

BIOGENIC AMINES IN CULTURED NEUROBLASTOMA AND ASTROCYTOMA CELLS

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ABSTRACT

The presence of biogenic amines in cultured cells of mouse neuroblastoma C-1300 (clone NB-2a) was suggested by fluorescence-microscope histochemistry. Incubation in media containing L-[¹⁴C]tyrosine and L-[¹⁴C]tryptophan for 24 h, followed by high-voltage electrophoresis, radiochromatogram scanning, and scintillation counting, confirmed the presence of [¹⁴C]dopamine, [¹⁴C]norepinephrine, [¹⁴C]epinephrine, [¹⁴C]serotonin, [¹⁴C]tyramine, and [¹⁴C]octopamine. Dopamine, norepinephrine, epinephrine, and serotonin were demonstrated spectrophotofluorometrically in concentrations, expressed as micrograms amine per milligram protein, of 1.19, 0.027, 0.038, and 0.148, respectively, for cells in a stationary growth phase.

Fluorescence-microscope histochemistry also suggested the presence of biogenic amines in cultured astrocytoma cells (cell line C6). Spectrophotofluorometric assay of cells in a stationary growth phase demonstrated intracellular dopamine, norepinephrine, epinephrine, and serotonin in concentrations significantly lower than those of neuroblastoma cells.

Mouse neuroblastoma cells (C-1300) adapted to tissue culture conditions have many of the properties of nerve cells *in vivo*. These cells, which have axon-like processes (neurites) (3), have the appearance of typical neurons when grown under appropriate conditions and can generate action potentials spontaneously (23) in response to electrical stimulation (23), or in response to iontophoretically applied acetylcholine (16). Cholinergic clones, with the enzymatic capability for acetylcholine synthesis, and adrenergic clones, which contain the enzymes necessary for synthesizing catecholamines have been isolated (1).

Initial histochemical fluorescence studies suggest that at least some clones of mouse neuroblastoma cells contain catecholamines (13). The presence of these amines intracellularly has been substantiated by following the incorporation of radioactive tyrosine into dopamine and norepi-

nephrine in neuroblastoma cells (27). In addition, it has been shown that neuroblastoma cells possess the capacity to accumulate [³H]norepinephrine ([³H]NE), [³H]dopamine ([³H]DA), and [³H]epinephrine ([³H]E), from the media, although the kinetics of these uptake mechanisms have not been elaborated (25). In view of these data it is of some interest that published electron micrographs of these cells give little indication of the intracellular localization of these amines (24).

As a first step in relating cellular ultrastructure to the intracellular accumulation, storage, and release of catecholamines, we have attempted to characterize in a more definitive way the catecholamine content of a cloned line of neuroblastoma cells *in vitro*. Biogenic amines were localized by the fluorescence histochemical method of Falck and Hillarp (14, 15), isolated electrophoretically after labeling with radioactive precursors, and

quantitated by spectrophotofluorometric assay. These methods were used also to characterize the biogenic amines of a cloned line of astrocytoma cells adapted to tissue culture.

MATERIALS AND METHODS

Experiments were performed with neuroblastoma cells derived from a clone established by Klebe and Ruddle (20), designated NB-2a, and an astrocytoma cell line established by Benda et al. (4), obtained from Dr. A. Dorfmann (University of Chicago, Chicago, Ill.) and Dr. S. E. Pfeiffer (University of Connecticut, Bridgeport, Conn.), and designated C6 and C6P, respectively. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Grand Island Biological Co., Grand Island, N. Y.) supplemented with: 10% gamma globulin-free newborn calf serum; penicillin, 10 U/ml; and streptomycin, 10 μ g/ml. Cells were grown in either 250-ml Falcon flasks (Falcon Plastics, Div. of B.-D. Laboratories, Los Angeles, Calif.), or 100 \times 20-mm Optilux tissue culture dishes (Falcon), or 3-ml multidish Disposotrays (Linbro Chemical Co., Inc., New Haven, Conn.), and were maintained at 37°C in a humidified, 95% air-5% CO₂ incubator. The media were changed on alternate days and the cells were subcultured every 6-8 days using a 0.25% Viokase solution (Grand Island Biological Co.) to free the cell monolayer, which was adherent to the bottom of the dish.

