*Proc. Nat. Acad. Sci. USA*
Vol. 68, No. 7, pp. 1436–1440, July 1971

# Regulation of the Synthesis of Choline-*O*-Acetyltransferase and Thymidylate Synthetase in Mouse Neuroblastoma in Cell Culture

**(confluent cells/rapidly-dividing cells/specific activity)**

ROGER N. ROSENBERG, LARRY VANDEVENTER, LAURA DE FRANCESCO, AND MORRIS E. FRIEDKIN

The Departments of Neurosciences and Biology, University of California, San Diego School of Medicine, La Jolla, Calif. 92037

*Communicated by Clifford Grobstein, April 19, 1971*

**ABSTRACT** **The specific activity of mouse neuroblastoma choline-*O*-acetyltransferase (EC 2.3.1.6.) increased 5.7-fold when the rate of cell division was restricted (as compared to cells kept rapidly dividing for 9 days); the specific activity of mouse neuroblastoma thymidylate synthetase increased 2.4-fold when nondividing cells again entered the logarithmic phase of cell growth. The highest specific activities for choline-*O*-acetyltransferase and lowest specific activities for thymidylate synthetase were obtained from cultures where cell division was restricted; the opposite result was observed when the cells were growing rapidly. Thus, the regulation of these two enzymes is out of phase with respect to each other and is dependent on the rate of cell division. The inverse relationship for the regulation of these two enzymes is discussed in relation to the needs of mitotic versus differentiated neuroblastoma cells.**

Mouse neuroblastoma C-1300 tumor cells in culture synthesize acetylcholine and norepinephrine and also generate spontaneous action potentials and action potentials in response to acetylcholine and electrical stimulation (1–4). Further, the tumor cells in culture possess a permease for dopamine uptake, and electron micrographs show clusters of dense-core vesicles in nerve terminals (3). Blume *et al.* (5) recently demonstrated the regulation of mouse neuroblastoma acetylcholinesterase specific activity in cell culture when the rate of cell division was restricted, and showed that the regulatory mechanism for acetylcholinesterase was inversely related to the rate of cell division. Highest acetylcholinesterase specific activity was obtained in cultures containing nondividing cells.

We report here that mouse neuroblastoma choline-*O*-acetyltransferase (EC 2.3.1.6) and thymidylate synthetase (the enzyme that converts dUMP to dTMP) activities are regulatable and that the regulatory mechanism is out of phase for these two enzymes. In confluent cultures in which cell division is restricted, choline-*O*-acetyltransferase specific activity is the highest and thymidylate synthetase specific activity is the lowest. The opposite is true in cultures where cells are rapidly dividing.

## MATERIALS AND METHODS

### Cell line

The mouse (Ajax strain) neuroblastoma C-1300 was originally obtained from the Jackson laboratories, Bar Harbor, Me., and clone N-18 was derived from a single-cell clone from tissue-culture adapted cells by Dr. T. Amano. Clone N-18 was a gift of Dr. Marshall W. Nirenberg, Laboratory of Biochemical Genetics, National Heart Institute, Bethesda, Md. (June 30, 1970).

### Culture conditions

Clone N-18 of mouse neuroblastoma C-1300 was grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum, sodium penicillin G (25 units/ml), and streptomycin sulfate (25 $\mu$g/ml) in Falcon flasks or Petri dishes at 37°C in an atmosphere of 10% $CO_2$–90% air at 100% humidity.

For the choline-*O*-acetyltransferase experiments, 250-ml Falcon flasks (75 $cm^2$) were incubated with $2 \times 10^6$ cells, grown to confluency and maintained confluent for 3 weeks. The medium was changed in these flasks on alternate days for the first 2 weeks and then daily for the last 7 days. At day zero of the choline-*O*-acetyltransferase experiment, the medium in each of the flasks was aspirated, and the cells were dissociated from the plastic surface by incubation in $Ca^{++}$- and $Mg^{++}$-free saline D-1 medium, supplemented with 5.6 mM glucose and 59 mM sucrose (modified D-1) for 15 min. About half of the cells from each flask could be tapped off into this medium. The cells were then centrifuged, resuspended in modified Eagle's Medium, and counted with a hemocytometer by the nigrosin method to determine viability (6). Plastic Petri dishes, 100-mm size, containing 10 ml of modified Eagle's Medium, 10% serum, 25 units per ml of sodium penicillin G, and 25 $\mu$g per ml of streptomycin sulfate, were prepared 24 hr earlier and kept at 37°C in 10% $CO_2$–90% air at 100% humidity. Into each dish were placed $3.7 \times 10^5$ viable neuroblastoma cells. Medium was changed on alternate days, beginning on day 2, for the first 8 days, and then daily. At day 7 in culture, several Petri dishes were dissociated in 0.1% trypsin (GIBCO 1:250) after aspirating off growth medium and washing the plate in modified D-1 medium. New, preincubated 100-mm Petri dishes were inoculated with $3.7 \times 10^5$ viable neuroblastoma cells and are referred to as split-cell plates. Medium was changed on the split-cell plates on alternate days.

