relative catecholamine content[a]

Days in vitro	Medium supplements	Epinephrine	Nor-epinephrine	Dopamine	Total	
0	None	1,030± 90	240± 30	20± 20	1,286	pmol/dish (suspension)
		80	19	1	100	% of total
30	None	7.7± 0.7	12.3± 0.3	6.0± 1	26.0	pmol/dish
		2.6± 0.2	4.1±	2.0± 0.3	8.7	fmol/cell
		30	47	23	100	% of total
30	Dexamethasone (10^{-5} M)	40.0± 3.0	14.0± 1.0	19.7± 13	73.7	pmol/dish
		10.0± 0.8	3.5± 0.3	4.9± 0.3	18.4	fmol/cell
		54	19	27	100	% of total
30	NGF (50 ng/ml)	5.7± 0.3	12.3± 0.3	4.7± 0.7	22.7	pmol/dish
		1.5± 0.1	3.3± 0.1	1.3± 0.2	6.1	fmol/cell
		25	54	21	100	% of total
30	Dexamethasone +NGF	50.0± 3.0	18.5± 1.5	19.5± 0.5	88.0	pmol/dish
		12.5± 0.8	4.6± 0.4	4.9± 0.1	2.2	fmol/cell
		57	21	22	100	% of total

[a] Catecholamine contents per cell are based on mean number of surviving chromaffin cells per dish for each separate set of growth conditions. All values represent mean± SEM of triplicate determinations except those for dexamethasone+NGF, which represent mean± SEM of duplicate determinations

Table 4. Effects of dexamethasone and NGF on rate of Dopa production[a]

Days in vitro	Medium supplements	Dopa production
0	None	24.0± 2.0 pmol/dish/h
30	None	6.3± 1.3 pmol/dish/h
		2.1± 0.4 fmol/cell/h
30	Dexamethasone (10^{-5} M)	30.7± 8.3 pmol/dish/h
		7.6± 2.1 fmol/cell/h
30	NGF (50/ng/ml)	7.0± 0.7 pmol/dish/h
		1.9± 0.2 fmol/cell/h
30	Dexamethasone +NGF	26.5± 2.5 pmol/dish/h
		6.6± 0.6 fmol/cell/h

[a] Calculations of dopa production per cell are based on mean number of surviving chromaffin cells per dish for each separate set of growth conditions. All values represent mean± SEM except those for day 0 and for dexamethasone +NGF, which represent mean± SEM of duplicate determinations. Disproportionately low values for Dopa production/dish/h in relation to catecholamine content/dish on day 0 probably reflect measurement of catecholamines stored in non-viable cells

Cumulative mean sizes of LGV cores in neurones were significantly lower than cumulative mean sizes of granules in chromaffin cells ($p < 0.001$), but individual neurons were sometimes indistinguishable from individual chromaffin cells on this basis (Tables 1, 2). LGV in neurons, however, were always markedly sparser than granules in chromaffin cells.

Catecholamine content and tyrosine hydroxylase activity

At the start of the experiment, 80% of total catecholamine stores consisted of E and 19% of NE, in approximate agreement with the ratio of E- to NE-type cells in electron micrographs of the cell suspension.

After 30 days in vitro all of the cultures showed decreases in total catecholamine stores, and in amounts of stored E in relation to NE and DA. Calculated per dish, total catecholamine stores were decreased about 50-fold in cultures maintained with no additives. Allowing for an approximately 5-fold reduction in chromaffin cell number, the decrease was approximately 10-fold per cell. The proportion of total catecholamine stores comprised by E in cultures with no additives concomitantly decreased to 30%, and that comprised by NE increased to about 50% (Table 3).

Cultures maintained in the presence of dexamethasone showed 3–4 fold higher total catecholamine contents than those with no additives, whether calculated per dish or per cell, with E comprising 50–60% of the total. Dexamethasone also produced 3–5 fold increases in content of DA and in rate of dopa production in culture, while not significantly affecting content of NE. NGF did not significantly affect either catecholamine content or rate of dopa production (Tables 3, 4).

Discussion

The present findings confirm and extend a number of observations made by Unsicker and Chamley (1977) and Unsicker et al. (1978, 1980 b) on adrenal medullary cultures of postnatal rats. In addition, they suggest that adrenal medullary cells of young adult rats differ greatly from those of postnatal animals in their responses to NGF and dexamethasone.