Cells were prepared for fluorescence histochemistry according to the method of Falck and Hillarp (14, 15). Sterile glass cover slips 22 \times 22 mm (Scientific Products Div., American Hospital Supply Corp., McGaw Park, Ill.) were placed in tissue culture dishes and cells were added. After 6-8 days the cover slips with attached cells were removed, rinsed with buffer (144 mM NaCl, 10 mM tricine, pH 7.0), and frozen in liquid Freon-22 prechilled to -154°C with liquid nitrogen. After drying in vacuo at -40°C for at least 2 days, the specimens were exposed to formaldehyde vapors generated from paraformaldehyde (equilibrated at 60% relative humidity) at 80°C for 1 h, and then examined with a fluorescence microscope. Details of the formaldehyde exposure and microscopy have been described previously (8).

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A qualitative analysis of amines visualized by formaldehyde-induced fluorescence was achieved with neuroblastoma cells plates in 3-ml multidish Disposotrays and grown in DMEM. After 24-48 h the media were replaced with DMEM deficient in tyrosine and tryptophan, and L-[¹⁴C]tryptophan (2,500 μ Ci/mg) and L-[¹⁴C]tyrosine (U), (227 μ Ci/mg) (both from New England Nuclear, Boston, Mass.) were added to each well to give a total of 10 μ Ci of labeled compound per milliliter of media. Cells were then incubated for 24 h, rinsed with nonradioactive media, and harvested in 150 μ l of electrophoresis buffer

(see below) which contained unlabeled DA, NE, E, 5-HT, tyramine (TY), and octopamine (OT), each in a concentration of 2 mg/ml. These unlabeled compounds served as markers during the electrophoretic separation. The cells were then alternately frozen and thawed, and electrophoresed using methods modified from Hildebrand et al. (18). High-voltage electrophoresis was carried out on Whatmann 3 MM paper in a Savant tank (Savant Instruments, Inc., Hicksville, N. Y.) with a 0.47 M formic acid, 1.4 M acetic acid buffer at pH 1.9, using Varsol as a coolant. Voltage was maintained at 2.5 kV for 1 h with a Savant power source. During this time the current increased from approximately 80 to 110 mA. After electrophoresis and drying at 60°C the paper was sprayed with a potassium ferricyanide-ethylene diamine solution (26) to visualize the catecholamines and indoleamines, and with diazotized sulfanilic acid to visualize TY and OT (12). The paper was cut into strips and scanned with a Packard model 7201 radiochromatogram scanner (Packard Instrument Co., Inc., Downers Grove, Ill.). The strips were then cut into 0.5-1-cm segments and placed in toluene-Liquifluor scintillation fluid. Radioactivity was measured in a Packard scintillation counter. Medium from each well was also analyzed by electrophoresis, radiochromatogram scanning, and scintillation counting.

For quantitative determination of DA, NE, E, and 5-HT, cells grown in 8-10 tissue culture dishes were pooled by scraping with a rubber policeman, rinsed with buffer (144 mM NaCl, 10 mM tricine, pH 7.0), and centrifuged at low speed. The buffer was discarded and the cells were homogenized in acidified *n*-butanol. A sample of the butanol phase was assayed for protein by the method of Lowry et al. (21). The amines were further extracted and assayed using methods modified from Chang (9), and Maickel et al. (22). The catecholamines were oxidized with an iodine reagent and the 5-HT with *o*-phthalaldehyde (Regis Chemical Co.; Morton Grove, Ill.). Fluorescence was determined with an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Travenol Laboratories, Inc., Silver Spring, Md.) with the following excitation/emission settings: DA, 320 nm/370 nm; NE, 385 nm/485 nm; E, 410 nm/500 nm, and 5-HT, 360 nm/470 nm. Standards, blanks, and recoveries were prepared, and after final calculations the concentrations of the amines were expressed as micrograms amine per milligrams per protein.