For the thymidylate synthetase experiments, cells were kept rapidly dividing in monolayer culture adherent to the surface of a Falcon flask for seven generations. At day zero, these rapidly dividing cells were tapped off into $Ca^{++}$- and $Mg^{++}$-free modified D-1 medium, centrifuged, resuspended in modified Eagle's Medium, and counted with nigrosin to determine viability. Plastic Petri dishes, 150-mm size, were

prepared 24 hr prior to the addition of cells with 20 ml of the serum-containing medium described above, warmed to 37°C, and gassed with 10% $CO_2$–90% air at 100% humidity. Into each plate were added $1.9 \times 10^6$ viable cells. Medium was changed on alternate days for the first 7 days, and then daily. At day 8 in culture, several plates were dissociated in 0.1% trypsin after aspirating off the medium and washing each plate in modified D-1 medium. New dishes that contained 20 ml of warmed, gassed growth medium were prepared, and to each were added $1.9 \times 10^6$ viable cells. These are referred to as split-cells no. 1. On day 1 in culture, split-cells no. 1 were split again, and $5.5 \times 10^5$ viable cells were added to new dishes and are referred to as split-cells no. 2. At various times in culture, cell counts were performed by dissociating the adherent cell monolayer with 0.1% trypsin and counting viable cells by the nigrosin method in a hemocytometer.

#### Homogenates

The medium from each dish was aspirated, and the dish surface was washed twice with modified D-1 medium. Dishes were tilted and drained, and cell monolayers were recovered by scraping. For choline-*O*-acetyltransferase assays, the cells were scraped with small amounts of solution containing 50 mM potassium phosphate buffer (pH 6.8)–1 mM EDTA, and the scraped cells were sonicated at 4°C for 5 min. For thymidylate synthetase assays, the cells were scraped with small amounts of a solution containing 0.05 M Tris buffer–0.01 M mercaptoethanol–1 mM EDTA, 25% sucrose, pH 7.4, and freeze–thawed three times (minus 78°C to 37°C) in ethanol–dry ice. All assays were performed on the same day as scraping.

#### Assays

Choline-*O*-acetyltransferase activity was determined by a modification of the method of Schrier and Shuster (7). Each reaction contained the following, in a final volume of 0.05 ml: 0.05 M potassium phosphate buffer (pH 6.8), 1 mM EDTA, 0.5% Triton X-100 (Packard Instrument Co.), 0.2 M NaCl, $2.5 \times 10^{-3}$ M choline iodide, $1 \times 10^{-4}$ M neostigmine methyl sulfate, $2.18 \times 10^{-4}$ M [1-$^{14}$C]acetyl coenzyme-A, and 0–1.4 mg of homogenate protein. Reactions were incubated for 10 min at 37°C, and stopped by the addition of 1.0 ml of water at 4°C. In all cases, the rate of reaction was proportional to homogenate concentration. The diluted reaction mixture, and an additional 2.0 ml of cold water, were passed through a disposable 0.5 × 5 cm column of Bio-Rad-1-chloride (100–200 mesh) washed with water. The eluates were collected in a scintillation vial, and 15 ml of scintillation fluid (188 ml of Triton X-100, 750 ml of toluene, 9.4 g of 2,5-diphenyl oxazole) was added; the radioactivity was determined in a Beckman Liquid Scintillation Counter. Most values represent the average of quadruplicate homogenates. Each homogenate was assayed at four concentrations. One unit of choline-*O*-acetyltransferase specific activity is defined as 1.0 pmol of $^{14}$C-product formed/mg of protein per 10 min at pH 6.8 and 37°C. Protein was determined by a modification of the method of Lowry *et al.* (8).

