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Markers for Gene Expression in Cultured Cells from the Nervous System

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SAMUEL H. WILSON,* BRUCE K. SCHRIER,‡ JOHN L. FARBER,§ EDWARD J. THOMPSON, ROGER N. ROSENBERG,¶ ARTHUR J. BLUME,‖ AND MARSHALL W. NIRENBERG

From the Laboratory of Biochemical Genetics, National Heart and Lung Institute, National Institutes of Health, Bethesda, Maryland 20014

SUMMARY

Methods are presented for preparation of extracts from cultured cells from the nervous system and for study of choline acetyltransferase, acetylcholinesterase, glutamate decarboxylase, and catechol *O*-methyltransferase activities. These enzyme activities are markers that can be used for studying gene expression in neurons. The methods are sufficiently sensitive so that all assays can be performed with protein harvested from one Petri dish. Activities of the marker enzymes were assessed in surface cultures of newborn mouse brain cells, and in glial and nonbrain cell lines. Low activities of choline acetyltransferase, acetylcholinesterase, and glutamate decarboxylase were detected in all the cells tested. All of these activities, and particularly glutamate decarboxylase, were higher in cultured brain cells from newborn animals than in non-neuronal cell lines. Glutamate decarboxylase activity in glial cells and in brain cells was inhibited more than 95% by 1 mM amino-oxyacetic acid.

Techniques have been developed in this laboratory and others for culture of differentiated neurons (1–10). Activities of enzymes important in neuronal cell metabolism are useful parameters for following cell maturation and exploring steps in differentiation in such cultures.

The purpose of this communication is to describe a set of methods used to explore the expression of genes that determine the metabolism of molecules involved in intercellular communication in the nervous system. Simple, convenient methods are presented for preparing cell-free extracts from surface cultures and for assays of acetylcholinesterase (EC 3.1.1.7), catechol *O*-methyltransferase (EC 2.1.1.1), choline acetyltransferase (EC 2.3.1.6), and glutamate decarboxylase (EC 4.1.1.15) activities. The assays presented are extensive modifications of previously published procedures.

Activities of the marker enzymes were assessed in a variety of glial and nonbrain cell lines as well as in surface cultures of newborn mouse brain cells. The results document properties and usefulness of the methods presented and also show that newborn brain cells in surface culture attained levels of glutamate decarboxylase activity significantly higher than in non-neuronal cell lines. A preliminary report of this work has been presented (11).

METHODS

Isolation and Culture of Newborn Mouse Brain Cells

Whole brains from newborn Balb/c (National Institutes of Health stock) mice were placed in Solution D (137 mM NaCl, 5.4 mM KCl, 0.17 mM Na_2HPO_4, 0.22 mM KH_2PO_4, 5.5 mM glucose, and 5.9 mM sucrose) pH 7.2, 340 mOsM (modified Puck's D1 solution (12)) at 0–4°, weighed, washed several times with Solution D, and minced to about 1-mm³ pieces with iris scissors. The minced tissue was subjected to two 15-min treatments with 0.25% crude trypsin (Nutritional Biochemicals 1:250 or Difco 1:300) in Solution D (100 ml per g of tissue) at 37° with constant mild swirling. After each treatment, tissue pieces were allowed to sediment and the dissociated cells that remained suspended were collected by decantation. Tryptic activity in both the cell suspension and undissociated tissue was inhibited by the addition of an equal volume of growth medium containing 10% (v/v) fetal bovine serum (Colorado Serum Co.). The undissociated tissue was then triturated five times by gravity flow through a 10-ml serological pipette and cells were again recovered by decantation. Cells were pooled and sedimented at 250 × g_{max} for 10 min at 3° and resuspended in Solution D. Cell number and viability were determined by counting in a hemocytometer with 0.5% (w/v) nigrosin (Allied Chemical, Morristown, N. J.). Only those cells which excluded the dye were considered viable. Usual cell recoveries for dissociation of newborn brain were 90 to 100 × 10^6 cells per g of tissue (98 to 100% viable). Cells were inoculated at 10^7 viable cells per 150-mm Falcon polystyrene Petri dish (145 cm² surface area) in 20 ml of DMEM

* Present address, National Cancer Institute, Bethesda, Md. 20014.
‡ Present address, National Institute of Child Health and Human Development, Bethesda, Md. 20014.
§ Present address, Department of Pathology, Health Science Center, Temple University, Philadelphia, Pa. 19140.
¶ Present address, Division of Neurosciences, University of California at La Jolla, San Diego, Calif. 92122.
‖ Present address, Roche Institute of Molecular Biology, Nutley, N. J. 07110.

(90% Dulbecco-Vogt modification of Eagle's medium, 10% fetal bovine serum with 50 units of sodium-penicillin and 10 μg of streptomycin-SO_4 per ml). Dishes were maintained at 37° in an humidified atmosphere of 10% CO_2-90% air. Changes of medium were performed on the 3rd day of culture, every 2nd day thereafter, and 16 to 20 hours before enzyme assay.

Other Cell Lines

The C-6 rat astrocytoma cell line was obtained from the American Type Culture Collection (No. CCL 107). HeLa cells were from Flow Laboratories, Rockville, Md. The 3T3 and Balb/C 3T3 cell lines were from Dr. George Todaro. The RG-179 line was a permanent cell line obtained after multiple passages of brain cells from 5-day-old Fisher rats. The human astrocytoma CHB and rat glioma $C2_1$ were provided by Dr. S. Pfeiffer and Dr. H. Schein, respectively. The $C2_1$ cell was originally developed by Dr. G. Benda.

Preparation of Homogenates

Surface Cultures—The procedure was designed to wash cells free of serum protein and to recover cell enzymes reproducibly in high yield. Growth medium was removed from Petri dishes and discarded and the cell monolayer was gently washed twice with 10 ml of Solution D containing 0.14 mM $CaCl_2$ and once with 10 ml of Solution D. The Petri dish was drained for 90 s at a 45° angle, then the dish was scraped with a spatula 5 cm wide (the end of a flexible plastic ruler) covered with disposable Teflon tape (Scientific Specialties Services, Inc., Randallstown, Md.). Cells were recovered by aspiration with a large bore micropipette and transferred to a polyallomer tube, 3 × 0.5 inch. The surface of the dish was washed twice with 0.1 to 1.5-ml portions of Buffer A (50 mM potassium phosphate buffer, pH 6.8; 1 mM EDTA, potassium salt) at 3°. The amount of Buffer A added was adjusted so that the final protein concentration was 2 to 10 mg per ml of homogenate. Cells contained in the washes were recovered and combined with the scraped cells. The tubes were stoppered and placed in cold H_2O in the chamber of an ultrasonic oscillator (Raytheon, model No. DF101); cells were lysed by sonication at 1° for 5 min. Homogenate volumes were measured; each homogenate was divided into small portions which were frozen quickly in a Dry Ice-acetone bath and stored in the vapor phase of a liquid nitrogen freezer.

Mouse Brain—Brain tissue from Balb/c mice was washed with Solution D, blotted, minced in approximately 7 volumes of Buffer A, and homogenized at 1° with a Potter-Elvehjem homogenizer and then by sonication as described above. The homogenate was centrifuged at 35,000 × g_{max} for 15 min at 1°; the supernatant fraction (4 to 12 mg of protein per ml) was divided into 0.05-ml portions, quick frozen, and stored at −100°.

Protein concentration was determined by a modification of the method of Lowry *et al.* (13) with 3 to 20 μg of protein per reaction. DNA was determined by the spectrofluorophotometric method of Kissane and Robbins (14). Histochemical tests were kindly performed by Dr. Lloyd Guth.

Enzyme Assays

Homogenates in Buffer A were thawed shortly before use and portions were diluted with appropriate modifications of Buffer A to adjust the homogenates to the specific conditions of each assay. Homogenates were added to reactions last. Each homogenate was assayed at four concentrations; values were used only if the rate of reaction was proportional to the homogenate concentration. Triplicate homogenates were prepared and assayed routinely; the average values are shown.

