

INCREASED RATE OF ACETYLCHOLINESTERASE SYNTHESIS IN DIFFERENTIATING NEUROBLASTOMA CELLS

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ABSTRACT

When neuroblastoma cells (N18) *in vitro* are maintained in the absence of serum, the specific activity of AChE begins to rise rapidly after an initial lag period of about 2–3 days, reaching a maximum level (10–20-fold increase) by 7 days after induction. In order to clarify the mechanism of induction, it was necessary to measure the rate of AChE synthesis and its sensitivity to metabolic inhibitors. Return of enzymatic activity after irreversible inhibition of AChE in “differentiated” cells was blocked by cycloheximide, but not by cordycepin or actinomycin D, suggesting that protein but not mRNA synthesis was required for replacement. By using the initial rate of this replacement as a measure of the rate of synthesis of the enzyme, it was shown that cells which had differentiated in the absence of serum synthesized AChE 50-fold faster on a specific activity basis than their undifferentiated counterparts. In contrast, cordycepin effectively blocked the increase in the rate of AChE synthesis that occurs as a result of serum deprivation, indicating that the induction process itself requires the synthesis of new mRNA. Axonation, another index of differentiation, was not completely blocked by inhibition of RNA or protein synthesis and presumably utilizes only pools of pre-existing structural proteins.

Several laboratories (1–3) have shown that serial propagation *in vitro* of the C1300 mouse neuroblastoma is possible. Cloned cell lines derived from this tumor exhibit morphological characteristics of neurones, such as elaboration of processes of considerable length (3, 4). In addition, they have electrically excitable membranes (5, 6), and the complement of enzymes necessary for the synthesis and degradation of neurotransmitters (7). Both the morphological (4, 8) and enzymatic (9, 10) indices of neuronal differentiation are accentuated by inhibition of cell multiplication or by exposure to putative inducers (11, 12). However, it is not clear that the morphological changes or increases in

enzymatic activities represent true induction, since it has not been shown that they are dependent upon increases in the rate of synthesis of particular structural proteins or enzymes.

Acetylcholinesterase (AChE),¹ which is synthesized by neuroblastoma cells in tissue culture, is one of the enzymes whose activity increases dramatically upon manipulation of culture conditions (10). In this study we will describe a technique for measuring the rate of synthesis of AChE and show that the increase in specific activity that occurs

¹ *Abbreviations used in this paper:* acetylcholinesterase, AChE; pinacolylmethylphosphonofluoridate, soman.

after maintenance in serum-free medium parallels a large increase in the rate of enzyme synthesis. Also, data will be presented to implicate transcriptional control in the regulation of AChE synthesis.

MATERIALS AND METHODS

The N18 clone of neuroblastoma cells was obtained from Microbiological Associates Inc., Bethesda, Md., in approximately its 30th passage in vitro and was serially propagated in our laboratory until used (5–10 additional passages). The growth medium routinely used was Dulbecco's modification of Eagle's medium supplemented with 10% fetal calf serum and equilibrated with 10% carbon dioxide. Penicillin and streptomycin were added to concentrations of 100 U/ml. The components of the medium were obtained from Microbiological Associates. At each subcultivation, cells were released from the surface of the culture vessel by using either saline-D₁ (10), or EDTA 1:5,000 in buffered isotonic saline.

Cells which were to be cultivated in serum-deficient medium were first permitted to grow to a density of $1-2 \times 10^6$ per 75 cm² in the presence of serum, after which they were washed and cultivated in the medium containing 0.1% serum. Prolonged maintenance of cells in the serum-deficient medium was possible only if the medium was changed every 48 h. Increasing the interval between medium changes to 3 or 4 days resulted in complete loss of cells from the surface of the flask after about 3 wk.

AChE activity was measured by a modification of the method of Ellman (13), using 1% Nonidet P-40 (Shell Chemical Corp., New York) in both the lysis and assay buffers. Optical density increase at 412 nm was measured by a Beckman DK-1 recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.), with the sample compartment maintained at 35°C. When it was necessary to assay samples containing relatively large quantities of protein, acetylthiocholine was added only after incubation of the sample aliquot with the other components of the reaction system for 5–10 min. By the use of this modification it was possible to eliminate background due to sulfhydryl-containing materials in the samples, which permitted assay of samples containing much larger amounts of protein than would otherwise be possible. One unit of AChE activity is defined as that quantity of enzyme required to hydrolyze 1 nmol (10^{-9} mol) of acetylthiocholine per min at 35°C. The protein content of samples was measured by an automated adaptation of the Lowry procedure (14).

Soman (see footnote 1) was obtained from the Biochemistry Group, Biomedical Laboratory Dept. of the Army. Before use it was diluted in sterile saline to produce a 5×10^{-4} M stock. Since this compound is moderately unstable in aqueous solution, the concentration of active inhibitor present at any time, relative to the concentration present upon initial dilution, was calculated from the rate of inactivation of serum cholinesterase. The stock solution was discarded if less than 10% of the initial anticholinesterase activity remained.