Thymidylate synthetase activity was assayed by a modification of the methods of Roberts (9). Cells disrupted by the freeze–thaw procedure were centrifuged in a Sorvall SS-34 centrifuge at 17,000 × *g* for 10 min at 4°C, and the supernatant fluid was assayed for enzyme activity. The total incubation mixture (315 μl) consisted of 0.76 mM tetrahydrofolate, 10 mM formaldehyde, 0.038 mM [5-$^3$H]dUMP (0.23 μCi), 2.9 mM $MgCl_2$, 72 mM Tris buffer (pH 7.4), 64 mM β-mercaptoethanol, and neuroblastoma cell extract. Various aliquots of the supernatant fluid derived from the original disrupted cell preparation (50–250 μl) were used. In each case, the final volume was made up to 315 μl with sucrose buffer (see *Homogenates*). The reaction mixture was incubated at 37°C for 40 min. The tubes were then placed on ice and 500 μl of $8.0 \times 10^{-3}$ mM dUMP in water and 0.5 ml of a solution containing 136 mg/ml of Norite in 8% trichloroacetic acid (TCA) was added to stop the reaction. The tubes were agitated and allowed to stand on ice for 10 min. All tubes were then centrifuged in an IEC-PR-2 centrifuge at 1500 × *g* for 10 min at 4°C. An 800-μl aliquot of supernatant fluid was removed to a clean tube on ice, 250 μl of $16 \times 10^{-3}$ mM dUMP in 4% TCA and 500 μl of 168 mg/ml Norite in 4% TCA were added, and the tubes were mixed and centrifuged. 1-ml aliquots of supernatant fluid were pipetted into vials, 15 ml of counting fluid (100 g of naphthalene–5 g of 2,5-diphenyl oxazole–1000 ml of dioxane) were added, and radioactivity was determined in a Beckman Scintillation Counter. Most of the values represent an average of two or three extracts of different plates, and each extract was assayed at five different concentrations. One unit of thymidylate synthetase activity is defined as 1 nmol of thymidylate formed/μg of protein per 40 min at pH 7.4 and 37°C.

## RESULTS

#### Choline-*O*-acetyltransferase activity as a function of cell division

The specific activity of choline-*O*-acetyltransferase in relation to cell growth is illustrated in Fig. 1. Flasks containing confluent cells were subcultured on day 0, and $3.7 \times 10^5$ viable cells obtained from the splitting were put into each of a series of 100-mm Petri dishes. After a lag period, cells multiplied rapidly, with a generation time of 28 hr. On day 8 in culture, the maximum cell density of $17 \times 10^6$ cells was obtained. Cells obtained from confluent flasks had the highest choline-*O*-acetyltransferase specific activity. There was a two-fold reduction in specific activity in cells subcultured from day-zero flasks (131 units) to day-7 cells in culture (64.5 units), that had been in the phase of rapid division for at least 6 days. Cells split on day 7 and assayed 3 days later, thus extending their phase of rapid division to 9 days, had a 5.7-fold decrease in choline-*O*-acetyltransferase specific activity (23.0 units) as compared to confluent cells in flasks (131 units). Cells assayed on days 10, 12, and 14 in culture, having been confluent for 2, 4, and 6 days respectively, showed increased specific activities that approached the starting activities of the original confluent cultures. Thus, the highest specific activities were obtained in cultures in which neuroblastoma cell division was restricted.

#### Thymidylate synthetase activity as a function of cell division

The specific activity of thymidylate synthetase in relation to cell growth is shown in Fig. 2. Cells were kept in the logarithmic stage of cell growth for seven generations in Falcon flasks and then subcultured on day zero by tapping off the cells into $Ca^{++}$- and $Mg^{++}$-free modified D-1 solution. $1.9 \times 10^6$ viable cells were placed into each of the series of previously prepared 150-mm Petri dishes containing 20 ml of growth medium and, after a lag period, they divided rapidly with a generation time of about 24 hr. Between days 4 and 8