Acetylcholinesterase Assay

Acetylcholinesterase activity was assayed by a modification of the methods of Reed *et al.* (15) and Ehrenpreis *et al.* (16). Neuroblastoma clone N-18 homogenates were thawed immediately before use and adjusted to the assay conditions by mixing 4 volumes of homogenate with 1 volume of Buffer A containing 1.0 M NaCl and 2.5% (v/v) Triton X-100. The radioactive substrate, [2-^{3}H]acetylcholine chloride, 250 mCi per mmole (Amersham-Searle) or [1-^{14}C]acetylcholine iodide, 2.4 mCi per mmole (New England Nuclear) was dissolved in H_2O and lyophilized for 16 hours to remove possible volatile contaminants. The cation exchange resin AG 50W-X8 (H^+ form, 100 to 200 mesh, Bio-Rad Laboratories) was converted to the Na^+ form with 2 N NaOH at 25° for 45 min and then washed with H_2O until the pH of the effluent was 6.0. Columns (0.5 × 5.0 cm) of resin were formed over small plugs of glass wool in 9-inch disposable Pasteur pipettes and washed with H_2O.

Each reaction contained the following components in a final volume of 50 μl unless stated otherwise: 2.8 mM [2-^{3}H]acetylcholine chloride (0.15 μCi per reaction, 1.08 mCi per mmole), 200 mM NaCl, and 0.5% Triton X-100 in Buffer A; and 0 to 40 μl of neuroblastoma homogenate, or Buffer A containing 200 mM NaCl and 0.5% Triton X-100, or both. Reactions in disposable glass tubes, 10 × 75 mm, were incubated for 10 min at 37°, then transferred to an ice-water bath and diluted rapidly by the addition of 1.0 ml of H_2O at 1°. Each diluted reaction was immediately passed over a column of the cation exchange resin; the tube was washed with two 1.0-ml portions of H_2O at 1°, and the washes also were passed through the column. The column effluent was collected in a glass scintillation vial, then 10 ml of Triton X-100-toluene-Liquifluor (333 g:666 ml:55 ml) scintillation fluid were added and radioactivity was determined with a scintillation counter (32% counting efficiency).

Since reactions are not deproteinized, the column step must be performed rapidly. The rate of acetylcholinesterase activity after dilution at 1° was approximately 1.5% that of undiluted reactions at 37°. Therefore, the assay was performed in batches of 20 tubes or less so that all reactions could be passed through columns within 3 min. In some instances distinction was made between "true" and "pseudo" cholinesterases by employing 10^{-5} M BW 284C51 dibromide.

Catechol O-Methyltransferase Assay

The assay described was a modification of the method of Nikodejevic *et al.* (17). Neuroblastoma homogenates were adjusted to the conditions of the catechol *O*-methyltransferase assay immediately before use by mixing 9 volumes of homogenate with 1 volume of Buffer A containing 50 mM $MgCl_2$. The L-*S*-adenosyl[*methyl*-^{14}C]methionine, 58 mCi per mmole (obtained from Amersham-Searle in approximately 0.001 N H_2SO_4), was extracted twice with 10 ml of toluene; the aqueous phase was lyophilized before use. Nonradioactive L-*S*-adenosylmethionine iodide and 3,4-dihydroxybenzoic acid were obtained from Calbiochem Corp.

Each reaction contained the following components in a final

volume of 50 μl except where stated: 0.55 mM L-*S*-adenosyl-[*methyl*-^{14}C]methionine iodide (0.318 μCi per reaction, 11.55 mCi per mmole), 2.5 mM dihydroxybenzoic acid, and 5 mM $MgCl_2$ in Buffer A; and 0 to 40 μl of neuroblastoma homogenate, or Buffer A containing 5 mM $MgCl_2$, or both. Each reaction was incubated in a 12-ml conical glass centrifuge tube with ground glass stopper for 20 min at 37°, then transferred to an ice-water bath and 0.2 ml of 1.0 N HCl and 10 ml of toluene were added. Tubes were shaken vigorously for 1 min, and centrifuged for 5 min at 250 × *g*. Then 9 ml of the toluene phase were removed and transferred to a scintillation vial containing 5 ml of toluene-Liquifluor (958 ml:42 ml) scintillation solution and radioactivity was determined (87% counting efficiency). A correction was applied so that each value reported represented the entire toluene phase. Authentic 3-[^{14}C]methoxy-4-hydroxybenzoic acid and 4-[^{14}C]methoxy-3-hydroxybenzoic acid were prepared with purified rat liver catechol *O*-methyltransferase obtained from Dr. C. R. Creveling.

Choline Acetyltransferase Assay

The assay described was a modification of the method of Schrier and Shuster (18). Mouse brain extracts were thawed immediately before use and adjusted to the choline acetyltransferase assay conditions by mixing 4 volumes of brain extract with 1 volume of Buffer A containing 1.0 M NaCl and 2.5% Triton X-100. The [1-^{14}C]acetyl-CoA (50 mCi per mmole) (New England Nuclear) was lyophilized for 16 hours before use. Unlabeled acetyl-CoA (trilithium salt, trihydrate, A grade) and neostigmine methylsulfate were from Calbiochem; choline iodide was from Schwarz. The anion exchange resin AG 1-X8, (Cl^- form, 100 to 200 mesh, Bio-Rad Laboratories) was washed with H_2O until the effluent was pH 5.5, and columns of this resin for each assay were prepared as described for the acetylcholinesterase assay.

Each reaction contained the following components in a final volume of 50 μl, except where stated: 0.21 mM [1-^{14}C]acetyl-CoA (6 mCi per mmole), 2 mM choline iodide, 200 mM NaCl, 0.1 mM neostigmine methylsulfate, and 0.5% Triton X-100 in Buffer A; and 0 to 40 μl of homogenate, or Buffer A containing 200 mM NaCl and 0.5% Triton X-100, or both. Reactions were incubated in glass tubes, 10 × 75 mm, for 10 min at 37°, then transferred to an ice-water bath and diluted by the addition of 1.0 ml of H_2O at 1°. The contents of each tube were passed through an anion exchange column; the tube was washed with two 1.0-ml portions of H_2O at 1°, and the washes were also passed through the column. The column effluent was collected in a glass scintillation vial, then 10 ml of Triton-toluene-Liquifluor scintillation mixture (see above) were added and radioactivity was determined at a counting efficiency of 87%.

Glutamate Decarboxylase Assay

Two modifications of the method of Wingo and Awapara (19) were employed.

Method a—Homogenates were thawed prior to use and adjusted to the conditions of the glutamate decarboxylase assay by mixing 9 volumes of homogenate with 1 volume of Buffer A containing 10 mM 2-mercaptoethanol, 5% Triton X-100, and 5 mM pyridoxal phosphate. The L-[1-^{14}C]glutamic acid, obtained from Calbiochem, was neutralized with KOH and lyophilized before use. Amino-oxyacetic acid was obtained from Sigma.