RESULTS

Neuroblastoma cells approaching a stationary growth phase showed a small degree of yellow-green autofluorescence before exposure to formaldehyde vapors (Fig. 1). Fluorescence was enhanced significantly by condensation with formaldehyde vapors generated from paraformaldehyde. Most NB-2a cells exhibited a diffuse yellow-green

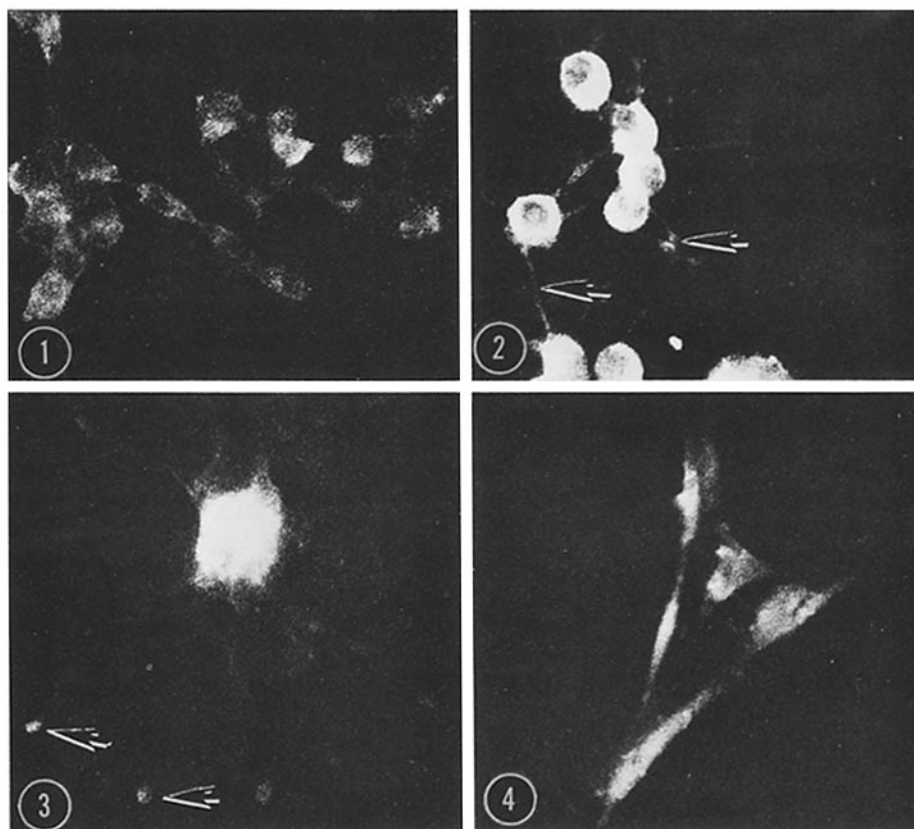


FIGURE 1 Fluorescent photomicrograph of NB-2a freeze-dried cells before condensation with formaldehyde vapors. A faint autofluorescence is visible. $\times 210$.

FIGURE 2 Fluorescent photomicrograph of NB-2a freeze-dried cells that were exposed to formaldehyde vapors for 1 h at 80°C. Intracytoplasmic fluorescence is enhanced in perikarya and processes (arrows). $\times 210$.

FIGURE 3 Fluorescent photomicrograph of NB-2a cells that were exposed to formaldehyde vapors for 1 h at 80°C. Bright fluorescent swellings at the terminations of cell processes (neurites) are indicated by arrows. $\times 525$.

FIGURE 4 Fluorescent photomicrograph of astrocytoma cells (C6P) after a 1-hour exposure to formaldehyde vapors at 80°C. A diffuse intracytoplasmic fluorescence is visible. $\times 210$.

fluorescence throughout the cytoplasm and processes (Fig. 2) with beads of particularly intense fluorescence irregularly distributed along the neurites and at their terminations (Fig. 3). Fluorescence intensity and color varied among NB-2a cells, some of which appeared more yellow than green. Similarly, C6P cells, which before formaldehyde condensation fluoresced minimally, emitted a variable yellow-green intracytoplasmic fluorescence after condensation with formaldehyde vapors (Fig. 4). This fluorescence appeared, in

general, less intense than that emitted by NB-2a cells. It was observed in both groups that those cells which fluoresced with a yellow color were not randomly distributed in the cultures, but occurred in patches of varying sizes. These yellow cells faded much more rapidly on exposure to UV light than those which emitted a green fluorescence.

