PRIMARY CULTURES OF DISSOCIATED SYMPATHETIC NEURONS

I. Establishment of Long-Term Growth in Culture and Studies of Differentiated Properties

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ABSTRACT

Rat sympathetic ganglia were disrupted by mechanical agitation to yield dissociated primary neurons, and the conditions for long-term growth in culture of the isolated neurons were examined. The neurons were grown with or without non-neural cells, simply by the addition or deletion of bicarbonate during growth in culture. Fluorescence histochemistry indicated that the isolated neurons contained catecholamines; incubations with radioactive precursors were used to verify the synthesis and accumulation of both dopamine and norepinephrine. The neurons also produced octopamine using tyramine as precursor, but not with tyrosine as the precursor. In the presence of eserine, older cultures synthesized and stored small amounts of acetylcholine. The cultures did not synthesize and accumulate detectable levels of radioactive γ-aminobutyric acid, 5-hydroxytryptamine, or histamine.

INTRODUCTION

Tissue culture of dissociated neurons offers certain opportunities for the study of the development and function of the nervous system. Control over the chemical environment in vitro should lead to a better understanding of the nutritional requirements and endocrine control of neurons. Specificity of cellular recognition and interaction may be investigated in tissue culture by confronting the neurons with both normal target cells (such as gland or muscle) and unusual cell types. Finally, neuronal culture may be useful for biochemical studies relating to the development of the nervous system which have been hindered by heterogeneous populations of neurons and by contamination with non-neural cell types.

All of these studies would be facilitated by a tissue culture system consisting of long-term cultures of dissociated primary neurons of known homogeneous origin and function which could be grown in the presence or absence of other cell types. There are several culture systems which meet some of these criteria using neurons from various embryonic sources (dorsal root ganglia: Scott et al., 1969, Miller et al., 1970, Okun, 1972; cerebrum: Varon and Raiborn, 1969; spinal cord: Fischbach, 1972). These neurons can be grown in the virtual absence of non-neural cells by use of separation procedures or antimitotic agents. Also available are tumor cells apparently of nervous origin (Augusti-Tocco and Sato, 1969; Schubert et al., 1969).

Sympathetic ganglia have several advantages

as a source of neurons for culture. These ganglia contain two distinguishable classes of neurons (e.g., Jacobowitz, 1970) whose natural history in vivo is well known. The extensive studies already available on norepinephrine (NE)[1] and dopamine (DA), used by sympathetic neurons as neurotransmitters, provide a rich store of information for use in analysis of the neurons in culture.

The investigations presented here were based on previous work carried out in this laboratory by Bray (1970) and E. J. Furshpan and D. D. Potter (unpublished). We describe conditions for establishing long-term (up to 13 wk) low-density cultures of dissociated primary neurons from the rat superior cervical ganglion (SCG). The presence or absence of non-neural cells in these cultures can be controlled by the addition or deletion of bicarbonate during growth in culture. Evidence is presented that the cultured neurons possess many of the properties expected of terminally differentiated sympathetic cells. The accompanying papers (Mains and Patterson, 1973 *a*, *b*), report our initial studies on the catecholamine (CA) metabolism of the cultured neurons and changes with age in the metabolism of the cultures.

MATERIALS AND METHODS

Cell Preparation

Neonatal rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) were killed by a blow to the head, and the superior cervical ganglia were removed under a stream of sterile 0.9% NaCl and placed in plating medium (see below) at 0–4°C. The ganglia were then cleaned of surrounding tissue, and teased apart as completely as possible with forceps to give cells, chunks, and small clusters of cells. Ganglia from pups more than 48 h old were very poor for this tearing procedure as they were tougher than those from neonates. The cells and chunks were vortexed in a test tube with a mixture of swirling and vibratory motions for 2–3 min, and the chunks were allowed to settle at room temperature for 5 min. The supernate containing the dissociated cells was removed and centrifuged at room temperature for 5 min at setting no. 3 in an International clinical centrifuge, model CL (International Equipment Co., Needham Heights, Mass.). The cells were resuspended in a small volume of plating medium and were added dropwise inside glass rings in culture dishes containing complete growth medium (see below).

The cultures were allowed to stand for 2 days, without examination, before the glass rings were removed and the first change of the medium made. Subsequently, they were fed twice weekly with 2 ml of growth medium. 30–35 cultures of about 2,000 viable neurons each could be prepared from 40 pups. The cell preparation required about 3 h from the death of the first of 40 animals until the last culture was in the incubator.

Cerebellum cultures were made by trypsinizing minced newborn rat cerebellum, using the method of Okarma and Kalman, 1971, except that the digestion mixture contained 100 mg Trypsin I-300 (Nutritional Biochemicals Corporation, Cleveland, Ohio), 10 mg collagenase (Worthington Biochemical Corp., Freehold, N. J.), and 15 mg Viokase (VioBin Corp., Monticello, Ill.) per 100 ml. After stopping the dissociation with an equal volume of plating medium containing 20% fetal calf serum (FCS), the cells were filtered through 48-μm mesh nylon (Bray, 1970) and plated into Leibovitz's medium (L-15)-CO_2 (see below).

Culture Media

BASAL L-15 MEDIUM: L-15 was the basal medium used in most of these studies. To 1080 ml water was added: one 1-liter packet of dry L-15 (North American Biologicals, Inc., Rockville, Md.), 60 mg imidazole (recrystallized from acetone), 15 mg aspartic acid, 15 mg glutamic acid, 15 mg proline, 15 mg cystine, 5 mg β-alanine, 2 mg vitamin B_{12}, 10 mg inositol, 10 mg choline chloride, 0.5 mg lipoic acid, 0.02 mg biotin, 5 mg *p*-aminobenzioic acid, 25 mg fumaric acid, and 0.4 mg coenzyme A. The pH was adjusted to 7.35 with 1 N HCl; the medium was then sterilized by filtration and stored up to 3 wk at 4°C.

PLATING MEDIUM: The medium used for collecting ganglia and dissociating cells consisted of 100 ml of the basal L-15 described above with the following additions: glucose (2 ml of a 30% wt/vol solution), glutamine (1 ml of a 200 mM solution; Microbiological Associates, Inc., Bethesda, Md.),

[1] *Abbreviations used in this series of papers:* ACh, acetylcholine; BH_4, tetrahydrobiopterin, BSA, bovine serum albumin; CA, catecholamine; DA, dopamine (3,4-dihydroxyphenethylamine); DBH, dopamine β-hydroxylase; $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine; dopa, 3,4-dihydroxyphenylalanine; FCS, fetal calf serum; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine (serotonin); L-15, Leibovitz's medium; MEM, Eagle's minimal essential medium; αMPT, α-methyl-*p*-tyrosine; NE, norepinephrine (1-[3,4-dihydroxyphenyl]-2-aminoethanol); NGF, nerve growth factor; PCA, perchloric acid; SCG, superior cervical ganglion; SDS, sodium dodecyl sulfate; SEM, standard error of the mean; TH, tyrosine hydroxylase.

pen-strep (2 ml of 10,000 U/ml penicillin and 10 mg/ml streptomycin; BioQuest Div., Becton, Dickinson & Co., Cockeysville, Md.), and nerve growth factor (NGF) (final concentration 1 μg/ml), whose preparation is described below.

GROWTH MEDIUM, L-15-AIR: The medium used to grow neurons with few non-neural cells consisted of 100 ml plating medium with the following additives described below: Methocel (0.7 g), fresh vitamin mix (1 ml), bovine serum albumin (BSA) (5 ml), and adult rat serum (5 ml). L-15-air cultures were incubated in a Wedco incubator (Wedco, Inc., Silver Spring, Md.) in a humidified air atmosphere at 36°C.