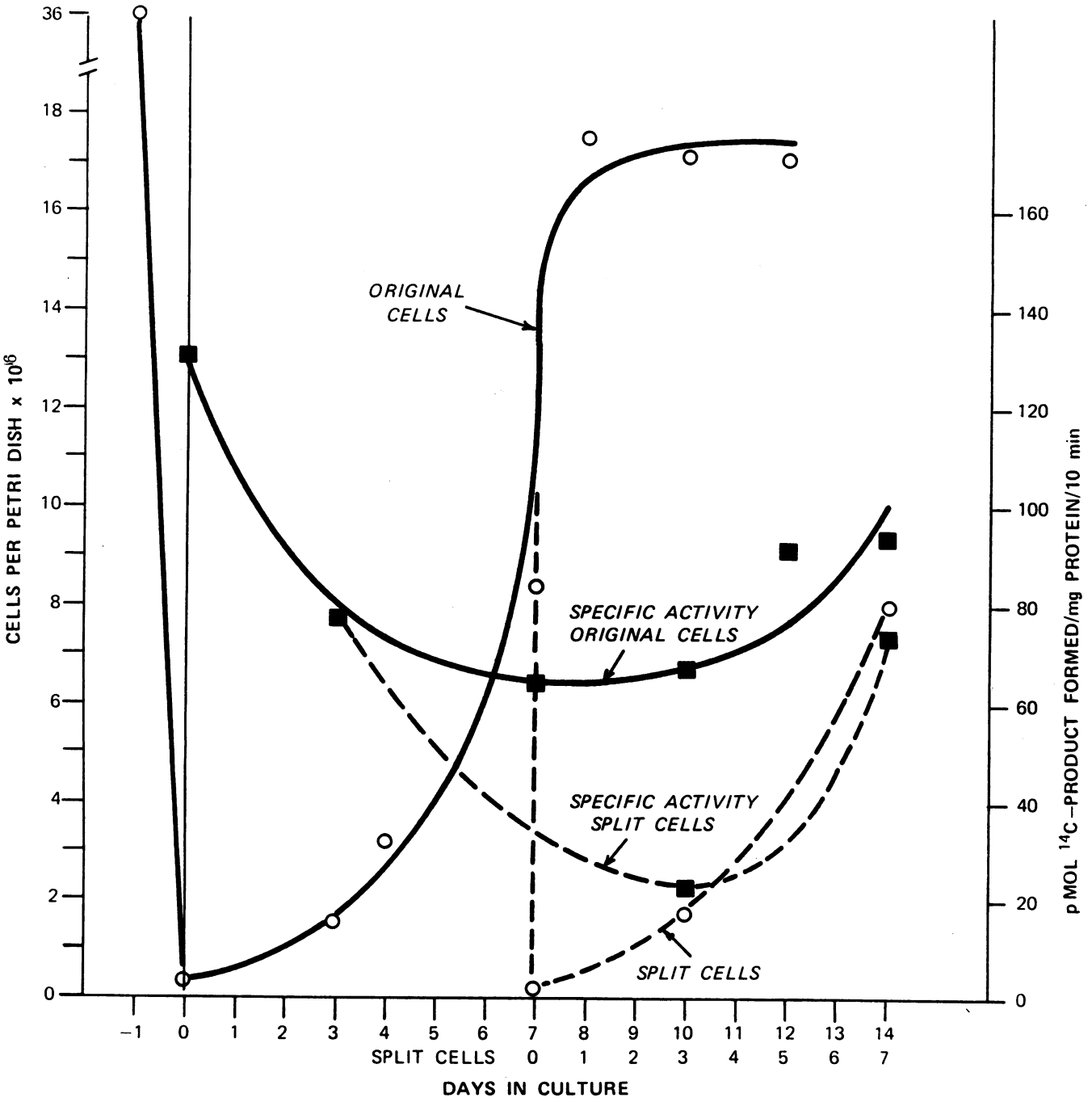


FIG. 1. The growth and number of cells present per Petri dish are shown in relation to the specific activity of choline-*O*-acetyltransferase. On the seventh day of incubation, some of the cells were dissociated with trypsin (as indicated by the *vertical broken line*). More than 90% of the cells were viable throughout the experiment. The rate of growth of cells incubated at day zero of the experiment and not subcultured is shown by the *solid line* and *circles* (—○—). Subcultured cell growth is shown by the *broken line* and *circles* (- -○- -). Choline-*O*-acetyltransferase specific activity of the cells incubated at day zero and not subcultured is shown by the *solid line* and *squares* (- -■- -), and by a *broken line* and *squares* for the subcultured cells (- -■- -).

in culture, the cells reverted from the phase of rapid division to static, nondividing cultures. Between days 8 and 11, cell division remained restricted. There was a progressive fall in thymidylate synthetase specific activity between days 4 and 8 and between days 8 and 11. On day 8, several Petri dishes were subcultured with 0.1% trypsin; split-cells no. 1, 1 day later, showed a decrease in thymidylate synthetase specific activity from 3.5 units to 1.9 units. 3 days after the split, however, split-cells no. 1 showed a 2.4-fold increase (from 1.9 units) to 4.5 units. Subculture of split-cells no. 1 into split-cells no. 2, and assay on day 2 before they could enter the phase of rapid division, gave high specific activity. Thus, the highest thymidylate synthetase specific activities were obtained from cultures in which neuroblastoma cells were rapidly dividing.