Each reaction contained the following components in a final volume of 50 μl, except where stated: 5 mM L-[1-^{14}C]glutamic acid, potassium salt (1.04 μCi per reaction, 4.16 mCi per mmole), 1 mM 2-mercaptoethanol, 0.5 mM pyridoxal phosphate monohydrate, and 0.5% Triton X-100 in Buffer A; and 0 to 40 μl of homogenate, or Buffer A containing 0.5% Triton X-100, 1 mM 2-mercaptoethanol, and 0.5 mM pyridoxal phosphate, or both. Reaction mixtures in glass tubes, 10 × 37 mm, were sealed with tightly fitting rubber stoppers that could be easily perforated with a needle. Reactions were incubated for 10 min at 37° and then transferred to an ice-water bath. Each tube was then placed in a glass scintillation vial containing 5 ml of Hyamine-toluene-Liquifluor scintillation solution (288 ml of 1 M Hyamine hydroxide in methanol to 640 ml to 42 ml). The vial was sealed with the reaction tube stopper; then 0.2 ml of a solution of 10 mM acetic acid in methanol was injected through the rubber stopper into the reaction in the tube with a hypodermic syringe. The injection needle was used to dislodge the glass reaction tube from the rubber stopper so that the tube fell to the bottom of the scintillation vial. The stoppered vial was incubated for 30 min at 24° to allow absorption of $^{14}CO_2$ by the Hyamine solution. Then the reaction tube was removed from the scintillation vial with forceps and the vial was capped with a scintillation vial top. Radioactivity was determined by scintillation counting at 80% efficiency.

Method b—This alternate method differed only in the technique of $^{14}CO_2$ collection. Each reaction was performed in the 6-mm diameter center well of a 10-ml Erlenmeyer flask (Kontes) equipped with a tightly fitting rubber stopper. Hydroxide of Hyamine at 37° (Packard Instruments, Downer's Grove, Ill.) was placed in the outer portion of the flask and reaction components were added to the center well. The flask was sealed with the stopper and incubated at 37° for 11 min. The reaction was terminated by injection of 0.2 ml of 10 mM acetic acid in meth-

TABLE I

Range and sensitivity of assays

Assay	Source of homogenate	Incubation time	Radioactive product	Usual range and sensitivity, amount of ^{14}C or ^{3}H product per reaction	Average amount of ^{14}C or ^{3}H product/min/mg protein
		min		*pmoles*	*pmoles*
Acetylcholinesterase	Neuroblastoma clone N-18	10	[^{3}H]Acetate	1,000–14,000	75,000
			[^{14}C]Acetate	75–14,000	75,000
Choline acetyltransferase	Mouse brain (age 35 days)	10	[^{14}C]Acetylcholine	5–700	1,500
Glutamate decarboxylase	Mouse brain (newborn)	10	$^{14}CO_2$	20–3,000	150
Catechol *O*-methyltransferase	Neuroblastoma clone N-18	20	3-[^{14}C]Methoxy-4-hydroxybenzoic acid[a]	10–1,200	75

[a] Approximately 5% of the ^{14}C-product is 4-[^{14}C]methoxy-3-hydroxybenzoic acid.

anol through the stopper into the center well. After an additional 90 min at 37° an aliquot of the Hyamine-$^{14}CO_2$ solution was transferred to a scintillation vial containing 10 ml of toluene-Liquifluor scintillation mixture. Radioactivity was determined by scintillation counting at 87% efficiency.

RESULTS

Assays for Marker Enzymes

The usual range and sensitivity observed for the four enzyme assays are shown in Table I. In evaluation of the assays neuroblastoma clone N-18 homogenate was used for acetylcholinesterase and catechol *O*-methyltransferase assays; mouse brain extracts were used for evaluation of the assays for choline acetyltransferase and glutamate decarboxylase, although these assays were also applicable to cell culture extracts. The range of linearity with protein for neuroblastoma choline acetyltransferase was about half that obtained with mouse brain homogenates (20).

Conditions were selected so that a homogenate could be prepared by harvesting a single Petri dish and stored frozen for future use. About 30 mg of protein could be harvested from a

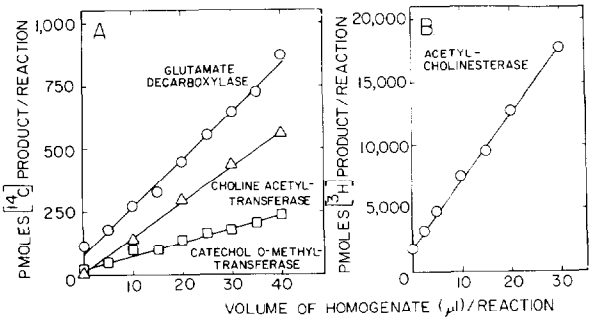

FIG. 1. The relation between concentration of enzyme extract and reaction velocity for glutamate decarboxylase, choline acetyltransferase, and catechol *O*-methyltransferase (Panel *A*) and for acetylcholinesterase (Panel *B*). Reactions contained the components described under "Methods," and enzyme extract protein at the following concentrations: 12.2, 1.06, 5.0, and 1.0 μg of protein per μl of extract for glutamate decarboxylase, choline acetyltransferase, catechol *O*-methyltransferase, and acetylcholinesterase, respectively. Glutamate decarboxylase was determined by Method *a*.

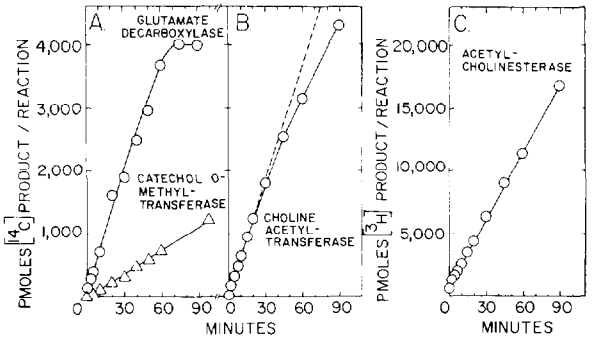

FIG. 2. The relation between length of incubation and amount of product formed. Each glutamate decarboxylase, catechol *O*-methyltransferase, choline acetyltransferase, and acetylcholinesterase reaction contained the components described under "Methods" and 488, 200, 42.4, and 8.0 μg of protein, respectively. The *dashed line* in Panel *B* represents the theoretical linear reaction rate. Glutamate decarboxylase was determined by Method *a*.

150-mm Petri dish (145 cm²) containing a confluent monolayer of neuroblastoma cells. Routinely, less than 1.0 mg of protein was used to determine the specific activity of each enzyme studied, and 40 to 100 assays were performed per day.

Kinetics of Reactions

As shown in Fig. 1, the rate of each reaction was proportional to concentration of homogenate protein within the range studied. Conditions were adjusted so that the proportion of radioactive substrate converted to product was <5% in the case of choline acetyltransferase and <10% with the other three enzymes.

The relation between time of incubation and reaction rate is shown in Fig. 2. Under the conditions employed, reactions catalyzed by glutamate decarboxylase, catechol *O*-methyltransferase, choline acetyltransferase, and acetylcholinesterase were linear for 60, 98, 20, and 90 min, respectively.

The relation between reaction mixture pH and enzyme activity is shown in Fig. 3. At pH 6.8 the activities of glutamate decarboxylase and catechol *O*-methyltransferase were essentially maximal (the latter was inhibited by Tris); however, choline acetyltransferase and acetylcholinesterase activities were 60 to 65% of the observed maxima. Although these latter two activities were optimal between pH 8 and 8.5, the four enzymes were routinely assayed at pH 6.8 because acetyl-CoA and acetylcholine are more stable at pH 6.8 than at pH 8. In addition, the same homogenate could be used to determine the activity of each enzyme without adjustment of pH.

Enzyme Characteristics

Enzyme stability and product recovery is shown in Table II. Enzyme activity was not affected appreciably by freezing and thawing homogenates or by keeping the enzyme extracts at 1° for 2 to 3 hours prior to assay. However, each enzyme tested was completely inactivated after incubation at 100° for 10 min.