GROWTH MEDIUM, L-15-CO_2: The medium used to grow non-neural cells as well as neurons consisted of 85 ml basal L-15 and 17 ml 150 mM $NaHCO_3$. To this modified basal medium were added the same ingredients listed above for growth medium L-15-air. L-15-CO_2 cultures were maintained at 36°C in a 5% CO_2-95% air atmosphere.

OTHER GROWTH MEDIA: In some experiments media other than L-15 were tested. Commercial Eagle's minimal essential medium (MEM), Dulbecco's modified MEM, L-15, F-10, and Medium 199 were supplemented with glucose, glutamine, pen-strep, NGF, Methocel, as well as BSA and various sera as described in Results. A 5% CO_2 atmosphere was employed with all of these media except 199 which was incubated in 1% CO_2 to keep the pH near 7, and commercial L-15, which was incubated as for L-15-air. The commercial media were obtained from Microbiological Associates, Inc. and Grand Island Biological Co., Grand Island, N. Y.

ADDITIVES: NGF was prepared through the Sephadex G-100 step of Bocchini and Angeletti (1969), followed by the DEAE step of Varon et al., (1967). The pooled DEAE fractions were concentrated by filtration (Diaflo, UM-10, Amicon Corp., Lexington, Mass.) to 1 mg/ml and stored at −20°C for periods up to 1 yr with no apparent loss of activity. The optimal concentration determined by out-growth assays with neonatal rat SCG explants was 0.2 to 1.0 μg/ml. Routinely 1 μg/ml was used in the media.

Adult rat serum was obtained from rats of either sex purchased from Charles River Breeding Laboratories, Inc. The rats were stunned and killed by decapitation, and blood was collected on ice. After clotting, the serum was separated by centrifugation at 35,000 *g* for 30 min. Serum was stored at 0°C for 12–16 h, recentrifuged to remove clots, and stored at −20°C. Amino acid analyses were performed on a Beckman model 120 C amino acid analyzer (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) by the method of Spackman et al. (1958). The tyrosine concentration in rat serum (from which protein was precipitated by mixing with an equal volume of 2 N HCl) was 75 ± 5 μM (N = 3), which is within the usual mammalian range of 60–90 μM (Altman and Dittmer, 1961; Yamasaki and Natori, 1972).

BSA stock solution was prepared from crystallized, lyophilized BSA (Sigma Chemical Co., St. Louis, Mo.) dissolved in L-15 salts with 0.75 mM imidazole as buffer, pH 7.1. The stock solution, 60 mg/ml in protein, was stored at −20°C.

Methocel (HG 65, 4,000 cps, Dow Chemical U. S. A., Membrane Systems Div., Midland, Mich.) was dissolved in the growth media by 10–24 h of vigorous stirring at 4°C.

Fresh vitamin mix was prepared in 20-ml lots as a 100× concentrate and contained 1 mg 2-amino-4-hydroxy-6,7-dimethyltetrahydropterine ($DMPH_4$) (Aldrich Chemical Co., Inc., Milwaukee, Wis.), 100 mg ascorbic acid (Sigma Chemical Co.), and 10 mg glutathione (Sigma). Before storage at −20°C, the pH was adjusted to 6.0 with 1 N NaOH.

Sera other than adult rat were purchased from Gray Industries (Fort Lauderdale, Fla.), Microbiological Associates, Inc., Grand Island Biological Co., and Flow Laboratories, Inc. (Rockville, Md.); pooled human serum was obtained from the Red Cross.

Culture Dishes

The dishes used to culture the neurons were 35-mm Falcon dishes, prepared as described by Bray (1970) except that plastic (Lux, 25 mm) cover slips were used instead of glass. The cover slip was coated with a collagen solution (Bornstein, 1958, or Wood and Keech, 1960), dried in a dessicator at room temperature for 24 h, and sterilized by ultraviolet irradiation. The cover slip was coated with 0.05-ml double-strength Tryptose Phosphate Broth (Difco Laboratories, Detroit, Mich.) about 15 min before addition of the growth medium. After the growth medium was added (1.9 ml), a glass ring (1.5 cm diameter microslide rings, Arthur H. Thomas Co., Philadelphia, Pa.) was placed in the medium enclosing the central well. The ring ensured that the cells added to the dish would settle on the cover slip. The dish was placed in the incubator a few hours before the cells were added. Tryptose was not included in subsequent feedings of the cultures.

Light Microscopy

Phase micrographs were taken using standard techniques. Nomarski pictures were taken by the method of McMahan and Kuffler (1971).

For studies of catecholamine histochemistry (Dahlstrom and Fuxe, 1964), cultures were washed in freshly prepared 155 mM $(NH_4)HCO_3$, and then were either air-dried or freeze-dried. The cover slips were incubated for 1 h at 80°C in paraformaldehyde equilibrated at 75% relative humidity; controls were

incubated as above but without paraformaldehyde. Glass cover slips were used for histochemistry. Photographs were taken on a Zeiss Universal microscope, with a Xenon light source, BG 12 exciter filter, and barrier filters to pass light primarily in the 410–470 nm range. For comparisons of HCHO-treated and control cultures, pictures were taken and prints were made by using standard exposure times instead of a light meter.

Incubations with Radioactive Compounds

ISOTOPES: The following isotopes were used: [U-^{14}C]tyrosine (455 mCi/mmol), [3,5-^{3}H]tyrosine (30–45 Ci/mmol), [U-^{3}H]glutamate (2.8 Ci/mmol), [2-^{3}H]dopamine (3.5 Ci/mmol), and [U-^{3}H]tyramine (7.3 Ci/mmol) from New England Nuclear, Boston, Mass.; and [2-^{14}C]tyramine (30 mCi/mmol), [U-^{3}H]-tryptophan (3.2 Ci/mmol), [*methyl*-^{3}H]choline (15 Ci/mmol), and [U-^{14}C]histidine (330 mCi/mmol) from Amersham/Searle Corp., Arlington Heights, Ill. All compounds were tested for radiochemical purity by high voltage paper electrophoresis at pH 2 (Hildebrand et al., 1971). Histidine and tryptophan were found to be satisfactorily pure. Some batches of radioactive tyrosine contained a contaminant (less than 0.1%) which electrophoresed in the catecholamine region, and radioactive choline always contained a contaminant (about 1%) that co-electrophoresed with acetylcholine. To remove contaminants, the isotope was taken to dryness under a stream of nitrogen, dissolved in pH 2 buffer, and submitted to high voltage electrophoresis. The appropriate region of the electrophoresis paper was eluted in 1–2 ml of 1 mM formic acid. After three extractions with cold ether to remove traces of the electrophoresis coolant, the sample was taken to dryness and redissolved in 0.1 M HCOOH three times (as suggested by E. A. Kravitz). Samples not treated with formate were toxic to the cultures, as monitored by catecholamine synthesis. Samples were stored at −20°C in 1 mM HCOOH and were found to be free of the original contaminant by electrophoretic analysis.

Glutamic acid commonly contained a contaminant which was uncharged at pH 2; this had to be removed because some contaminants of glutamic acid can be converted to γ-aminobutyric acid (GABA), bypassing the usual glutamic acid decarboxylase step (Molinoff and Kravitz, 1968). Boiling the isotope in 2 N HCl removed the contaminant, but it reappeared over a period of weeks at −20°C; therefore [^{3}H]glutamic acid was always boiled for 1 h in 2 N HCl immediately before use.