Choline-*O*-acetyltransferase and thymidylate synthetase specific activities were therefore out of phase with respect to each other. During the phase of rapid cell division, low

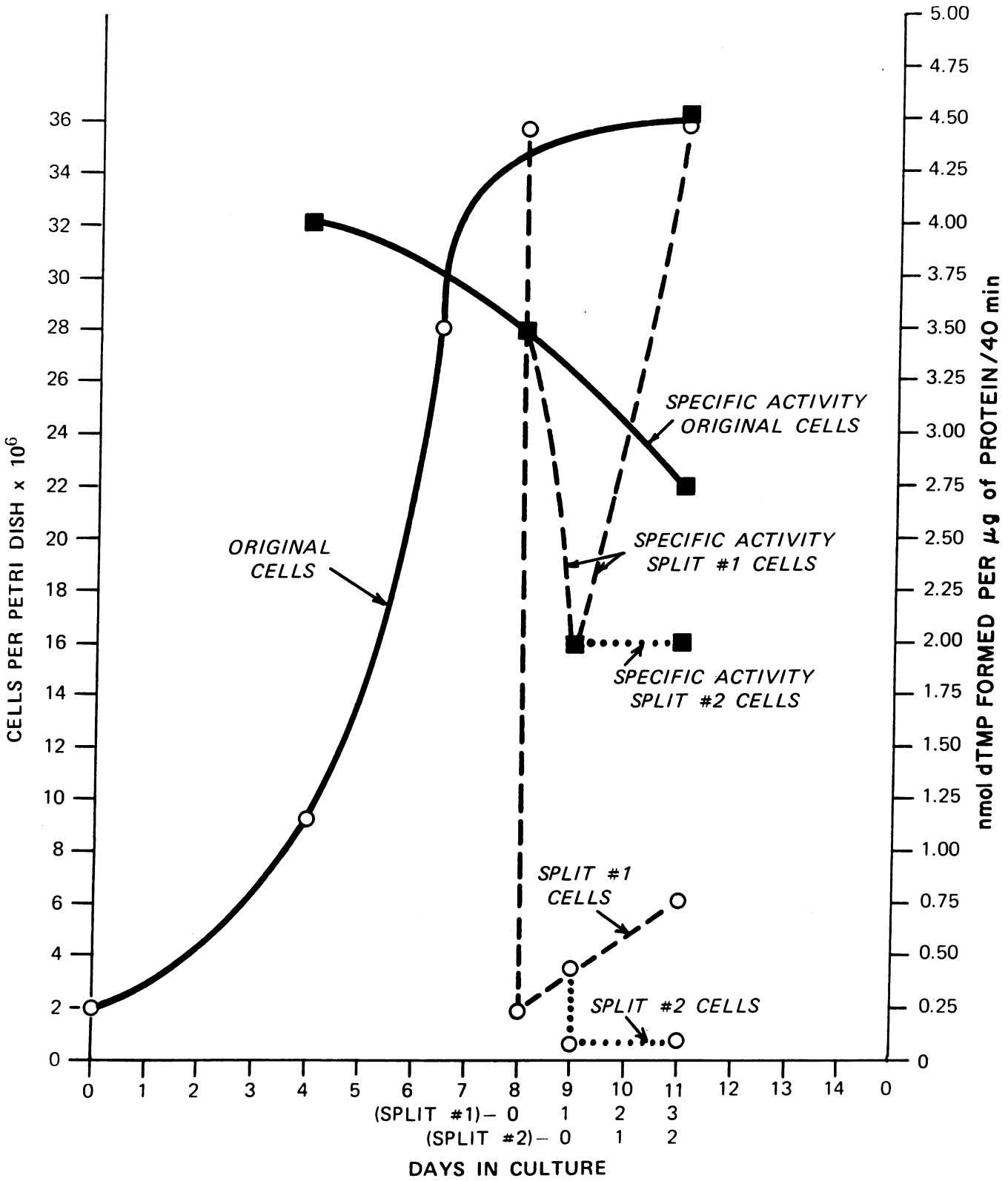


FIG. 2. Thymidylate synthetase specific activity is shown in relation to the growth rate and number of cells present per Petri dish. Some cells were subcultured with trypsin on day 8 of incubation, and some of these subcultured cells were subcultured again 1 day later (as indicated by the *vertical broken lines*). More than 90% of the cells were viable throughout the experiment. The rate of growth of the cells incubated at day zero of the experiment and not subcultured is shown by the *solid line* and *circles* (—○—). The rate of growth of cells subcultured on day 8 of incubation is shown by the *broken line* and *circles* (– –○– –), and the rate of growth of those cells subcultured again 1 day later is shown by the *dotted line* and *circles* (· ·○· ·). Thymidylate synthetase specific activities are shown as follows: *solid lines* and *squares* (—■—), cells incubated on day zero and not subcultured; *broken line* and *squares* (– –■– –), cells subcultured on day 8 of incubation; *dotted line* and *squares* (· ·■· ·), cells subcultured again 1 day later.

choline-*O*-acetyltransferase and high thymidylate synthetase specific activities were obtained; in cultures in which cell division was restricted, high choline-*O*-acetyltransferase and low thymidylate synthetase specific activities resulted.

## DISCUSSION

The results show that neuroblastoma choline-*O*-acetyltransferase and thymidylate synthetase activities are regulated and are inversely related to one another. When the rate of cell division was restricted, choline-*O*-acetyltransferase specific activity increased about 6-fold, compared to cells kept rapidly dividing for at least 9 days. Thymidylate synthetase specific activity, however, increased about 2.4-fold when confluent cultures were split and began to divide rapidly.

The opposite effects on these two enzymes are understandable in terms of the needs of dividing or differentiating neuroblasts. Dividing cells, which are replicating their DNA content, have a greater need for thymidylate and, thus, increased thymidylate synthetase specific activity could maintain the thymidylate pool under these conditions of greater demand. The thymidylate synthetase pathway is active in this tumor cell line and