Although little or no activities of the other enzymes were detected at 1°, acetylcholinesterase activity at 1° was approxi-

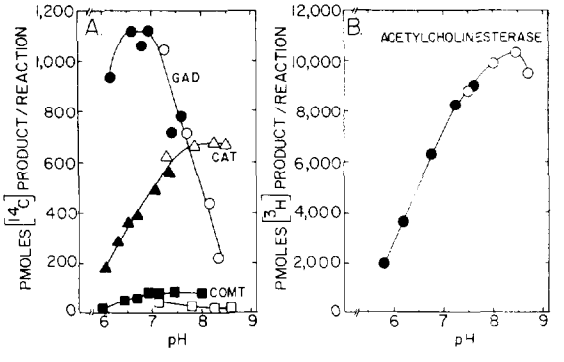

FIG. 3. The effect of pH upon reaction rates. Each glutamate decarboxylase, choline acetyltransferase, catechol *O*-methyltransferase, and acetylcholinesterase reaction contained the components described under "Methods," the specified buffer, and 122, 26.5, 50, and 10 μg of protein, respectively. Glutamate decarboxylase was determined by Method *a*. The formation of radioactive products was determined with standard 50-μl reactions; pH was determined in 0.5-ml reactions that were identical with the 50-μl reactions except that unlabeled substrates were employed. *Solid symbols* represent reactions with 50 mM potassium phosphate buffers; *open symbols* represent 50 mM Tris-HCl buffers. The abbreviations used are: *GAD*, glutamate decarboxylase; *CAT*, choline acetyltransferase; *COMT*, catechol *O*-methyltransferase.

TABLE II

Enzyme stability and product recovery

Each reaction contained the components described under "Methods" and 40, 15, 366, or 60 μg of protein for choline acetyltransferase, acetylcholinesterase, glutamate decarboxylase, and catechol *O*-methyltransferase reactions, respectively. The amount of radioactive product formed (picomoles) per complete reaction corresponding to 100% were 610, 11,300, 630, and 114 for choline acetyltransferase, acetylcholinesterase, glutamate decarboxylase, and catechol *O*-methyltransferase reactions, respectively. Glutamate decarboxylase was determined by Method *a* (see "Methods"). Product recovery was tested by adding the following compounds to reactions in place of radioactive substrate: choline acetyltransferase assay, 28 nmoles of [1-^{14}C]acetylcholine iodide (1.53×10^5 dpm); acetylcholinesterase, 208 nmoles of sodium [^{14}C]acetate (8.05×10^6 dpm); glutamate decarboxylase, 35 nmoles of $NaH^{14}CO_3$ (3.97×10^5 dpm); catechol *O*-methyltransferase, 0.133 nmole of 3-[^{14}C]methoxy-4-hydroxybenzoic acid (3.4×10^3 dpm) and 0.015 nmole of 4-[^{14}C]methoxy-3-hydroxybenzoic acid (350 dpm).

Modification	Choline acetyltransferase	Acetylcholinesterase	Glutamate decarboxylase	Catechol *O*-methyltransferase
	%	%	%	%
Enzyme stability				
Complete reaction	100	100	100	100
Minus enzyme	2	9	8	9
Enzyme frozen and thawed three times	98	99	109	108
Enzyme held at 1° for 2–3 hrs	100	100	116	104
Enzyme held at 100° for 10 min	2	9	8	8
Reaction incubated at 1°	9	31	10	7
Product recovery				
Radioactive product added instead of substrate	95	110	94	83

mately 20% of that found at 37°. For this reason acetylcholinesterase reactions were performed in batches (<20 tubes) so that reactions could be chilled immediately after incubation, diluted, and passed through the ion exchange columns in <3 min.

Radioactive product was added to reactions instead of substrate to determine the percentage of product recovered. Greater than 94% of the radioactive products of the choline acetyltransferase, acetylcholinesterase, and glutamate decarboxylase reactions and 83% of the radioactive product of the catechol *O*-methyltransferase reaction were recovered.

The effect of reaction components and other compounds on the rate of enzyme activity is shown in Table III. Choline acetyltransferase from mouse brain was completely dependent upon choline for activity and was stimulated by 200 mM NaCl. Enzyme activity was not affected appreciably by omission of Triton X-100 or by the addition of 2 mM $MgCl_2$.

Neuroblastoma acetylcholinesterase activity was reduced in the absence of Triton X-100; however, omission of NaCl or EDTA in the presence or absence of $MgCl_2$ was without effect. Marked inhibition was observed by 10^{-5} M BW 284C51, a potent inhibitor of acetylcholinesterase (EC 3.1.1.7).

The activity of glutamate decarboxylase from mouse brain was reduced slightly in the absence of pyridoxal phosphate but was not affected appreciably by omission of EDTA or 2-mercaptoethanol. The reaction was stimulated slightly by omission of Triton X-100 or by the addition of 1 mM $MgCl_2$. Marked inhibition was observed in the presence of iodoacetamide, hydroxylamine or amino-oxyacetic acid, as expected (21).

The activity of neuroblastoma catechol *O*-methyltransferase was almost completely dependent on the substrate, dihydroxybenzoic acid. *p*-Hydroxyphenylacetic acid did not serve as a substrate for the enzyme and did not inhibit methyl transfer to

TABLE III

Assay conditions

Each reaction contained the components described under "Methods," except as noted, and the following: 40, 15, 366, and 60 μg of homogenate protein for the choline acetyltransferase, acetylcholinesterase, glutamate decarboxylase, and catechol *O*-methyltransferase reactions, respectively. Enzyme specific activities, in the order stated above were: 1,480, 64,400, 140, and 88 pmoles of product per min per mg of protein. Glutamate decarboxylase was measured by Method *a* with [1-^{14}C]glutamic acid as substrate.

Modification	Amount of radioactive product formed per min	Percentage
	pmoles	%
Choline acetyltransferase		
Complete	64	100
Minus enzyme (mouse brain)	2	3
Minus choline	2	3
Minus NaCl	40	62
Minus Triton X-100	61	94
Plus 2 mM $MgCl_2$	59	91
Acetylcholinesterase		
Complete	1130	100
Minus enzyme (neuroblastoma)	105	9
Minus Triton X-100	750	66
Minus NaCl	1140	101
Minus EDTA	1160	102
Minus EDTA, plus 1 mM $MgCl_2$	1130	100
Plus 10^{-5} M BW 284C51	135	12
Glutamate decarboxylase		
Complete	63	100
Minus enzyme (mouse brain)	5	8
Minus pyridoxal phosphate	55	87
Minus EDTA	68	110
Minus 2-mercaptoethanol	60	96
Minus Triton X-100	87	140
Plus 1 mM $MgCl_2$	76	120
Plus 10 mM iodoacetamide	18	29
Plus 10 mM hydroxylamine	8	12
Plus 1 mM amino-oxyacetic acid	8	12
Catechol *O*-methyltransferase		
Complete	5.7	100
Minus enzyme (neuroblastoma)	0.48	9
Minus dihydroxybenzoic acid	0.69	12
Minus dihydroxybenzoic acid, plus 1 mM *p*-hydroxyphenylacetic acid	0.76	13
Plus 1 mM *p*-hydroxyphenylacetic acid	6.5	115
Minus $MgCl_2$	0.98	17
Minus EDTA	5.7	100
Minus $MgCl_2$ and EDTA	3.0	52
Plus 0.5% (v/v) Triton X-100	6.6	115
Plus 1 mM 2-mercaptoethanol	7.9	140
Plus 0.25 mM dihydroxymethoxybenzoic acid	1.3	23
Plus 1 mM tropolone	0.94	17

dihydroxybenzoic acid. Hence, the enzyme was capable of distinguishing between mono- and dihydroxy substrates. Omission of $MgCl_2$ markedly reduced the rate of reaction; omission of EDTA was without effect. Enzyme activity was markedly inhibited in the presence of compounds known to inhibit catechol *O*-methyltransferase, such as dihydroxymethoxybenzoic acid (17) and tropolone.

Product Identification

The radioactive products of the choline acetyltransferase, acetylcholinesterase, and catechol *O*-methyltransferase reactions were characterized by thin layer or paper chromatography as shown in Table IV. Virtually all of the radioactive product of the acetylcholinesterase reaction was chromatographically indistinguishable from authentic sodium acetate added as carrier. Greater than 73% of the applied radioactive product was recovered after chromatography.