For incubations, all isotopes were taken to dryness under a stream of nitrogen and redissolved in medium lacking the compounds in question.

INCUBATIONS: L-15 lacking tyrosine, choline, phenylalanine, glutamine, tryptophan, folic acid, and histidine was purchased from Grand Island Biological Co. The substances not under study as radioactive precursors of neurotransmitters were added back to the medium. With two exceptions, all of the additives in L-15 growth medium (except Methocel, which was never included) were present. For experiments with [^{3}H]glutamate, glutamine was omitted from the medium; in two cases, alanine (which can substitute for glutamine as an amino donor; Nagle and Brown, 1971) was used in place of glutamine. Preliminary experiments with [^{14}C]histidine indicated that imidazole might compete with histidine for uptake, so glycerol-2-phosphate was used as the buffer in experiments with [^{14}C]histidine. Incubations always included radioactive tyrosine.

Cultures were incubated 15 min in growth medium lacking both Methocel and the precursor(s) to be examined, and then were incubated in 100 μl of medium containing radioactive tyrosine at 30 μM, and (when appropriate) another radioactive amino acid at 60 μM, labelled choline at 5 μM, or tyramine at 1 μM. The concentrations of choline and the amino acids except tyrosine approximate blood levels in mammals (Altman and Dittmer, 1961; Yamasaki and Natori, 1972; our own studies), and 30 μM tyrosine is near the saturation value for these cultures (Mains and Patterson, 1973 *a*). The incubations were carried out at 36°C, usually for 8 h.

Sample Analysis

At the end of the incubation, the cultures were washed in 2 ml of warm plating medium containing BSA for about 10 min, and scraped off the dish into 20 μl of pH 2 buffer containing unlabeled standards (in 1000× excess) for the products being studied. Samples were frozen and thawed, and 10 μl of 1% sodium dodecyl sulfate (SDS) was added in pH 2 buffer. After an hour at room temperature, an aliquot was taken to determine the total radioactivity in the sample and the remainder was electrophoresed as described by Hildebrand et al. (1971).

Measurements of the radioactivity remaining on the cover slips showed that 94 ± 3% of the label was routinely scraped off the cover slips, and that cover slips without cells do not retain label. Recoveries of both [^{14}C]tyramine and [^{3}H]dopamine added to the extracts of cultures were 95 ± 3%. In other studies, catecholamines are usually extracted from tissues in 0.4 N perchloric acid (PCA), but in our hands PCA released only 80 ± 5% of the catecholamines extracted by pH 2 buffer and SDS.

Paper electrophoresis strips were cut into 1-cm sections after visualization of the compounds of interest (Hildebrand et al., 1971), eluted with 0.5 ml of 0.1 N HCl, and counted after addition of 4 ml of Aquasol (New England Nuclear). On a Packard model 3320 scintillation counter (Packard Instru-

ment Co., Inc., Downers Grove, Ill.) the counting efficiency was consistently 25% for tritium and 95% for carbon 14. Occasionally, electrophoretograms were scanned as described by Hildebrand et al. (1971).

The identities of NE and DA synthesized by the cultures were established by pH 2 electrophoresis on paper, followed by ascending chromatography perpendicular to the direction of electrophoresis in five solvents: I, methyl ethyl ketone:propionic acid:water (200:65:55); II, *n*-butanol:isopropanol:acetic acid:water (20:40:10:20); III, *sec*-butanol:pyridine:acetic acid:water (300:5:20:50); IV, phenol:0.1 N HCl (100 g:15 ml) saturated with SO_2; and V, *n*-butanol:ethanol:1 N acetic acid (280:80:80). Whenever the conditions of growth or incubation were altered, the identities of NE and DA were verified in solvents I through III. Likewise acetylcholine and γ-aminobutyric acid were identified by electrophoresis followed by chromatography in *n*-butanol:acetic acid:water (60:15:25). Acetylcholine (ACh) was also treated with acetylcholinesterase as described by Hildebrand et al. (1971) and GABA with GABA:glutamic aminotransferase (GABAase) as described by Lam (1972).

RESULTS

Growth of Isolated Neurons

As first demonstrated by Bray (1970) using a simpler L-15 culture medium, sympathetic neurons from superior cervical ganglia of the newborn rat isolated by mechanical dissociation can adhere to a collagen-coated cover slip and grow processes. Neuronal cell bodies are 10–15 μm in diameter immediately after plating, and are virtually devoid of processes except for occasional short remnants (10 μm or less). In our modified L-15-air medium, the cells adhered to the dish and produced processes less than 1 μm thick, with growth cones (Bunge, 1973) (Figs. 1 A, B). Some cells began growth within a few hours of plating and grew processes longer than 100 μm in their first 12 h in culture; others did not begin to elaborate processes for almost a day. After a few days the tendency of growth cones to follow existing nerve processes made isolated growth cones more difficult to find than in younger cultures. As the cells got older, their cell bodies grew to 25–40 μm in diameter. Nuclei often remained eccentric for many weeks (Fig. 1 C) as is also seen in vivo for several weeks after the efferents from the SCG are cut (Matthews and Raisman, 1972). Less than 10% of the nuclei in cultures grown in L-15-air belonged to non-neural cells, even after three or more weeks in culture. "Non-neural cells" here refers to all cells lacking the rounded cell body, clear nucleus and nucleoli, and processes characteristic of neurons (e.g., Fig. 1). The vast majority of cells in these cultures were neurons, and much of the surface of cover slip was covered with an intricate meshwork of nerve processes (Fig. 1 C). An example of the networks formed between cells is shown at lower magnification in Fig. 2. The processes seen in older cultures, which were as much as 30 μm thick, were probably bundles of small fibers, rather than very thick single processes. This is illustrated by Nomarski optics in Fig. 3.

Bray, Potter, and Furshpan observed that SCG cultures obtained using the mechanical dissociation procedure but grown in MEM developed a monolayer of non-neural cells as well as neurons (unpublished results). We extended these findings to cultures grown in L-15-CO_2, in which non-neural cells proliferated rapidly to form a monolayer in 2–3 wk (Fig. 1, D–F). To determine if the growth of non-neural cells in a CO_2 atmosphere and the lack of non-neural cells in an air atmosphere was due to the presence and absence of bicarbonate, some cultures were grown in L-15-CO_2 growth medium (i.e., L-15-air plus isotonic $NaHCO_3$) and some in a modified L-15-air growth medium diluted with isotonic NaCl and maintained in an air atmosphere. Basal L-15 in both media was thus diluted by 17%. The cultures grown in the air medium were identical to the normal L-15-air cultures but the CO_2 cultures developed a monolayer of non-neural cells over a period of 2–3 wk, indicating that bicarbonate strongly stimulated multiplication of non-neural cells. While the neurons in L-15-CO_2 tended to be more flattened than those grown in L-15-air, the neurons in the two media were indistinguishable in their capacity to synthesize and accumulate catecholamines (see below).