that thymidylate formation is not entirely dependent on preformed thymine and thymidine kinase. Nondividing neuroblastoma cells are capable of neuronal differentiation, and four differentiated properties have been found thus far to increase when cell division was restricted: acetylcholinesterase (5), formation of acetylcholine receptors (4), axon–dendrite development (10), and the presence of electrically excitable plasma membranes (2). We now report an additional differentiated function, increased choline-*O*-acetyltransferase specific activity with restricted cell growth.

The finding of reciprocal choline-*O*-acetyltransferase and thymidylate synthetase specific activities in neuroblastoma cell cultures in which cell division was restricted may be analogous to how these enzymes function during the embryogenesis of the central nervous system. Autoradiographic data from developing mouse brain indicate that periventricular cells divide a specific number of times, migrate outward as postmitotic young neurons, and then generate axons (11). *In vivo*, embryonal neuroblasts, like neuroblastoma cells in culture, may be committed to a similar program of either dividing and inducing enzymes essential for this process, or become postmitotic and differentiated, with induction of neuronal enzymes for neurotransmitter synthesis.

This work was supported by grant IN-39A of the American Cancer Society and by grants RR05665-04 and CA 11449 of the USPHS, National Institutes of Health, Bethesda, Md.

1. Augusti-Tocco, G., and G. Sato, *Proc. Nat. Acad. Sci. USA*, **64**, 311 (1969).
2. Nelson, P., W. Ruffner, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **64**, 1004 (1969).
3. Schubert, D., S. Humphreys, C. Baroni, and M. Cohn, *Proc. Nat. Acad. Sci. USA*, **64**, 31 (1969).
4. Harris, A. J., and M. J. Dennis, *Science*, **167**, 1253 (1970).
5. Blume, A., F. Gilbert, S. Wilson, J. Farber, R. Rosenberg, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **67**, 786 (1970).
6. Phillips, H. J., and J. E. Terryberry, *Exp. Cell Res.*, **13**, 341 (1957).
7. Schrier, B., and L. Shuster, *J. Neurochem.*, **14**, 977 (1967).
8. Lowry, O. H., N. F. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9. Roberts, D., *Biochemistry*, **5**, 3546 (1966).
10. Seeds, N. W., A. G. Gilman, T. Amano, and M. Nirenberg, *Proc. Nat. Acad. Sci. USA*, **66**, 160 (1970).
11. Rakic, P., and R. Sidman, *J. Neuropathol. Exp. Neurol.*, **27**, 246 (1968).