Similarly, virtually all of the radioactive product formed in the choline acetyltransferase reactions with a mouse brain homogenate was chromatographically indistinguishable from authentic acetylcholine chloride. It has been shown (20), however, that with some homogenates possessing low choline acetyltransferase activity, values may be spuriously high due to the formation of radioactive products other than acetylcholine. Thus, the assay as described is valuable as a rapid screening technique, but must be validated by product identification.

Most of the radioactive product formed in the presence of neuroblastoma catechol *O*-methyltransferase preparations co-

TABLE IV

Chromatographic mobility of radioactive products

Chromatographic systems—Solvent 1, ethanol-H_2O-concentrated NH_4OH (8:1:1) (Reference 22), thin layer chromatography with MN Polygram Cel 300; Solvent 2, 1-butanol-cyclohexane-ethylene glycol-H_2O-concentrated NH_4OH-cyclohexylamine (30:30:10:3.7:0.07:0.05), thin layer chromatography with MN Polygram Cel 300; Solvent 3, 1-propanol-0.1 N acetic acid (3:1) (Reference 23), Whatman 3MM paper; Solvent 4, 1-propanol-formic acid-H_2O (8:1:1) (Reference 23), Whatman 3MM paper; Solvent 5, 1-butanol-1-propanol-H_2O (4:2:1) (Reference 23), MN Polygram Cel 300 thin layer; Solvent 6, 1-propanol-benzyl alcohol-H_2O (5:2:2) (Reference 23), MN Polygram Cel 300 thin layer; Solvent 7, 2-propanol-concentrated NH_4OH-H_2O (8:1:1), Silica Gel G thin layer; Solvent 8, 1-butanol-formic acid-benzene-H_2O (15:25:5:1:1.5), Silica Gel G thin layer; Solvent 9, benzene-glacial acetic acid-methanol (10:1:5), Silica Gel G thin layer.

Acetylcholinesterase—The reaction contained the components described under "Methods" except that 2.78 mM [2-^{3}H]acetylcholine chloride (0.43 μCi) and 48 μg of neuroblastoma clone N-18 protein were present (61,200 pmoles per mg of protein per min). The column effluent was collected and NaOH was added to a final concentration of 0.18 M. Authentic sodium [^{14}C]acetate was added and portions were applied to thin layer chromatograms. The chromatograms were developed by ascending chromatography, dried, covered with cellophane tape, cut into 0.5-cm horizontal strips, and each was placed in a scintillation vial with 20 ml of toluene-Liquifluor scintillation solution (see "Methods") for determination of radioactivity at efficiencies of 54% (^{14}C) and 16% (^{3}H).

Choline Acetyltransferase—The reaction contained the components described under "Methods" except that 8.1 nmoles of [2-^{3}H]acetyl-CoA (1.24 Ci per mmole) were used as substrate, choline chloride was added in place of choline iodide, 168 μg of homogenate protein from mouse brain were added, and the reaction was incubated for 20 min at 37° in a final volume of 0.1 ml. Authentic [^{14}C]acetylcholine chloride was added to the reaction after incubation. Acetic acid was added to the column effluent to a final concentration of 0.06 M. Portions of the column effluent were subjected to ascending paper or thin layer chromatography. Developed chromatograms were cut into 1.0- or 0.5-cm strips and each was placed in a scintillation vial containing 20 ml of toluene-Liquifluor scintillation solution for determination of radioactivity as described above.

Catechol O-Methyltransferase—The reaction mixture contained the components described under "Methods" in a final volume of 0.25 ml with 750 μg of neuroblastoma N-18 homogenate protein. The reaction was incubated for 60 min and extracted with toluene; the toluene phase was evaporated to dryness under a stream of nitrogen at 24° and the residue then was dissolved in 0.35 ml of methanol. Of the radioactivity originally present in the toluene extract, 81% was recovered in the methanol solution. Authentic 3-methoxy-4-hydroxybenzoic acid and 4-methoxy-3-hydroxybenzoic acid were added and portions of the extract were subjected to ascending thin layer chromatography. Chromatograms were dried at 24° and marker compounds were located by exposure to ultraviolet light. Chromatograms were cut into strips, 1 × 3 cm, and each was placed in a scintillation vial containing 10 ml of Triton-toluene-Liquifluor scintillation solution for determination of radioactivity.

Enzyme	Chromatographic solvent	Percentage of radioactive product with R_F of authentic compound	Percentage of applied radioactivity recovered	R_F
		% Sodium [^{3}H]acetate	%	
Acetylcholinesterase	1	72	73	0.70
	2	90	90	0.25
		[^{3}H]Acetylcholine chloride		
Choline acetyltransferase	3	80	82	0.66
	4	80	81	0.77
	5	86	87	0.49
	6	103	104	0.89
				0.65[a]
		3-[^{14}C]-Methoxy-4-hydroxybenzoic acid		
Catechol *O*-methyltransferase	7	84	92	0.38
	8	73	78	0.91
	9	76	81	0.86
		4-[^{14}C]-Methoxy-3-hydroxybenzoic acid		
Catechol *O*-methyltransferase	7	5	92	0.76
	8	4	78	0.75
	9	5	81	0.75

[a] With Solvent 6 two peaks of radioactivity were found, both with authentic [^{14}C]acetylcholine chloride and with the tritiated reaction product. This phenomenon has been attributed to the effect of salts on the chromatographic mobility of acetylcholine (23).

chromatographed with authentic 3-methoxy-4-hydroxybenzoic acid; 4 to 5% of the reaction product exhibited the chromatographic mobility of 4-methoxy-3-hydroxybenzoic acid. Greater than 78% of the applied radioactive material was recovered after chromatography. No discrete contaminants were detected.

Characterization of the products of the glutamate decarboxylase reaction is shown in Table V. In addition, the amounts of γ-amino[U-^{14}C]butyric acid and $^{14}CO_2$ formed during incubation with L-[U-^{14}C]glutamic acid as substrate are compared. The results show that γ-amino[U-^{14}C]butyric acid was formed during incubation and also that similar amounts of γ-aminobutyric acid and CO_2 were formed. However, as shown below, this 1:1 ratio of products was not found with all tissues assessed. Because of the possibility that $^{14}CO_2$ may not accurately reflect production of γ-aminobutyric acid, quantitative identification of the latter product is required to validate the glutamate decarboxylase assay.

Substrate Concentration

The relations between substrate concentrations and enzyme activities are shown in Fig. 4. The apparent Michaelis constants, determined by the method of Lineweaver and Burk (24), were as follows: acetylcholinesterase, 9.1×10^{-4} M acetylcholine-chloride, choline acetyltransferase, 1.5×10^{-5} M acetyl-CoA (with 2×10^{-3} M choline iodide), and 9.1×10^{-4} M choline iodide (with 2.1×10^{-4} M acetyl-CoA); glutamate decarboxylase, 3.7×10^{-3} M L-glutamic acid; and catechol *O*-methyltransferase, 2.3×10^{-4} M dihydroxybenzoic acid (with 5.5×10^{-4} M L-*S*-adenosylmethionine), and 4.0×10^{-5} M L-*S*-adenosylmethionine (with 2.5×10^{-3} M dihydroxybenzoic acid). Substrate inhibition was detected only in the case of acetylcholinesterase, similar to observations that have been reported with that enzyme from other tissues (25).

In standard reactions (Fig. 1) substrates were present in concentrations well above their respective enzyme K_m values and less than 10% of the radioactive substrates were converted to product during reactions. High substrate concentrations were used to maintain zero order kinetics and to reduce error due to endogenous substrates or inhibitors and other reactions competing for substrate.