For measurements of rates of AChE synthesis, enzyme present in a culture before initiation of an experiment was inactivated by adding sufficient soman to make the final concentration 1×10^{-6} M. The cells were incubated for 1 h at room temperature, then the monolayer was washed once with medium, 10 ml of fresh medium per 75 cm² were added, and the cultures were incubated at 37°C until assayed. If log-phase cells were being used, the medium was supplemented with 10% fetal calf serum whose cholinesterase activity had been inhibited by previous treatment with soman followed by storage until residual anticholinesterase activity was no longer detectable.

Actinomycin D was a gift from Merck & Co., Inc., Rahway, N. J., and cycloheximide was obtained from Sigma Chemical Co., St. Louis, Mo. Preliminary experiments established that the concentration of each of these compounds necessary to inhibit 99% of RNA or protein synthesis, respectively, was 2 µg/ml of culture medium. The effect of these antibiotics on synthesis of AChE was ascertained after preincubation of the cells in the presence of the appropriate compound for 1 h before treatment with soman. After an additional 1 h of exposure to both soman and the antibiotic, the monolayer was washed, fresh medium containing the antibiotic at the initial concentration was added, and the cells were incubated at 37°C until assayed.

Cordycepin was obtained from Sigma and was used at a concentration of 20 µg/ml, which has been reported to eliminate the appearance of mRNA in HeLa polysomes (15). Treatment with this antibiotic was as described for other inhibitors of RNA or protein synthesis.

RESULTS

Morphological Differentiation and AChE Levels After Serum Removal

The morphological changes and time course of AChE accumulation in neuroblastoma after serum removal are shown in Fig. 1 and Fig. 2 A, respectively. The complexity and extent of process formation did not progress appreciably after 7 days of maintenance in the absence of serum, and the level of AChE activity increased only slowly after this time. The pattern of AChE increase was quite reproducible with respect to the duration of the lag period, but the level of activity finally attained in different experiments varied between 500 and 1,000 units/mg. The AChE activities which we found in both log-phase and serum-deprived cells were three- to fivefold higher than reported for the N18 clone by Blume et al. (10). These higher specific activities may have resulted from the presence in our assay system of dithiobis (nitrobenzoic acid) which is believed to be an allosteric activator of AChE (16). Also, we have