In the present experiments adult rat chromaffin cells did not show significant NGF-induced process outgrowth. A small number of chromaffin cells in all cultures spontaneously formed short processes, as also noted by Unsicker and Chamley (1977), and this spontaneous process outgrowth was not inhibited by 10^{-5} M dexamethasone. In contrast, adrenal medullary neurons survived and/or formed processes only in the presence of NGF. Process outgrowth from these neurons was also unaffected by dexamethasone. Chromaffin cells cultured for 30 days showed marked decreases in total catecholamine content and in ratios of E to NE and DA. These decreases were paralleled by decreases in mean granule size and increasing numbers of NE-type granules. Dexamethasone caused increases in total catecholamine content and in relative content of E, but not to levels as high at the start of the experiment. Dexamethasone also increased mean granule size, but not in proportion to increases in catecholamine content. Heterogeneity in mean granule size was observed from cell to cell both at the start of the experiment and after 30 days in vitro. TH activity was increased by dexamethasone in the presence or absence of NGF, but not apparently by NGF alone.

In contrast to these findings, chromaffin cells from postnatal rats show extensive process outgrowth in response to NGF, and this outgrowth is abolished by 10^{-5} M dexamethasone (Unsicker et al. 1978, 1980 b; Lillien and Claude 1981). Although effects of dexamethasone on adrenal medullary neurons have not been

previously commented upon, dexamethasone has been noted to impair markedly NGF-induced process outgrowth in cultures of sympathetic neurons from the superior cervical ganglia (SCG) of neonatal rats (Unsicker et al. 1978). Rat adrenal medullary neurons are similar to other sympathetic neurons morphologically, and also show weak catecholamine-specific formaldehyde-induced fluorescence (Unsicker et al. 1978; Tischler et al. unpublished). If adrenal medullary neurons in neonatal rats respond to dexamethasone comparably to SCG neurons, in adult rats they may exhibit a loss of dexamethasone responsiveness analogous to the loss of NGF responsiveness by adult rat chromaffin cells. Aloe and Levi-Montalcini (1979) have also noted a loss of the ability of NGF to transform rat chromaffin cells in vivo into neuron-like cells in the period shortly after birth.

Changes which we have observed in granule size and morphology and in catecholamine storage after 30 days in vitro are for the most part comparable to those noted in cultured postnatal rat chromaffin cells (Unsicker and Chamley 1977; Unsicker et al. 1978, 1980). Unlike the latter, however, cultured adult rat chromaffin cells do not acquire exclusively NE-type granules. Neither do they completely lose their abilities to synthesize and store E or to respond to dexamethasone with increased E synthesis. The presence of E in cultures without dexamethasone in the present studies might result from small quantities of steroids in the serum added to the culture medium, small numbers of surviving cortical cells in the cultures, or other mechanisms. Adult and postnatal rat chromaffin cells also differ significantly from each other in that TH activity is increased by NGF but not by dexamethasone in the postnatal cells (Unsicker et al. 1978), while the reverse is apparently true in those of adults.

Although some of the differences between our present findings and those of Unsicker and his co-workers might result from differences in animal strains or culture conditions, the above comparisons suggest that chromaffin cells and neurons in the rat adrenal medulla may show a number of different types of structural and functional plasticity in animals of different ages. This phenomenon is of particular interest in light of recent studies of pheochromocytomas. Human and rat pheochromocytoma cells in primary cultures (Tischler et al. 1976; Tischler and Greene 1975) and clonal PC 12 rat pheochromocytoma cells (Greene and Tischler 1976, 1982) show extensive NGF-induced process outgrowth. In the case of human pheochromocytomas, this outgrowth appears to reflect capabilities retained by large numbers of normal chromaffin cells in adult life (Tischler and Greene 1980; Tischler et al. 1980). In contrast, the present experiments were performed under culture conditions in which rat pheochromocytoma cells show florid NGF-induced process outgrowth, and no statistically significant NGF effects were observed. Under other conditions both we and Unsicker et al. (1980 a) have noted apparently variable responses of adult rat chromaffin cells to NGF, but the proportion of cells with processes has never approached that in cultures of adult human chromaffin cells. The structural responses of rat pheochromocytoma cells to NGF, unlike those of their human counterparts, may therefore reflect properties of chromaffin cells which constitute an extremely small subpopulation in the normal young adult rat, or may reflect characteristics of immature chromaffin cells which are regained during neoplastic transformation. It may be of interest that NGF-induced process outgrowth from rat pheochromocytoma cells, unlike that from immature