Recovery of Protein and Enzyme Activity from Petri Dishes

The effectiveness of the harvesting procedure was examined by comparing the protein yield obtained by harvesting replicate plates by the scrape-wash method with the amount of protein recovered by the addition of 0.1 N NaOH to dishes (Table VI). The results show that 95% of the protein was recovered by the standard harvesting procedure. In experiments with a variety of cells in culture the reproducibility between replicate plates was ±15% with respect to the specific activity of the four enzymes and ±25% with respect to the yield of protein recovered per plate.

The recovery of enzyme activity and cell protein also was studied by adding to Petri dishes known quantities of a mixture of neuroblastoma N-18 and brain homogenates which had previously been characterized with respect to enzyme activity and protein concentration. The homogenate was then recovered by the usual procedure. A portion was set aside for future assay of enzyme activity and protein concentration; the remainder was added to a fresh Petri dish and recovered again. The harvesting cycle was performed four times. The results, presented in Fig. 5, show that recovery for each harvest cycle was 96% for protein, 87% for glutamate decarboxylase, 95% for choline acetyltransferase, 83% for catechol *O*-methyltransferase, and 88% for acetylcholinesterase. Thus, enzyme activity released from cells during the scraping procedure would be recovered in high yield and with little alteration of enzyme-specific activity.

TABLE V

$^{14}CO_2$ and γ-amino[^{14}C]butyric acid synthesis in glutamic acid decarboxylase reaction

The reaction contained 2.44 mg of protein from mouse brain (156 pmoles of $^{14}CO_2$ formed per min per mg of protein) and the components described under "Methods" except that L-[U-^{14}C]-glutamate was employed as substrate and the final volume was 0.25 ml. Incubation was for 45 min at 37°. The reaction was terminated by the addition of 0.175 ml of 10 mM acetic acid in methanol. The evolved $^{14}CO_2$ was collected for 60 min by Method *a*. The acidified reaction then was centrifuged at 15,000 × *g* for 10 min and the supernatant fraction was collected. Unlabeled γ-aminobutyric acid, glutamic acid, and glutamine were added and portions were applied to thin layer chromatograms of cellulose MN Polygram Cel 300 and subjected to ascending thin layer chromatography with Solvent Systems 1, 2, and 3 (two-dimensional chromatography with System 3) and to Whatman 3MM paper for high voltage electrophoresis (45 min at 61 volts per cm and 180 ma) with Solvent 4. Solvent systems were: Solvent 1, 2-propanol-methanol-concentrated NH_4OH (9:7:4); Solvent 2, phenol saturated with 6 M NH_4OH; Solvent 3, 2-propanol-1-butanol-1 N HCl (6:1.5:2.5) for the first dimension and phenol saturated with H_2O for the second dimension; and Solvent 4, pyridine-acetic acid-H_2O, pH 3.95 (1.8:5.0:144.5). Chromatograms and electropherograms were dried, stained with 0.2% ninhydrin in acetone, and cut into 0.5- or 1.0-cm strips. Each strip was placed in a scintillation vial with 10 ml of Triton-toluene-Liquifluor scintillation solution and radioactivity was determined. Specific activities of $^{14}CO_2$ and γ-amino[U-^{14}C]butyric acid reaction products were 1,580 and 6,320 dpm per nmole, respectively.

Solvent system	Amount of γ-amino [^{14}C]butyric acid recovered	Amount of $^{14}CO_2$ recovered	R_F of marker γ-aminobutyric acid
	nmoles	*nmoles*	
1	29	22	0.42
2	33	22	0.88
3 (1st dimension)	28	22	0.77
(2nd dimension)			0.89
4	33	22	---

Marker Enzymes in Cell Culture

Non-neuronal Cells in Culture—In order to determine the specificity of the marker enzymes, established cell lines from brain and other tissues were grown in surface culture until several days after confluency and then tested for presence of the enzyme activities. As shown in Table VII, choline acetyltransferase, acetylcholinesterase, and glutamate decarboxylase activities were present in all of the cell lines examined. Activities of the three enzymes in established cell lines were considerably lower than corresponding activities in newborn brain.

Glutamate decarboxylase specific activity in L-929 cells and in glial cell lines C_6, $C2_1$, and RG-179 when measured by γ-amino-[^{14}C]butyric acid production was 10% or less of that measured by $^{14}CO_2$ production. The presence of 1 mM γ-aminobutyric acid in

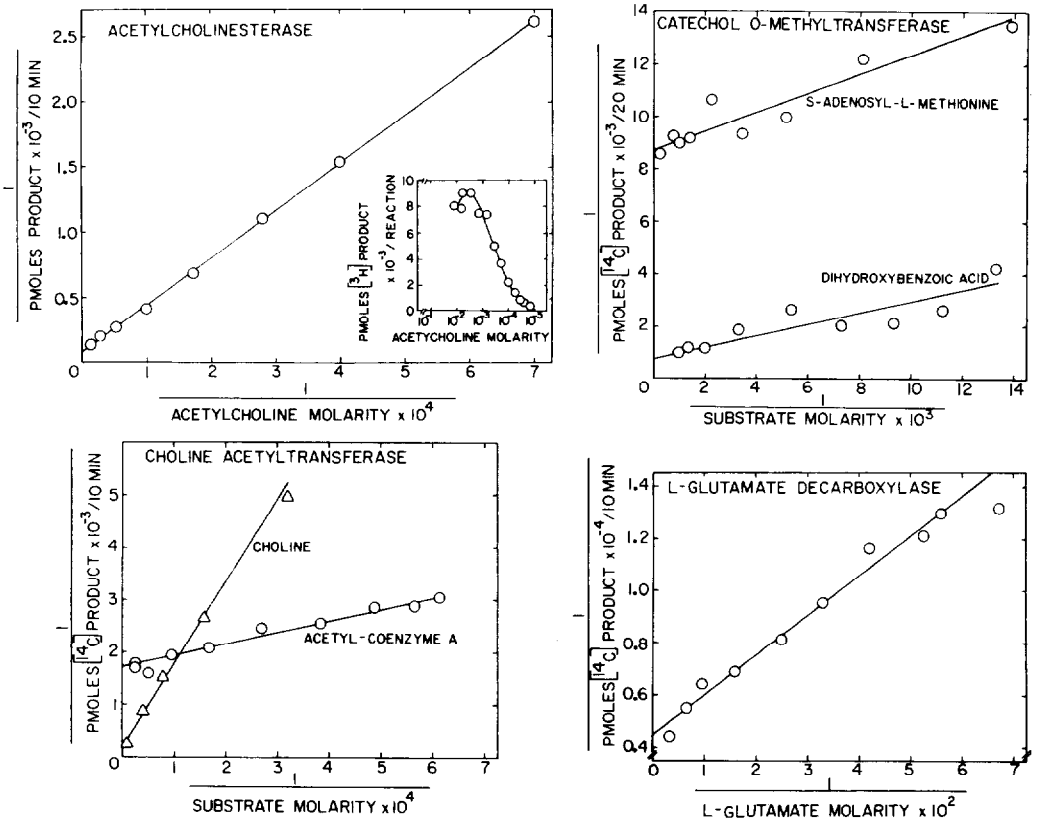

FIG. 4. Relation between reaction velocity and substrate concentration. Reactions for acetylcholinesterase, choline acetyltransferase, glutamate decarboxylase, and catechol *O*-methyltransferase contained the components described under "Methods" except for substrate concentrations as indicated and 15, 42.4, 488, and 200 μg of protein, respectively. In addition acetylcholinesterase and catechol *O*-methyltransferase reactions were incubated for 7 and 15 min, respectively. The data were plotted according to the Lineweaver and Burk method (24). Glutamate decarboxylase was determined by Methods *a* and *b* (Method *a* is shown).

TABLE VI

Recovery of cell protein from Petri dishes

Petri dishes (145 cm² surface area) containing neuroblastoma clone N-18 cells were incubated for 4 days and then harvested by the procedure specified in the table. Each method was tested in triplicate; values shown are averages. Harvesting Method 1a is the standard procedure described under "Methods." For Method 1b, 2.5 ml of 0.1 N NaOH was added to each dish that had been scraped and washed. The dish then was incubated at 4° for 15 min to dissolve protein. For Method 2, 10 ml of 0.1 N NaOH was added to each dish containing a confluent monolayer of cells and the dish was again incubated for 15 min at 4°.