The results of electrophoretic separation after incubation with radioactive precursors are shown in Fig. 5 A. The electrophoretogram from NB-2a cells shows separate peaks that correspond to DA,

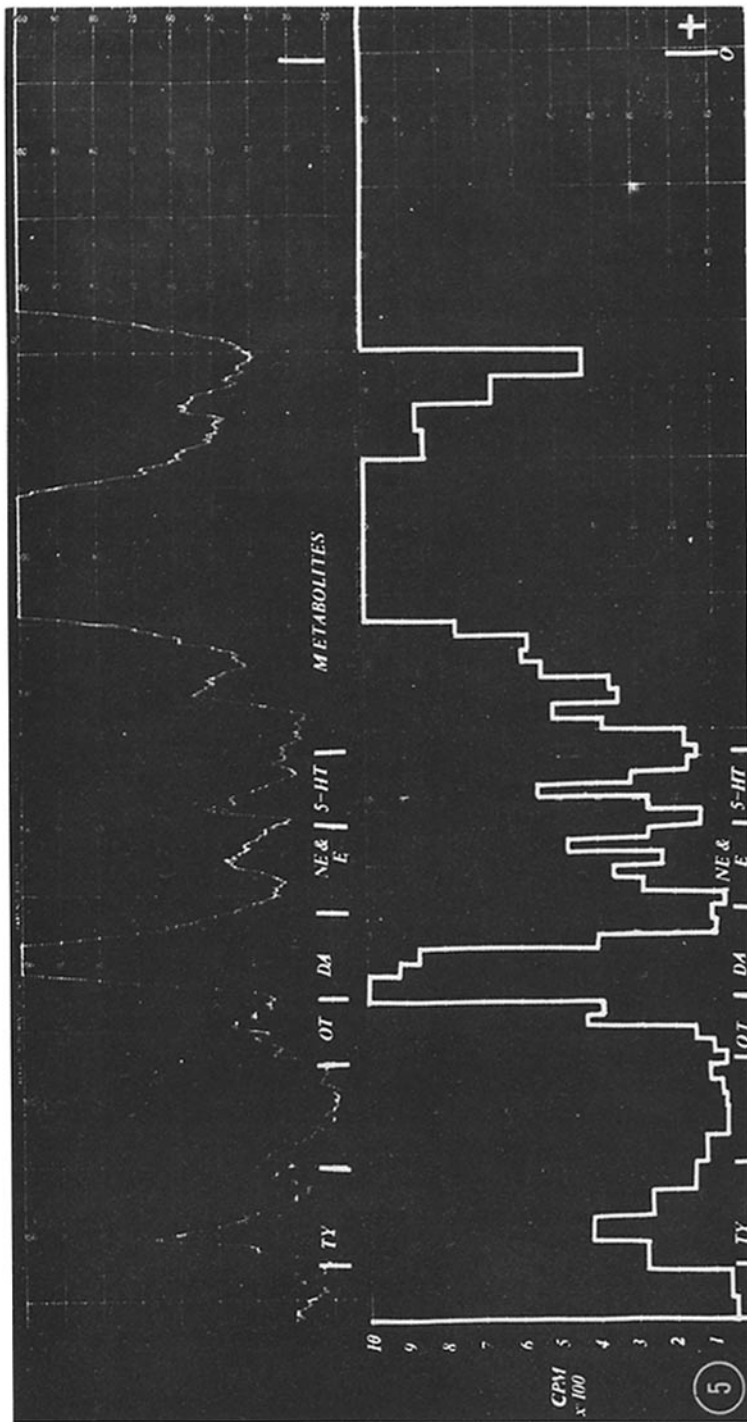


FIGURE 5 A Radiochromatogram scan of electrophoretogram of NB-2a cells following 24-h incubation in L-[¹⁴C]tyrosine and L-[¹⁴C]tryptophan. Peaks corresponding to DA, TY, OT, and 5-HT, and one peak for NE and E are visible (300 cpm full scale). FIG. 5 B Analysis by scintillation counting of electrophoretogram shown in Fig. 5 A. The single peak corresponding to NE and E in Fig. 5 A is now resolved into two peaks, one corresponding to NE, the other to E.