Comparisons of Media

In arriving at the final growth medium composition, many combinations of ingredients were employed. A few of these are listed in Table I in order to demonstrate the dependence of the survival and health of the dissociated neurons in low-density culture on the medium employed. The media were compared with respect to the ability of 1-wk old cultures to accumulate and utilize radioactive tyrosine (primarily for protein synthesis, but also for catecholamine production, metabolites, etc.) in an

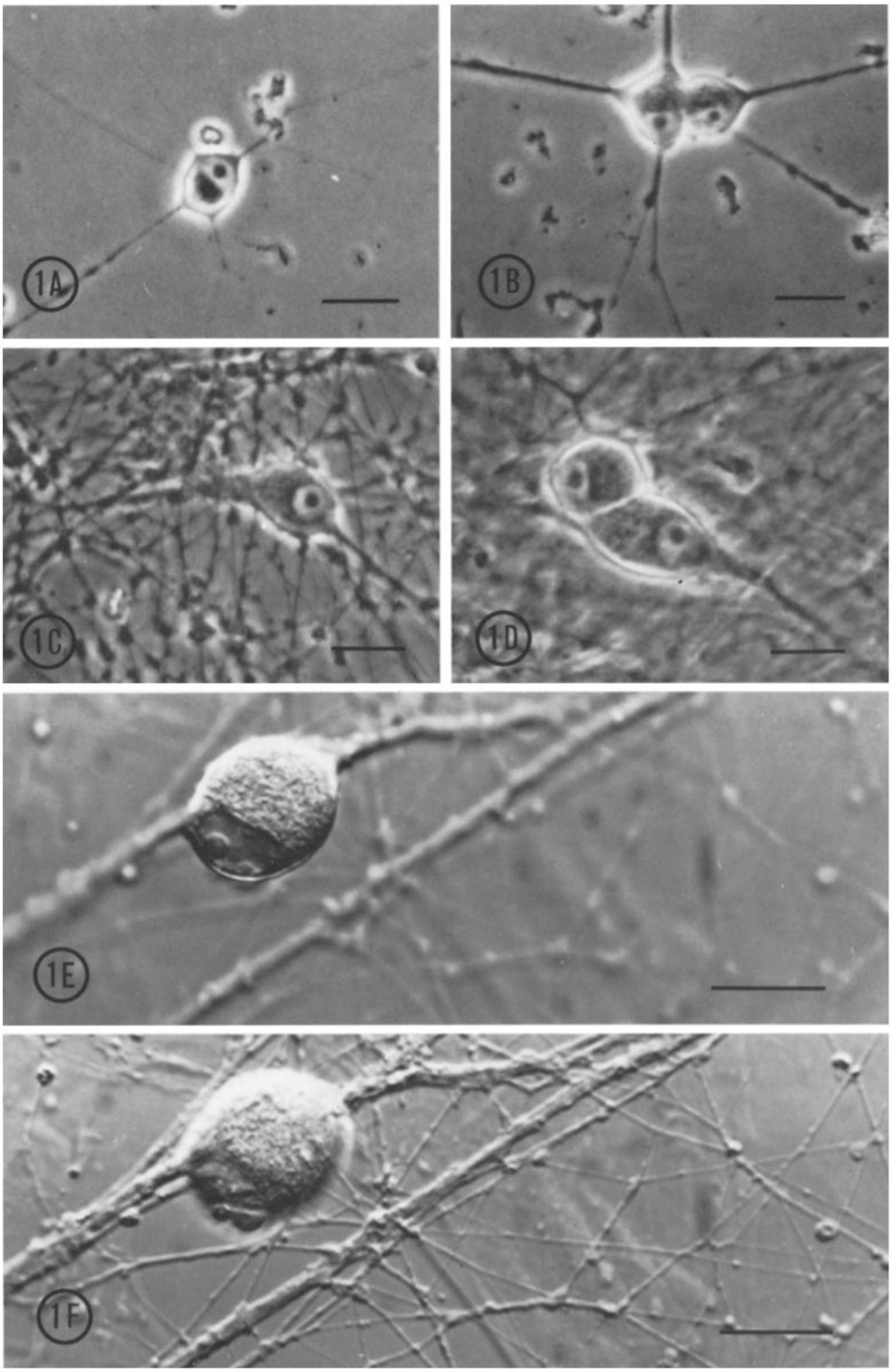

FIGURE 1 Microscopy of the cultures; A-D phase optics (× 420) and E-F Nomarski optics (× 640). (A) Neuron in 2-day old culture grown in L-15-air. (B) 6 days old, L-15-air. (C) Unusually small neuron, 2 weeks old, L-15-air. (D) 3 wk old, L-15-CO_2. (E and F) Different focal planes of a L-15-CO_2 culture, 2 wk old, showing the neuronal cell body, fiber network, and non-neural cells in background. In these and all other micrographs, the bar represents 25 μm.

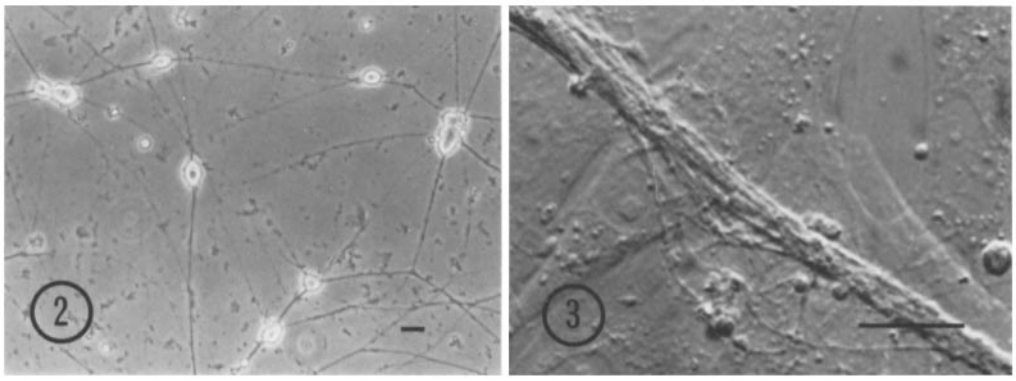

FIGURE 2 Interconnecting network of processes formed in 6-day old cultures grown in L-15-air. × 120.

FIGURE 3 A large nerve fiber crossing underlying non-neural cells; from a 3-week old L-15-CO_2 culture. The fiber appears to be a bundle of many fine processes. × 560.

8-h incubation. The concentration of tyrosine was not limiting and the incorporation was linear with time (Mains and Patterson, 1973 *b*). The comparison was based primarily on neuronal metabolism, as proliferation of non-neural cells was not appreciable in 1-wk old cultures (Figs. 1 *b*, 2). The data for L-15-air (neurons only) and L-15-CO_2 (neurons plus non-neural cells) support this visual observation.

The modified L-15 growth media with rat serum and Methocel were the best combinations found thus far for SCG cells (Table I). L-15-CO_2 was also very good for growth of cell lines such as 3T3 cells and for primary dividing cells such as atrium (data not shown). The pH of commercial L-15 was lowered by using imidazole buffer to maintain pH 7.3. The osmolarity of commercial L-15 plus glucose is about 340 mOsM; we routinely lowered this to about 315 mOsM, which is nearer the value for rat serum (300). Methocel was essential, and ficoll, thiolated gelatin, and polyvinylpyrrolidone would not substitute for Methocel (not shown). The mixture of amino acids and vitamins added to L-15 also promoted neuronal survival; the short-term growth of neurons without serum shown in Table I was not possible without the additive mixture. Fetal calf serum was toxic for the first week's growth of the neurons. In fact, no serum was required for 1 wk; the serum-free cultures also synthesized and accumulated catecholamines to the same extent as the normal cultures, and generated action potentials (R. E. Mains, unpublished). Serum was, however, necessary for growth beyond 1 wk. Dulbecco's MEM was the best alternative medium tested, but it was only one-third as good as the complete L-15 growth media. The high glucose concentration and the addition of penicillin and streptomycin have been employed by many other investigators. Nerve growth factor (1 μg/ml) was required for survival and growth; we observed no neurons in cultures plated into L-15-air medium without NGF. Neuronal survival was very poor (less than 10%, as judged by CA production) at 0.1 μg/ml; 10 μg/ml NGF was not better than 1 μg/ml NGF (as judged by morphology and CA production).