chromaffin cells, is not subject to inhibition by corticosteroids (Tischler and Greene 1975). Functionally, the in vitro characteristics of adult rat chromaffin cells are in some ways similar and in other dissimilar to those of rat pheochromocytomas. Dexamethasone increases TH activity (Edgar and Thoenen 1978; Otten and Towbin 1980; Schubert et al. 1980; Tischler et al., submitted for publication), total catecholamine content (Tischler et al., submitted for publication), total DA content (Tischler et al., submitted for publication) and mean granule size (Schubert et al. 1980) both in PC 12 pheochromocytoma cells and in adult rat chromaffin cells, while NGF alone does not appear to increase catecholamine synthesis or storage in either (Greene and Tischler 1976; Tischler et al., submitted for publication). Although dexamethasone has recently been reported to exert a permissive action allowing induction of TH by NGF in PC12 cells (Otten and Towbin 1980), this phenomenon is only detectable at lower dexamethasone concentrations (10^{-8}–10^{-9} M) than that employed in the present studies, and the possibility that normal adult rat chromaffin cells may behave similarly to PC 12 cells in this regard has therefore not been ruled out. The major functional difference so far detected between normal adult rat chromaffin cells and rat pheochromocytoma cells thus appears to be the ability to synthesize E. Reported rat pheochromocytoma cells which have been studied in culture have not been documented to produce E under any conditions (Greene and Tischler 1976; Chalfie and Perlman 1976; Tischler et al. submitted for publication).

It should be noted that the failure of most adult rat chromaffin cells to exhibit NGF-induced process outgrowth does not imply a loss of structural plasticity. It is well established that many of these cells form processes when transplanted to the anterior chamber of the eye (Olson 1970), and this outgrowth may be mediated by growth factors other than NGF (Ebendal et al. 1980). At present it is not known whether any of these factors mediate the "spontaneous" process outgrowth observed in the present investigation, or in studies of normal postnatal rat (Unsicker and Chamley 1977), normal adult human (Tischler and Greene 1980; Tischler et al. 1980) or neoplastic rat and human (DeLellis et al. 1973; Tischler and Greene 1975; Tischler et al. 1976) chromaffin cells in culture.

Our observations of factors modulating catecholaminergic functions in dissociated adult rat chromaffin cells are of interest in view of previously reported data. The selective induction of TH by NGF has been described as a characteristic functional response of adult rat chromaffin cells to added NGF in vivo and in organ culture (Otten et al. 1977), as well as of dissociated postnatal rat chromaffin cells in culture (Unsicker et al. 1978). Such an effect was not, however, observed in the present studies. One possible explanation for this discrepancy is that the concentration of NGF which we employed was not optimal for achieving a detectable TH response. This consideration might be critical in view of the fact that NGF-induced increases in chromaffin cell TH are small, and have not been reported to reach 200 % of control values either in vivo or in vitro (Otten et al. 1977; Unsicker et al. 1978). At least for postnatal rat chromaffin cells, however, these increases are observed with NGF concentrations lower than those which we have employed (Unsicker et al. 1978). Another possibility is that chromaffin cells might lose their functional NGF responsiveness after 30 days in vitro, but we have also failed to detect NGF-induced increases in TH activity in cells maintained for two

weeks in vitro (Tischler et al., unpublished). Possible additional sources of the discrepancy lie in the difference between the TH assay procedures in the present study and in previous reports. While previous studies have measured TH activity in cell extracts, in our experiments the enzyme is measured in intact, viable cells. The validity of this approach has been documented elsewhere (Vaccaro et al. 1980). The rate of dopa production in intact normal chromaffin cells or in PC 12 cells (Tischler et al., submitted for publication) is much less than the V_{max} of TH as measured in cell extracts (Markey et al. 1980). Since it is unlikely that the comparatively low rate of dopa production in intact cells is due to limited availability of tyrosine (Vaccaro et al. 1980), it appears that other features of the intracellular environment are not optimal for TH activity. Specifically, the intracellular pH is probably higher than the pH optimum of TH (5.8–6.2), and the intracellular concentration of tetrahydrobiopterin cofactor is likely to be below optimal levels. In addition, limited proteolysis which occurs during extraction and solubilization of TH might result in some degree of enzyme activation in assays performed on cell extracts (Petrack et al. 1968).