5-HT, TY, and OT, and a single peak that corresponds to both NE and E. Scintillation counting of the electrophoretogram resolved the single peak found on radiochromatogram scanning into two peaks, one corresponding to NE, the other to E (Fig. 5 B).

Results of spectrophotofluorometric assays of NB-2, C6, and C6P cells in a stationary growth phase are summarized in Table I. The average concentrations of the various amines, in micrograms per milligram protein, for NB-2a cells were: DA, 1.19; NE, 0.027; E, 0.038, and 5-HT, 0.148. Average concentrations of amines in C6 cells were: DA, 0.25; NE, 0.012; E, 0.021, and 5-HT, 0.019. Average concentrations of amines in C6P cells were: DA, 0.45; NE, 0.024; E, 0.040, and 5-HT, 0.040. These concentrations of monoamines were compared using the Student's *t* test. The differences in concentrations of DA, 5-HT, NE, and E were found to be statistically significant ($p < 0.001$) in cultures of NB-2a and C6 cells. For DA and 5-HT the differences between NB-2a and C6P were also found to be significant ($p < 0.001$). Finally, the concentration of DA, 5-HT, NE, and E found in the two clones of astrocytoma cells were significantly different ($p < 0.05$).

DISCUSSION

Catecholamines and indolamines condense with formaldehyde vapors to form fluorescent isoquinoline derivatives which emit a green-yellow fluorescence when activated by UV light (11, 14, 15). This has been well established and is the basis of the fluorescence histochemical reaction of Falck and Hillarp (14, 15). Although the wavelength of light emitted depends upon both the specific mono-

amine present and its intracellular concentration, it has been established that neurons containing NE form a relatively stable, green fluorophore while 5-HT forms a highly labile, bright yellow fluorophore, which characteristically fades quickly when irradiated with UV light (11).

After preparing cultures of NB-2a cells by the method of Falck and Hillarp we have found two distinct populations of cells, one fluorescing green, the other bright yellow. The latter cells faded quickly while being viewed with the fluorescence microscope. Astrocytoma cells tended to emit a more yellow fluorescence which also faded rapidly. These observations suggested that cloned lines of NB-2a and astrocytoma cells synthesize monoamines and that there are at least two cell populations in the NB-2a clone, one synthesizing primarily catecholamines, the other synthesizing primarily 5-HT. Although there are previous reports that neuroblastoma cells contain catecholamines and the enzymes required for their synthesis (1, 27), we know of no previous report that neuroblastoma cells in vitro contain 5-HT or that astrocytoma cells contain monoamines.¹ That catecholamines and indolamines are synthesized by these cells is corroborated by spectrophotofluorometric assays (Table I), which indicate intracellular concentrations, expressed as micrograms amine per milligram protein, to be comparable to those estimated for DA in nerve cell bodies of the rat substantia nigra (11) (i.e., 0.1–0.4 $\mu\text{g}/\text{mg}$ protein).

It is well established that NB-2a clones synthesize catecholamines (1, 27). Our electrophoretic isolation of 5-[¹⁴C]HT from NB-2a cells incubated for 24 h in monoamine-free media contain-

TABLE I
Concentrations* of Biogenic Amines in Cultured Neuroblastoma and Astrocytoma Cells

	DA	NE	E	5-HT
Neuroblastoma	1.22	0.025	0.042	0.168
	1.05	0.024	0.034	0.127
	1.09	0.026	0.038	0.167
	1.19 ± 0.16‡	0.027 ± 0.004	0.038 ± 0.004	0.148 ± 0.030
NB-2a†	1.13	0.033	0.035	0.106
	1.45	0.027	0.041	0.171
Astrocytoma	0.19	0.011	0.022	0.020
	0.27	0.012	0.022	0.018
	0.25 ± 0.05	0.012 ± 0.000	0.021 ± 0.001	0.019 ± 0.001
C6†	0.28	0.013	0.020	0.018
	0.33	0.030	0.034	0.041
C6P†	0.47	0.018	0.046	0.038
	0.45 ± 0.11	0.024 ± 0.006	0.040 ± 0.006	0.040 ± 0.002
	0.55	0.024	0.040	0.041

* μg amine per milligram protein.