It should be emphasized that the findings in Table I apply only to low-density cultures of fully dissociated neurons. Neuronal growth in cultures of clumps or explants from the SCG did not show this marked dependence on L-15 and the additives.

Catecholamine Histochemistry

Differentiated sympathetic neurons in vivo give a positive reaction in the Falck-Hillarp histochemical test for catecholamines. Therefore, the same histochemical procedure was applied to the dissociated sympathetic neurons in culture. An example of formaldehyde-treated and control cultures of the same age is shown in Fig. 4. A marked fluorescence was produced by formaldehyde treatment. Further, the neurons in cultures grown in L-15-CO_2 also exhibited catecholamine fluorescence, while the background cells did not (Fig. 5). The neural processes, as well as cell bodies, fluoresced, indicating the presence of catecholamines throughout the neurons. Given the positive histo-

TABLE I

Comparisons of Neuron Growth in Various Culture Media

Basal medium	Protein additives: 5% rat serum	5% BSA	10% FCS	Methocel	cpm, as %*
L-15-air	+	+	−	+	100 ± 15
	+	+	−	−	8 ± 3
	−	−	+	+	27 ± 5
L-15‡	+	+	−	+	20 ± 3
L-15-CO_2	+	+	−	+	91 ± 12
	−	+	−	+	101 ± 7
	+/2	+/2	+/2	+	29 ± 6
MEM	+	+	−	+	7 ± 2
	−	−	+	+	2 ± 1
Dulb. MEM	+	+	−	+	31 ± 11
	−	−	+	+	10 ± 4
F-10	+	+	−	+	11 ± 4
Med. 199	+	+	−	+	18 ± 1

All cultures were grown for 6 days in the medium indicated and then incubated in that same medium with [^{14}C]tyrosine. Cover slips were dissolved in 1 ml of Insta-Gel (Packard Instrument Co., Inc.) and counted after addition of 10 ml Aquasol (New England Nuclear). Counting efficiency was greater than 95%. All samples were run in triplicate in one or two separate experimental series.

* Total cpm accumulated in an 8-h incubation, expressed as % of the value for complete L-15-air ± SEM. 100% = 7,000 cpm.

‡ Unmodified, from commercially supplied bottles.

chemical results, it was of interest to determine which catecholamines were produced in the cultures, as well as to ascertain whether other potential neurotransmitters were also produced.

Synthesis and Accumulation of Catecholamines

PRODUCT IDENTIFICATION: In order to show directly that the cells synthesized and accumulated catecholamines, cultures were incubated with radioactive tyrosine, extracted with pH 2 buffer, and the products examined by paper electrophoresis. The cells took up the tyrosine and produced radioactive proteins which stayed near the origin of the electrophoresis strip (Fig. 6); peaks of radioactivity were also found in the free tyrosine, norepinephrine, and dopamine areas of the electrophoretogram. The identity of NE and DA were confirmed in five solvents, as listed in Materials and Methods. All of the radioactivity that co-electrophoresed with NE and DA in the first dimension comigrated with authentic NE and DA in ascending chromatography in the second dimension. The chromatographic pattern for solvent I, including positions of other major tyrosine derivatives, is shown in Fig. 6. The radioactivity data for a sample which was electrophoresed then chromatographed in solvent I is given in Fig. 7.

The presence of background or non-neural cells did not alter the synthesis and accumulation of catecholamines by the neurons, as the data in Table II indicate. The cultures with non-neural cells, accumulated substantially more radioactivity than did cultures without non-neural cells, but neither the total catecholamines produced nor the NE/DA ratio was altered by the presence of other cells.

PRECURSORS The hydroxylation of tyrosine to form dopa by tyrosine hydroxylase (TH) is gener-

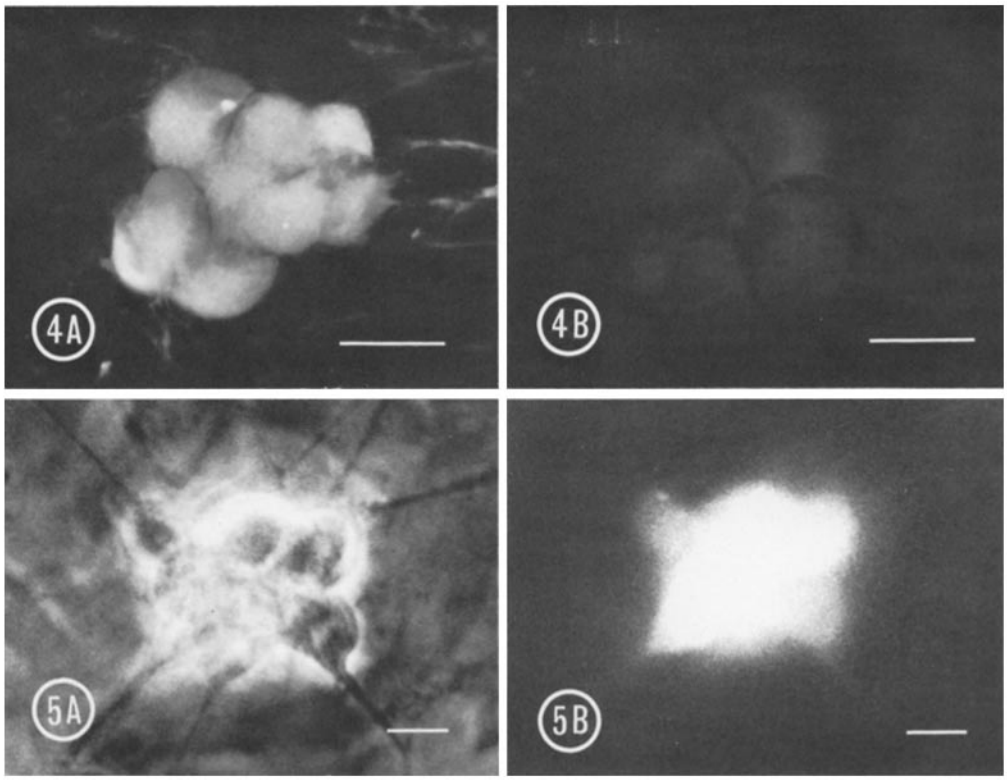

FIGURE 4 (A) Neurons treated by the Falck-Hillarp method for catecholamines; grown for 2 wk in L-15-air. (B) Control (without formaldehyde), otherwise identical to (A). × 560.

FIGURE 5 (A) Phase micrograph of a group of neurons grown for 2 wk in L-15-CO_2. Note non-neural cells in background. (B) The neurons shown in (A) display the fluorescence characteristic of catecholamines while the background cells do not. × 320.

ally accepted to be the rate limiting step in catecholamine biosynthesis (for review, see Molinoff and Axelrod, 1971). However, since [^{3}H]tyrosine can spontaneously oxidize to form [^{3}H]3,4-dihydroxyphenylalanine (dopa), it was possible that the cultures produced radioactive catecholamines from [^{3}H]dopa only, thereby bypassing the normal control step. Two types of experiments were done to address this problem.