Method of harvesting protein	Amount of protein recovered per Petri dish
	mg
1. a, Dish scraped and washed (standard method)	6.14
b, Protein remaining in dish that had been scraped and washed was recovered by the addition of 0.1 N NaOH	0.30
Total	6.44
2. 0.1 N NaOH added to Petri dish containing a cell monolayer	6.00

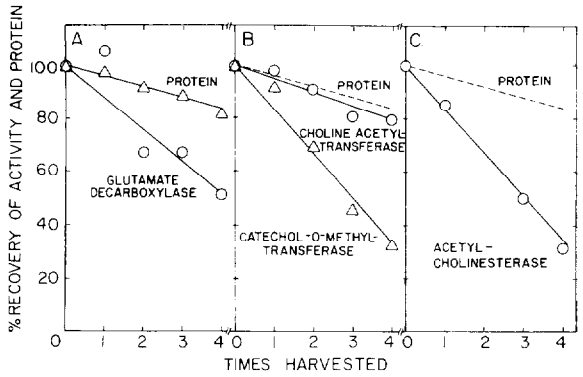

FIG. 5. Recovery of homogenate protein and total enzyme activities in multiple cycles of the scrape-wash procedure. Standard homogenates for assays of glutamate decarboxylase, catechol *O*-methyltransferase-acetylcholinesterase, and choline acetyltransferase were mixed in the proportion 10:4:1, added to culture dishes, and repeatedly cycled through the scrape-wash recovery procedure described in the text. The starting homogenate contained 20.3 mg of protein and enzyme specific activities of 145, 41.7, 31,850, and 118.4 pmoles of product formed per min per mg of protein for glutamate decarboxylase, catechol *O*-methyltransferase, acetylcholinesterase, and choline acetyltransferase, respectively. Glutamate decarboxylase was determined by Method *a*.

reaction mixtures had little influence on activity by either measurement. The presence of 1 mM amino-oxyacetic acid reduced CO_2 production by only 10 to 30%, yet γ-aminobutyric acid production was reduced by at least 95%. Thus, it appeared that $^{14}CO_2$ was being produced via pathways other than glutamate decarboxylase in the L-929 and glial cell lines tested.

Normal Brain Cells in Culture—Growth characteristics and marker enzyme activities were assessed in surface cultures of

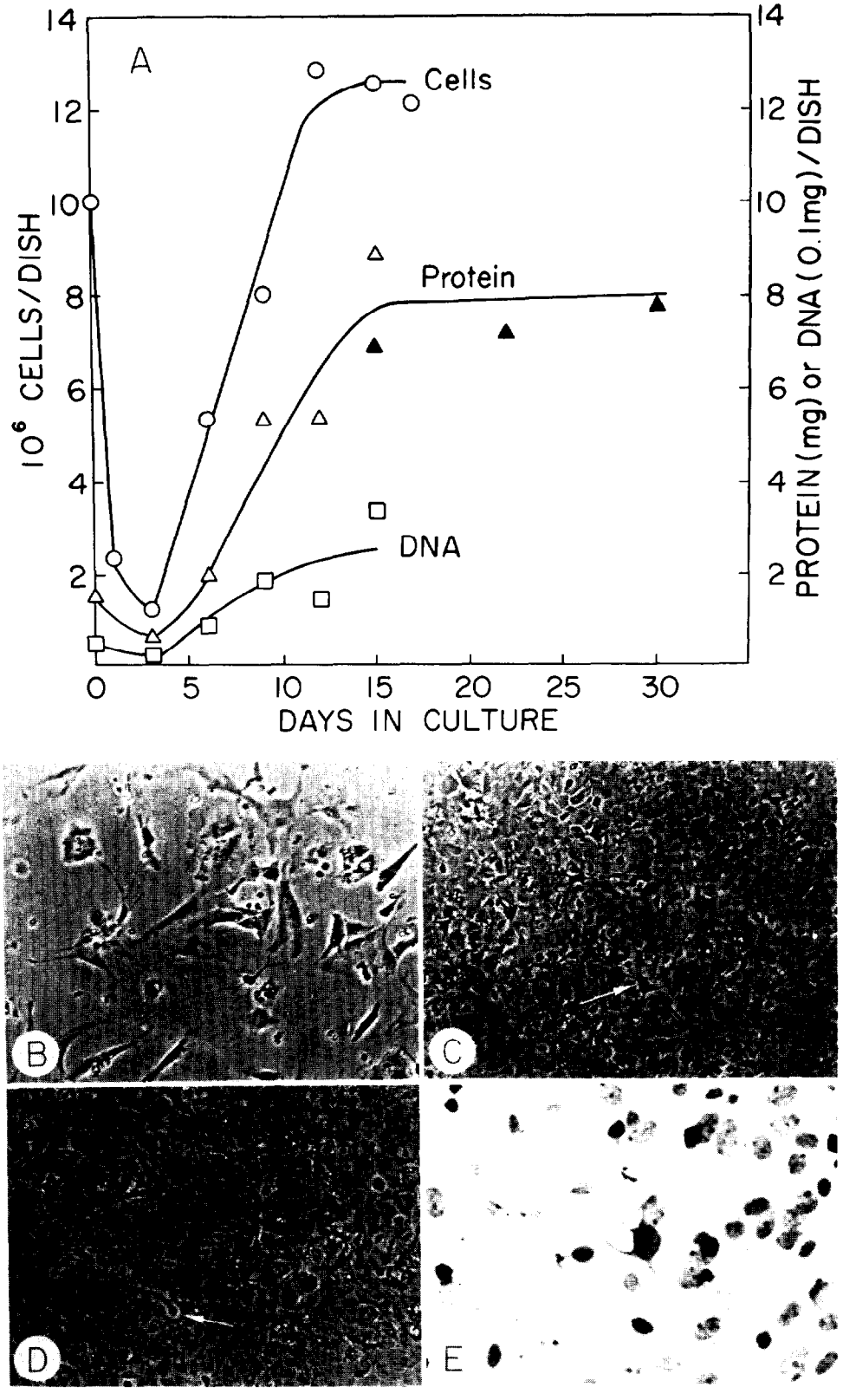

FIG. 6. Growth characteristics and morphology of cultured mouse brain cells. Mixed cells from brains of newborn mice were obtained and cultured as described under "Methods." *A*, (○) cell number (determined by counting trypsinized cells); (△, ▲) protein and (□) DNA contents of dishes at various times in culture. Values at zero time are those determined for the inocula. *Open symbols*, average of determinations on eight culture dishes (four from each of two separate, simultaneous time curves); *closed symbols*, average of determinations on four culture dishes from a third time curve. *B* to *D*, Phase-contrast photomicrographs of living cultures from the time curves. Magnifications of the 35-mm negatives were × 50. *B*, 3 days in culture. *C*, 11 days in culture, *arrow* indicates phase-dark cell (see text). *D*, 11 days in culture. *arrow* indicates phase-bright cell (see text). *E*, Bodian stain (modified protargol) of similar cells grown on cover glasses for 12 days. Bright field microscopy; magnification of the 4 × 5 inch negative was × 600.

newborn mouse brain cells. The effect of time in culture upon viable cell number, protein, and DNA content is shown in Fig. 6A. Each Petri dish was inoculated with 1×10^7 viable cells; however, after 3 days only 1.2×10^6 viable cells remained. Viable cell number and protein content increased during the 3rd to the 15th day, although the mixed population had formed a confluent monolayer by the 7th day in culture. The morphological appearance of the cultures is shown in Fig. 6, *B* through *E*. After 3 days of culture, cells with relatively small cell bodies and short processes were present along with a variety of large flat cells without processes (Fig. 6*B*). The confluent layer of cells present after 11 days of culture was composed of large flat cells,