† stationary growth phase.

‡ mean ± SD.

ing L-[¹⁴C]tryptophan indicates that certain NB-2a cells not only contained 5-HT, but synthesized it as well. Although these cells were not specifically identified, it is probable that they corresponded to those NB-2a cells which formed a labile, bright yellow fluorophore when condensed with formaldehyde vapors. These data suggest mixed populations of cells synthesizing both catecholamines and indolamines, a situation seemingly analogous to that reported by Amano et al. (1), who found neuroblastoma clones with the enzymes required for synthesis of both acetylcholine and norepinephrine. Because the enzyme activities of these were lower than in pure adrenergic or pure cholinergic clones, the existence of a mixed population of adrenergic and cholinergic clones seemed more likely than that of a pure clone synthesizing both catecholamines and acetylcholine.

The fluorescence of astrocytoma cells after formaldehyde condensation was shown by spectrophotofluorometric analysis to be due to significant amounts of intracellular catecholamines and 5-HT (Table I). The intensity of fluorescence, however, was considerably less than that of neuroblastoma cells under comparable *in vitro* conditions (compare Figs. 1 and 5), as was their concentration of intracellular monoamines (Table I). These astrocytoma cells resembled normal astrocytes both in their morphology (5) and in their capacity to synthesize the brain-specific protein, S-100 (5). Normal astrocytes have not been shown to synthesize catecholamines or indolamines, but monoamine oxidase and catechol-*O*-methyl transferase activities have been demonstrated in astrocytes (28), which also have been shown to accumulate exogenous catecholamines from their immediate extracellular environment (7). These data and the related observation that astrocytoma cells respond to exogenous catecholamines by increasing intracellular levels of cyclic 3':5'-adenosine monophosphate (10) have led to speculations as to the physiological role of glia with regard to the metabolism of monoamines in nervous tissue (17). Our demonstration of monoamines in astrocytoma cells *in vitro* (Table I) prompts further speculation as to the supposed relationship between glial cells and neurotransmitter substances in nervous tissues.

Amano et al. (1) have identified three types of clones of neuroblastoma C-1300: (a) clones characterized by high choline acetyltransferase activity or cholinergic clones; (b) clones characterized by high tyrosine hydroxylase activity or adrenergic

clones; and (c) clones that have neither choline acetyltransferase nor tyrosine hydroxylase activities. Such enzymatic activities are not unusual for derivatives of neural crest and anticipate our finding of catecholamines in neuroblastoma cells, which can be presumed to be of neural crest origin (19). Our finding of 5-HT in these cultures, however, was not anticipated and is somewhat surprising in a cell lineage in which genes required for tyrosine hydroxylase and choline acetyltransferase activities are normally expressed, but in which the synthesis of serotonin is not commonly found. As it is likely that in their metabolic capabilities, clonal lines resemble some common parent cell, clonal lines of neuroblastoma cells can be expected to resemble their stem cell (sympathogonia or sympathoblast) or a known progeny of the stem cell. Sympathogonial progeny, which synthesize DOPA, derivatives of DOPA (i.e., DA, NE, and E), tyramine and its derivatives, and acetylcholine have been identified (1, 2, 26). Although uncommon, a neural crest derivative (a human neuroblastoma) synthesizing 5-HT *in vivo* has been identified (6). In addition, it has been argued that the entire enterochromaffin system may derive from the neural crest (29). Our finding of neuroblastoma clones which synthesize 5-HT *in vitro* supports this argument and suggests a rarely occurring alteration in normal gene expression, heretofore unknown in clones of murine neuroblastoma.¹

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