First, medium containing [^{3}H]tyrosine at 30 μM was incubated for 8 h without cells, and then analyzed for dopa by paper electrophoresis in pH 6.2 borate buffer (B. R. Talamo, personal communication). The concentration of [^{3}H]dopa was always found to be less than 10 nM (less than 20 cpm above a background of 30 cpm). Therefore isotope dilution experiments were performed to determine if excess unlabeled dopa (100 nM) in the medium would suppress the production of [^{3}H]catecholamines in cultures incubated in 30 μM [^{3}H]tyrosine. 100 nM dopa had no effect on the synthesis and accumulation of [^{3}H]catecholamines, indicating that the [^{3}H]dopa formed spontaneously was not the major precursor of the [^{3}H]catecholamines under study.

A second way to investigate the precursors was to use the tyrosine hydroxylase inhibitor α-methyl-p-tyrosine (αMPT) (Levitt et al., 1967). αMPT depressed the synthesis and accumulation of [^{3}H]-catecholamines in the cultures. The inhibitor also had a definite, though less marked, effect on the overall utilization of [^{3}H]tyrosine by the cultures, although no suppression of the uptake of [^{3}H]tyrosine into the free tyrosine pool was observed. For example, at 10 μM αMPT, the total incorporation of radioactivity was 47 $\pm$ 9% of control, and CA synthesis and accumulation was 15 $\pm$ 4% of control values. While this experiment also supports the

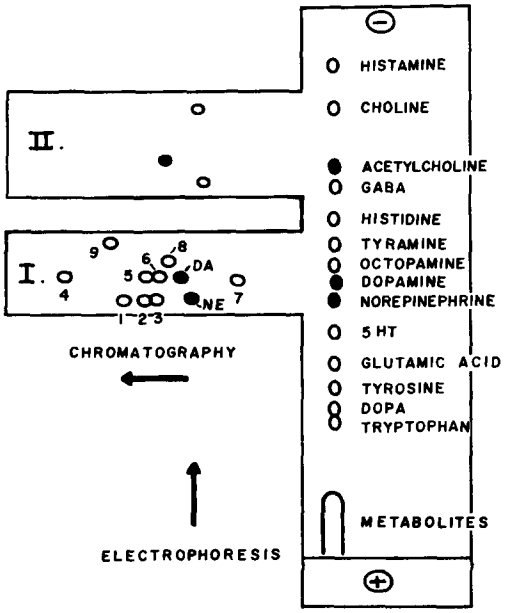

FIGURE 6 Separation scheme employed to identify transmitter candidates. Electrophoresis was carried out in the first dimension and was followed by chromatography in the second dimension as described in Materials and Methods. The chromatography solvent used in this example was number I. (1) *o*-methylepinephrine; (2) *o*-methylnorepinephrine; (3) epinephrine; (4) synephrine; (5) *o*-methyldopamine; (6) epinine; (7) alanine; (8) octopamine; (9) tyramine. Electrophoresis step modified from Hildebrand et al., 1971.

13	11	3	5	3
	8	1	1	0
	9	5	0	0
	6	2	0	3
	6	260	3	3
	10	370	570	3
5	2065	945	25	3
10	575	34	3	0
B	41	7	1	2
A	8	5	2	1

FIGURE 7 A single 10-day old L-15-air culture was incubated with [^{3}H]tyrosine for 8 h as described in Materials and Methods. The sample was electrophoresed at pH 2 in direction A, followed by ascending chromatography in direction B in solvent I. The shaded areas mark the positions of authentic NE and DA; DA is the upper spot. The numbers are cpm above a background of 25 cpm. The solvent front was in the top row of squares. All the radioactivity found in the catecholamine region of the electrophoretogram is shown in the figure.

contention that the cultured neurons possess an active tyrosine hydroxylase, the evidence is not as strong as in the isotope dilution case since αMPT did not act selectively on catecholamine synthesis.

ISOTOPE EFFECT: It was necessary to check whether the activity of tyrosine hydroxylase was the same with the two substrates used in these experiments, [U-^{14}C]- and [3,5-^{3}H]tyrosine, since there could be substantial isotope effects modifying the activity of tyrosine hydroxylase toward the two substrates. Experiments were therefore performed using mixtures of the two isotopes, and the ^{3}H/^{14}C ratio in the catecholamines produced was determined. In all cases, the ratio was within 5% of the predicted value, which is the limit of the accuracy of our double-label determinations. Therefore isotope effects on tyrosine hydroxylase activity are not significant under the present conditions.

Other Neurotransmitters

Mature, differentiated sympathetic neurons synthesize and accumulate catecholamines; it is not clear from in vivo studies whether SCG cells make other neurotransmitters as well (see Discussion). Therefore cultures were incubated in media containing radioactive tyrosine plus the labeled precursor of one other potential neurotransmitter. The results could then be presented as ratios of the production of other neurotransmitters to the production of catecholamines. However, for the comparison to be valid, there must have been significant uptake of both radioactive tyrosine and the other precursor under study. In one example, using [^{3}H]-glutamate and [^{14}C]tyrosine, roughly equimolar amounts of glutamate and tyrosine were accumulated by the cultures. Catecholamine synthesis from [^{14}C]tyrosine was 3,300 cpm, but no more than 10 cpm of GABA above a background of 50 cpm were present. By applying the appropriate correction factors for the difference in specific activity of the precursors, quenching, and the loss of label when precursor is converted into product, it was possible to calculate the molar ratio of [^{3}H]-GABA produced from [^{3}H]glutamate to [^{14}C]catecholamines produced from [^{14}C]tyrosine. The cal-

TABLE II

The Effect of Non-Neural Cells on Catecholamine Synthesis and Accumulation

Exp.	Medium	Total	NE	DA
		cpm × 10^{-3}	*cpm*	*cpm*
1	Air	97 ± 9	4,630 ± 250	1,375 ± 300
	CO_2	360 ± 55	4,590 ± 600	1,730 ± 200
2	Air	110 ± 17	3,100 ± 300	1,980 ± 250
	CO_2	290 ± 45	3,250 ± 450	2,100 ± 200

3 (exp. 1)- and 2 (exp. 2)-wk old sister cultures grown in L-15-air or L-15-CO_2 media were incubated 8 h with radioactive tyrosine. In exp. 1, [^{3}H]tyrosine (30 Ci/mmol) was present at 30 μM, and was supplemented to a final concentration of 200 μM. In exp. 2, a similar procedure was used to make the final tyrosine concentration 100 μM. The elevated concentrations of tyrosine used in these experiments were necessary to provide sufficient tyrosine for CA synthesis in the presence of a monolayer of non-neural cells (unpublished observations). The results are expressed as mean ± SEM of quadruplicate determinations; total cpm is the total radioactivity accumulated by the culture in the 8-h incubation.

culation of molar ratios in this manner provided a convenient method of comparing data between experiments. In this particular example, the ratio of cpm

$$\frac{\text{GABA}}{\text{catecholamines}} < \frac{10}{3300}$$

became the apparent molar ratio

$$\frac{\text{GABA}}{\text{catecholamines}} < \frac{1}{600}$$

For justification of the term "apparent molar ratio", see the Discussion.

Table III shows the results of a number of experiments of this type, assaying for the production of transmitters other than NE and DA. In the case of amino acid precursors, the cultures accumulated more moles of those precursors than of labeled tyrosine but choline was accumulated only about 70% as fast as tyrosine on a molar basis. No detectable radioactivity was found in other transmitters, with the exception of small amounts of acetylcholine and octopamine.