TABLE VII

Enzyme specific activities in newborn mouse brain and cultured cells

Specific activities of glutamate decarboxylase, choline acetyltransferase, and acetylcholinesterase were determined on extracts of cultured cells as described under "Methods." An uncentrifuged homogenate of newborn Balb/c mouse brain was used for comparison. The maximum content of homogenate protein in the assays was: 237 μg for mouse brain homogenates, 110 μg for C-6, 906 μg for $C2_1$, 852 μg for CHB, 258 μg for RG-179, 1306 μg for L-929 (B-82 clone), 936 μg for HeLa, 533 μg for 3T3-S (Swiss mouse 3T3), and 580 μg for mouse brain cells cultured 30 days. Formation of γ-aminobutyric acid was determined in assays with L-[U-^{14}C]glutamate as substrate, followed by electrophoresis and chromatography as described above, with both Methods *a* and *b* for collection and determination of evolved CO_2. All determinations on cultured cells were performed when cells were 7 to 20 days postconfluency. Glutamate decarboxylase activity shown for C-6 represents the highest activity (at 31.5 mg of cell protein per 150-mm dish) found among four separate points on a growth curve.

Determination	Newborn mouse brain homogenate	Cultured cells							Newborn mouse brain cells
		Brain, non-neuronal				Nonbrain			
		C-6	$C2_1$	CHB	RG-179	L-929	HeLa	3T3-S	
	pmoles product/min/mg protein								
Glutamate decarboxylase									
By CO_2 production	487	79	71	84	43	55	13	8	93
By γ-Aminobutyrate production	496	8	5	1	3	0.2		6	86
Choline acetyltransferase	75	9			2	10	3	5	16
Acetylcholinesterase	25,900	732	701		280	625	299	102	1,120

on top of which were smaller cells that were dark in the phase microscope (phase-dark cells) (Fig. 6*C*). Also present were phase-bright cells with large cell bodies and long processes (Fig. 6*D*). As seen in Fig. 6*E*, silver impregnation was detected (with a modified Bodian stain) in cells with multiple long processes and large cell bodies.

The effect of time in culture on marker enzyme activities is shown in Fig. 7. Catechol *O*-methyltransferase activity did not reach measurable levels during culture. Glutamate decarboxylase specific activity (Fig. 7*A*) did not begin to rise until the 15th day in culture, when increase in cell number had ceased. In contrast to glial cell lines (Table VII), cultured brain cell extracts produced γ-aminobutyric acid and CO_2 in equal amounts. Choline acetyltransferase specific activity (Fig. 7*B*) fell in the first 3 days to less than 7 units (the minimum that could have been detected). The specific activity then increased from the 3rd to the 15th day; this rise was coincident in time and rate with the increase in cell number. After the culture had reached a stationary cell number, choline acetyltransferase specific activity continued to rise, but at a slower rate.

The specific activities of choline acetyltransferase and glutamate decarboxylase attained in these cultures were 47 and 43%, respectively, of the levels in dissociated newborn brain cells.

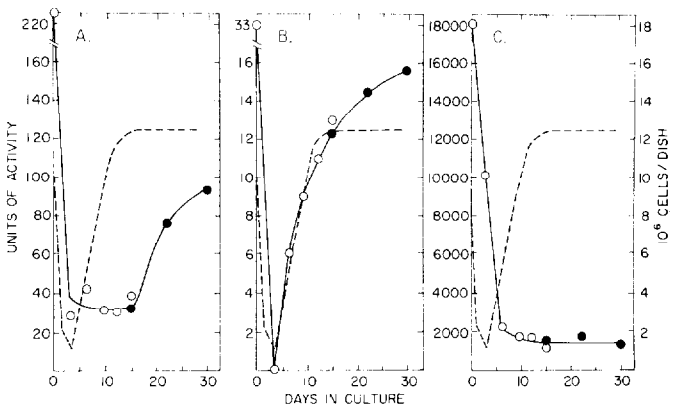

FIG. 7. Development of marker enzyme specific activities in surface cultures of newborn mouse brain cells. Each homogenate of Fig. 6*A* was evaluated for the marker enzyme activities as described under "Methods." Changes in specific activities (○, ●) and cell number (*dashed lines*) with time are shown for: *A*, glutamate decarboxylase (assessed by $^{14}CO_2$ evolution by Method *b*); *B*, choline acetyltransferase; and *C*, acetylcholinesterase. One unit of activity is defined as 1 pmole of product formed per min per mg of homogenate protein. *Open* and *closed symbols* are explained in the legend to Fig. 6.

From the 3rd to the 30th day in culture, total activities of these enzymes (picomoles per min per culture dish) increased by 48-fold for choline acetyltransferase and 21-fold for glutamate decarboxylase. Specific activity of glutamate decarboxylase was at least 10-fold higher in these cultures than in the nonbrain and glial cell lines tested (Table VII).

Acetylcholinesterase activity (Fig. 7*C*) decreased until the 6th day in culture, and remained constant for the remainder of the culture period. In all cultures, including the established cell lines, esterase activities were inhibited 80 to 95% by 10^{-5} M BW 284C51 dibromide, an inhibitor (25) of acetylcholinesterase (EC 3.1.1.7). Hydrolysis of acetylcholine by mouse brain was also inhibited 95% by this compound.

DISCUSSION

The results show that the procedure for preparation of extracts from cells in surface culture is reproducible and gives excellent recovery of protein and the enzyme activities measured. Various other procedures for preparation of extracts were not extensively studied; however, trypsinization, EDTA treatment, or scraping followed by washing of cells resulted in lower recoveries than the scraping procedure. The enzyme assays are rapidly and easily performed and are applicable for routine use with a large number of samples. Important features in applying the assays are identification of reaction products and determination of the specificity of the enzyme activities. In the glutamate decarboxylase assay, evolution of $^{14}CO_2$ was not always a measure of concomitant production of γ-aminobutyric acid by glutamate decarboxylase. In the choline acetyltransferase assay, enzyme systems utilizing acetyl-CoA may produce products other than acetylcholine that are recoverable in ion exchange column effluents (20). There are similar potential difficulties with the acetylcholinesterase and catechol *O*-methyltransferase assays. Hence, methods for product identification and enzyme characterization were presented as integral parts of the assays.

The enzyme activities studied were selected because they are important in neurotransmitter metabolism and also because acet-

ylcholinesterase, choline acetyltransferase, and glutamate decarboxylase activities are higher in brain than in most other tissues. Relatively low levels of these enzyme activities were found, however, in several nonbrain and glial cell lines. Thus, the enzyme activities are markers for neurons only when specific activities are considerably higher than those of the non-neuronal cell lines shown in Table VII. The low levels of the activities exhibited by these non-neuronal cell lines serve as a useful baseline for assessment of nerve cell cultures.

Properties of growth of normal newborn brain cells in surface culture were investigated as a first step toward establishing clonal cell lines of mammalian neurons. Primary surface cultures of newborn brain cells contained cells that appeared to be differentiated neurons both morphologically and histochemically. Activities of choline acetyltransferase, acetylcholinesterase, and catechol *O*-methyltransferase did not reach levels that were significantly higher than non-neuronal cell lines. In contrast, brain cultures attained levels of glutamate decarboxylase activity that were at least 10-fold higher than non-neuronal cells, and the increase in activity occurred after increase in cell number in the cultures had ceased. Thus, primary brain cell cultures possess several properties of differentiated neurons.

It is important to note that with improved culture techniques and with cells from younger animals, brain cell cultures have been obtained with activities of these enzymes which exceed those of newborn brain homogenates[1] (26). In addition, other sensitive radiochemical methods recently developed by Hildebrand *et al.* (27) also may be applicable to cells in culture.

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