Our finding of increased E storage in adult rat chromaffin cells treated with dexamethasone is not entirely surprising in view of the well-established relationship between E-production and corticosteroids in vivo (Wurtman and Axelrod 1966; Fuller 1973). Direct corticosteroid-induced increases in E synthesis have also been noted in immature rabbit (Coupland and MacDougall 1966) and adult human (Tischler et al., in preparation) chromaffin cells in culture. The relationships between TH and steroids, however, have been less certain. TH activity in rat adrenal glands is markedly reduced by hypophysectomy (Wurtman and Axelrod 1966). This decrease, however, is reversed by administration of ACTH, but not of glucocorticoids (Mueller et al. 1970). Although it is currently believed that TH activity in the adrenal gland is regulated to a greater degree by neural than by humoral influences (Fuller 1973), the present observations demonstrate that corticosteroids also directly increase both TH activity and total catecholamine storage in normal chromaffin cells. Comparable effects of corticosteroids have also been reported in neuroblastoma cell cultures (Sandquist et al. 1979; Williams et al. 1981).

The heterogeneity in mean granule size which was documented to exist from cell to cell in this study has also been noted in adult human chromaffin cell cultures (Tischler et al. 1980). Recent studies also suggest that subpopulations exist within classes of normal adrenal chromaffin cells in vivo (Gorgas and Böck 1976; Kobayashi 1977; Millar and Unsicker 1981), and it has not yet been determined to what extent these differences are intrinsic or extrinsic, transient or permanent. One possibility is that different granule sizes could reflect different stages in a secretory cycle (Kirshner and Viveros 1972). All of the cells in the present study, however, were presumably in a basal state. Since the heterogeneity could not be accounted for by contact with or proximity to other cells, the present observations suggest either that adult rat adrenal glands exhibit a wide spectrum of intrinsic heterogeneity with regard to granule sizes in individual cells, or that individual cells possess intrinsic secretory rhythms. A practical implication of this heterogeneity is that morphometric studies of cultured chromaffin cells should be designed to account for variations from cell to cell in order to detect small effects of drugs or trophic substances. Some

intrinsic heterogeneity may also exist with regard to granule morphology in adult rat adrenals, since some cells contained almost exclusively NE-type granules even in the presence of dexamethasone, while others contained granules of E-, NE-, and indeterminate types.

In these studies dexamethasone caused significant increases in mean sizes of chromaffin granules, but these increases were not nearly large enough to account for parallel increases in catecholamine content. Possible interpretations of this observation are that dexamethasone alters granule composition, increases number of granules per cell, or promotes incorporation of catecholamines into a non-granular pool. Dexamethasone also appeared to cause small increases in the mean sizes of LGV in adrenal medullary neurons. Although these increases were statistically significant, however, they must be interpreted with caution in view of limited sampling.

Adrenal medullary neurons in this study had two unusual features. First, while these cells appeared to be dependent on NGF for process outgrowth, they did not require added NGF for survival or attachment in the 15-h period immediately after plating. The cultures were plated at very low densities, and the majority of neurons were not in contact with other cells which could have provided NGF-like substances. With regard to their NGF requirements for survival these neurons may thus be similar to the "short" adrenergic neurons which innervate the urogenital tracts of rats and other species (Owman et al. 1971). In additional preliminary studies in which cultures were plated at high densities and contained numerous fibroblast-like cells, we have observed both survival and process outgrowth from at least some of these neurons in the absence of added NGF (Tischler et al., unpublished). Unsicker and Chamley (1977) also observed survival of these neurons without added NGF in explant cultures. Secondly, LGV in some of the adrenal medullary neurons were larger than those generally found in sympathetic ganglia (Grillo 1966), and were indistinguishable in mean size from granules in some of the chromaffin cells. The possibility that some of the cells which we identified as neurons might have in fact been chromaffin cells transformed into neuron-like cells by NGF has not been ruled out. It is of interest, however, that short adrenergic neurons are also known to possess unusual granules (Euler and Lishajko 1966). Further studies of adrenal medullary neurons are currently in progress.

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