[^{3}H]acetylcholine was produced from [^{3}H]choline in cultures older than 2.5 wk when eserine was present; eserine presumably blocked the intracellular breakdown of acetylcholine, as has been seen in other systems (Birks and MacIntosh, 1961; Potter, 1970). The identity of the acetylcholine formed was confirmed by electrophoresis followed by chromatography; furthermore, pretreatment of the extract with acetylcholinesterase quantitatively converted the radioactive acetylcholine into choline.

Table III also shows that production of acetylcholine is not due to non-neural cells, since confluent cultures containing only non-neural cells do not make acetylcholine, even in the presence of eserine. The non-neural cultures were produced by growing SCG explants without NGF in L-15-CO_2.

Octopamine was not detected using tyrosine as precursor, even when pargyline was included to retard breakdown. Small amounts of octopamine were made from tyramine, though the importance of tyramine as a biological precursor is not clear (see Discussion). Serum dopamine β-hydroxylase (DBH) was not likely to be the source of the octopamine in the cultures, since medium containing [^{3}H]tyramine, pargyline, and rat serum was incubated without cells and did not contain [^{3}H]octopamine.

To determine whether the failure to observe synthesis of other transmitters was due to the particular culture conditions used, GABA synthesis by neurons known to contain GABA in vivo was tested (Otsuka et al., 1971). 2-week old cultures of dissociated cerebellar cells were incubated with [^{3}H]glutamate; the samples from the cultures were pooled, split into equal aliquots, and one-half was treated with GABAase. The samples were analyzed by electrophoresis followed by chromatography. Each culture produced about 2,000 cpm which comigrated with GABA; at least 85% of the radioactivity was sensitive to GABAase. No similar tests

TABLE III

Neurotransmitter Synthesis

Transmitter	Precursor	No. of expts.	Ratio: Apparent Moles of Other Transmitter* / Moles of CA	Culture age (weeks)
Octopamine	Tyrosine	25‡	<1/4,000	1–4
	Tyramine	12§	1/100–1/30‖	4
ACh	Choline	13¶	<1/2,500	>2.5
		10**	<1/1,200	<2.5
		10**	0–1/30	>2.5
		4‡‡	<10 cpm‡‡	4
GABA	Glutamate	4	<1/600	3
Histamine	Histidine	4	<1/1,000	3
5-HT	Tryptophan	3‡	<1/670	3

* Based solely on radioactivity data. Precursor pools are assumed to be fully equilibrated (see Discussion).

‡ Usually electrophoresis was followed by chromatography, to lower background. Sometimes pargyline-HCl (Ciba Ltd., Duxford, Cambridge, Eng.), 0.5 mM, prepared fresh in saline, was included.

§ Samples were split into halves; one was electrophoresed at pH 2, one at pH 10.

‖ No radioactive tyrosine in the medium. Assuming the usual catecholamine synthesis for this age (Mains and Patterson, 1973 *b*), the 150 to 400 cpm observed correspond to 1/100 to 1/30 of the CA production.

¶ No eserine present during incubations; 4 of the 13 were washed after incubation in radioactive medium with medium containing 50 μg/ml eserine sulfate (Merck Chemical Div., Merck & Co., Rahway, N. J.).

** Incubation included eserine, 15 μg/ml.

‡‡ Non-neural cells only (SCG explants grown without NGF); 15 μg/ml eserine included. No radioactive tyrosine in the medium.

have yet been made for histamine and 5-hydroxytryptamine (5-HT).

DISCUSSION

Growth of the Cultured Neurons

Growth of sympathetic nervous tissue in vitro has been greatly facilitated by the discovery of nerve growth factor, and considerable work has been carried out on explants from sympathetic ganglia. However, in order to improve visualization and electrical recording from the neurons, to ensure elimination of any preexisting nerve-nerve synapses, and to obtain control of the medium surrounding the cells, we developed methods for obtaining and growing dissociated neurons in low-density cultures. These dissociated neurons, unlike explants and even small clumps of as few as 50 cells, displayed rather strict growth medium requirements for long-term growth.

The growth medium described above permitted the establishment of large numbers of cultures of dissociated neurons with variations in cell number from culture to culture of less than 20%. Close control of cell number was essential for biochemical comparisons between cultures. L-15 is richer in several categories of ingredients than many other commercial media, and our data show that the dissociated neurons grew better in the richer medium (Table I). Methocel (methylated cellulose) made the medium more viscous, protecting the cells from vibrations and movements of the culture dish, and may also have provided certain heavy metal ions (Thomas and Johnson, 1967). The fresh vitamin mix added to the medium supplied cofactors and reducing power necessary for catechola-

mine biosynthesis as described in an accompanying paper (Mains and Patterson, 1973 *a*).

We used rat serum for several reasons; the foremost was that the cells grew better with the homologous type than with fetal calf, calf, horse, rabbit, or swine (recent experiments have shown that pooled human serum supports neuronal growth as well as rat serum, however). Since we prepared the rat serum ourselves, reproducible methods were ensured. Also, the animals used as a source are from an inbred strain which will be permanently available, thus avoiding the well-known problem of the inconstancy of commercial sera. Adult serum was used, since preliminary work indicated that serum from younger rats was not as good as adult serum. 3T3 cells also grew better in rat serum than in fetal calf serum (data not shown), but the serum selectivity was not as marked as for neurons; Paul et al., 1971, also found that 3T3 cells grew better in rat serum than in FCS. The fact that FCS mixed with rat serum gave the same poor growth seen with FCS alone is consistent with the suggestion that FCS contains factors inhibitory to the expression of some differentiated functions (Bissell and Tilles, 1971).

Non-neural Elements

Phase microscopy showed that the neurons grew in culture, both in the diameter of the cell body and length of the processes. However, even after many weeks in L-15-air, less than 10% of the cells seen in phase microscopy were non-neural (Figs. 1, 2). Further analysis using Nomarski optics and electron microscopy (P. Claude, personal communication) has as yet revealed no glial or satellite cells wrapped around or adhering closely to the neurons. Thus the neurons may be conveniently grown in the virtual absence of non-neural cells without subjecting them to rigorous separation procedures or antimitotic poisons. Since the growth of non-neural cells is supported by making a relatively simple change in the medium, investigation of the role of these cells should be possible. It should also be noted that this change in the medium did not affect the ability of the neurons to synthesize and accumulate catecholamines (Table II). The lack of a bicarbonate requirement on the part of the neurons may be related to the reported lack of the enzyme carbonic anhydrase in the nerve cells of the central nervous system (Giacobini, 1961).

Characteristics of the Cultured Neurons Resembling those of Mature, Differentiated Sympathetic Neurons

Considerable progress has been made in recent years in the development of techniques for the maintenance of differentiated properties of various cell types in culture (e.g. Cahn et al., 1967; Yaffe, 1969; Sato et al., 1970; Yasamura, 1968), and detailed comparisons of cultured cells with corresponding cells in vivo are being made. A comparison of the cultured sympathetic nerve cells with SCG cells in vivo is discussed below.

CELL TYPES: As mentioned previously, the SCG contains two distinguishable nerve cell types in vivo. Given our observations, the neurons in these cultures resemble the "principal" or NE cells of the SCG in the size and shape of the cell body, and the number and size of the processes. Furthermore, the changes in the appearance and size of the nerve cell bodies parallel the changes in principal cells seen in vivo (Eranko, 1972). The data on the turnover and synthesis of catecholamines in the cultures presented in the accompanying paper (Mains and Patterson, 1973 *a*) support the hypothesis that there are few if any "small, intensely fluorescent" cells present and functioning normally in the cultures.

CELL DIVISION: One of the distinguishing characteristics of mature nerve cells is that they do not divide. Therefore it was not surprising that the number of neuronal cell bodies in these cultures did not appear to increase with time. Counts of nerve cell bodies (not shown) indicated that no more than 20% of the nerve cells could have divided in culture, but experimental scatter made a more rigorous determination difficult. Preliminary experiments with [^{3}H]thymidine (Patterson, Furshpan, and Mains, unpublished) have shown heavy nuclear labeling in non-neural cells; of approximately 4,000 neurons examined, none had grains over their nuclei. It is not clear whether neuronal cell division occurs after birth in the rat SCG in vivo.

NEUROTRANSMITTER BIOSYNTHESIS AND ACCUMULATION: Another characteristic property of neurons is their ability to synthesize and store high concentrations of neurotransmitters. In the sympathetic nervous system, NE and DA are known to be synthesized and accumulated, but there is evidence that at least two other potential transmitters are also present. Some postganglionic sym-

pathetic neurons utilize acetylcholine as their transmitter (Uvnas, 1954); further, denervation does not remove all the choline acetylase or aetylcholine from the SCG (Black et al., 1972; Brown and Feldberg, 1936). In addition, sympathetic endings in various target tissues contain octopamine, a noncatechol tyrosine derivative which is released by nerve stimulation (Molinoff and Axelrod, 1972). Our cultures produced all four substances, but it has not yet been possible to determine whether single sympathetic neurons synthesize, accumulate, and release several of these substances or whether there are distinct classes of sympathetic neurons, each utilizing only one or a few of these compounds.

The fluorescence histochemical method of Falck and Hillarp, which is specific for catecholamines, was used to demonstrate the accumulation of catecholamine in the cultured neurons. In order to verify that this fluorescence actually represented catecholamine synthesized and accumulated in vitro, we applied the method of Hildebrand et al., 1971, to the cultures. This procedure, with added chromatography steps, demonstrated that the neurons were synthesizing and accumulating NE and DA in culture (Figs. 6 and 7).

The same techniques were used to demonstrate the synthesis and accumulation of small amounts of acetylcholine in older cultures (Table III). Eserine blocks intracellular breakdown of ACh in the rat diaphragm (Potter, 1970) and presumably in the preganglionic endings of the SCG (Birks and MacIntosh, 1961). A similar effect was observed in our cultures. We think it most unlikely that the ACh synthesis occurred elsewhere than in the neurons, because cultures consisting of a monolayer of non-neural ganglionic cells without neurons (produced from explants grown without NGF) showed no ACh synthesis and accumulation in the presence or absence of eserine (Table III). Also unlikely is the possibility that the ACh was produced by residual presynaptic endings adhering to the cultured neurons; acetylcholine synthesis was not detectable until the cultures were 2–3-wk old, at which time the endings would presumably have degenerated. The presynaptic endings in vivo degenerate within 3 days after preganglionic denervation (Hamori et al., 1968). Furthermore preliminary electron microscopy of young cultures has revealed no evidence of residual synaptic endings on the neurons (P. Claude, personal communication). Experiments are in progress to determine whether the same neurons synthesize acetylcholine and catecholamines.

Synthesis and accumulation of octopamine was not detected in the cultures with radioactive tyrosine as the precursor, even in the presence of the breakdown inhibitor pargyline. However, the neurons converted labeled tyramine to octopamine. This is consistent with experiments in vivo (Brandau and Axelrod, 1972) which showed that injected tyramine stimulates octopamine accumulation in the heart far better than tyrosine. Furthermore, incubations of fresh rat atrium with radioactive tyrosine and tyramine in vitro (slices) showed tyramine to be the more effective precursor for octopamine synthesis (Mains and Patterson, unpublished data). Kopin et al., 1965, suggested that tyrosine is converted to tyramine only outside the nervous system.

Analysis using the same methods produced no evidence for synthesis and accumulation by the cultures of the other transmitter candidates GABA, 5-HT, and histamine. However, there are several reasons why the apparent lack of production of these transmitters could be deceiving. The neurons could have large unlabeled pools of some of the precursors, and the labeled precursor would then be isotopically diluted. An indirect argument suggests this is unlikely, however. Studies of a variety of other cell types in culture have shown that intracellular amino acid pools are in rapid equilibrium with the extracellular medium (Eagle, 1959). As is shown in an accompanying paper (Mains and Patterson, 1973 *a*) the uptake of exogenous radioactive tyrosine into the neuronal intracellular free tyrosine pool reaches steady state in less than 1 h. The labeled pool can also be chased in less than 1 h. Therefore, it is unlikely that the synthesis of GABA, for example, could have proceeded in the cultures for 8 h, drawing only from a pool of unlabeled glutamate (substantial amounts of all the radioactive precursors were accumulated by the neurons). A similar argument applies to the other potential transmitters derived from amino acids. Likewise, the intracellular choline pool is sufficiently small to be chased rapidly by [^{3}H]choline in the medium; less than 1% of the radioactive choline accumulated by the cultures in 8 h is present as free choline. Thus the isotopic data, presented as apparent molar ratios in Table III, may reasonably be taken as representative of the net synthesis and accumulation by the cultures in 8-h incubations.

Another possible cause for the lack of synthesis and accumulation of transmitters other than NE, DA, octopamine, and ACh is the absence of a cofactor or cosubstrate in the medium, rather than an inherent inability of SCG cells to make and store other transmitters. In the case of GABA at least this is unlikely, since cerebellar cultures grown and incubated in the same way as the sympathetic cells produced substantial amounts of GABA.

As the form of the data in Table III implies, these studies can only place limits on the amount of other transmitter candidates synthesized; the limit of detectability depends on the specific activity of the precursors. Nothing can be said from our data about the potential function of common cell metabolites such as glutamate, aspartate, and glycine as neurotransmitters in these cultures.

COMPARISON OF RATES OF CATECHOLAMINE SYNTHESIS IN VIVO AND IN VITRO: A rough estimate can be made of the catecholamine synthesizing ability of sympathetic cells in vivo, for comparison with the cells in culture. Combining the data of Brodie et al., 1966, for NE synthesis in adult rat SCG (1.5 μg/g wet weight/h) and the data of Larrabee, 1970, on the size and cell count in the adult rat SCG (wet weight = 1 mg; 40,000 neurons), the rate of NE synthesis per cell per hour in vivo is estimated as 0.2 fmol/cell/h. Similarly, the data in Table II (and the accompanying paper; Mains and Patterson, 1973 *b*) for three week old SCG cultures provides an estimate of catecholamine synthesis (allowing for quenching and loss of one tritium, ignoring possible synthesis of unlabeled catecholamines, and using 2,000 neurons per dish) as 0.3 fmol/cell/h.

We do not yet know whether there are adrenergic synaptic endings in the cultures, so a comparison of the cultures only to the cell bodies in vivo may be appropriate, in which case the cultured cells surpass their counterparts in vivo. On the other hand, in vivo there is much more NE in the terminals of a sympathetic neuron than in the cell body (Dahlstrom and Haggendal, 1966) while the rate of turnover is far slower in the periphery than in sympathetic ganglia (Brodie et al., 1966). Together, these facts suggest that more than 10% of the catecholamine produced by a sympathetic neuron is made at or near the cell body. Thus, if the cultures contain endings as well as cell bodies, the cultures are within an order of magnitude of the in vivo case. From this very rough comparison, the cultures appear capable of levels of synthesis similar to that in vivo. Experiments are in progress to examine the effect of target cells on the catecholamine metabolism of the cultured SCG cells.

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