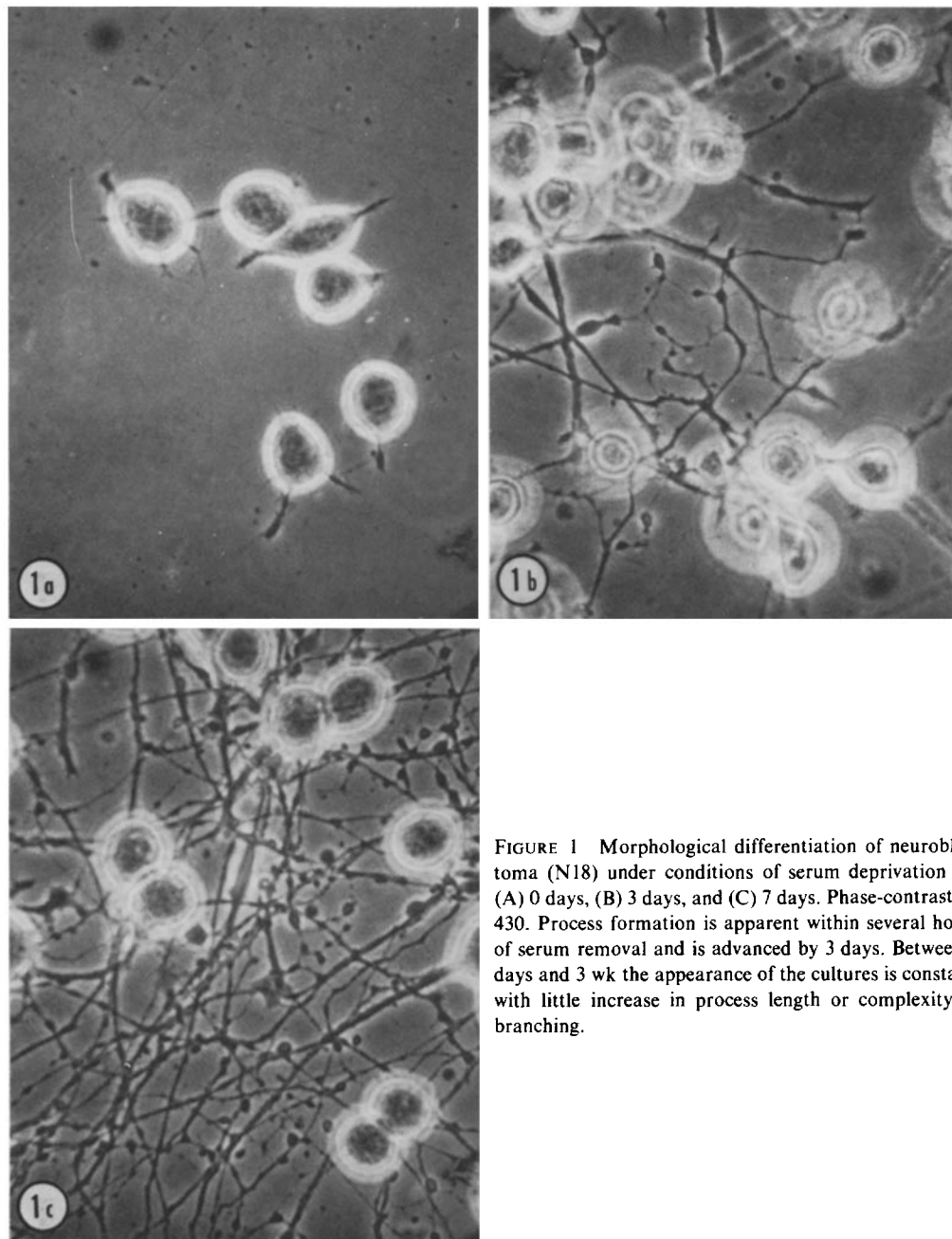


FIGURE 1 Morphological differentiation of neuroblastoma (N18) under conditions of serum deprivation for (A) 0 days, (B) 3 days, and (C) 7 days. Phase-contrast. $\times 430$. Process formation is apparent within several hours of serum removal and is advanced by 3 days. Between 7 days and 3 wk the appearance of the cultures is constant, with little increase in process length or complexity of branching.

found that NP-40 preserves AChE activity better than does Triton X-100.

Rate of AChE Reappearance After Soman Inhibition

Rates of reappearance of AChE in neuroblastoma cultures were measured after inhibition of

pre-existing enzyme activity with soman. Preliminary experiments indicated that use of a soman concentration greater than 1×10^{-6} M delayed reappearance of AChE activity. The duration of this lag period was directly proportional to the soman concentration initially used and was probably caused by the persistence of residual inhibitor. Treatment with 1×10^{-6} M soman for 1 h

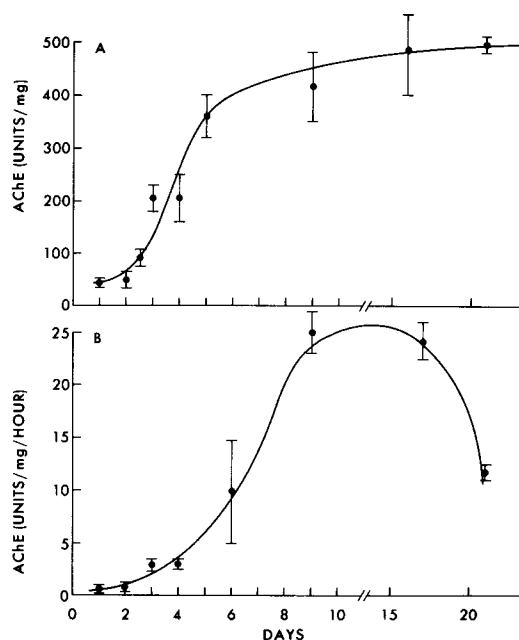


FIGURE 2 (A) Level of AChE activity in neuroblastoma cultures induced by serum removal. Cultures containing 2×10^6 log-phase cells per 75 cm² dish were incubated in medium containing 0.1% fetal calf serum and assayed in duplicate for AChE activity. (B) Rate of AChE synthesis was determined in parallel cultures at the indicated times. Pre-existing enzyme was inactivated by a 1-h exposure to 1×10^{-6} M soman and cells were assayed for AChE activity after a 9-h incubation in serum-deficient medium. Rate of synthesis is expressed as units AChE per milligram protein per hour. Data points indicate means and ranges of assays on two to four cultures.

reduced AChE activity to 3–5% of its initial level in both log-phase and stationary-phase cultures. The reappearance of enzymatic activity in such cultures occurred with the time course shown in Fig. 3. The initial rate of increase in log-phase cells is approximately $1.1 \text{ units/h}^{-1}/\text{mg}^{-1}$, and that for serum-deprived cells is $54 \text{ units/h}^{-1}/\text{mg}^{-1}$.

Effects of Cycloheximide, Actinomycin D, and Cordycepin on AChE Synthesis

In order for the rate of appearance of AChE activity after inhibition by soman to be considered a true measure of the rate of AChE synthesis, it was necessary to show that protein synthesis was required and that synthesis of AChE was not accelerated by treatment with soman. Fig. 4 shows the effects of cycloheximide and actinomycin D on recovery of AChE activity. Cycloheximide com-

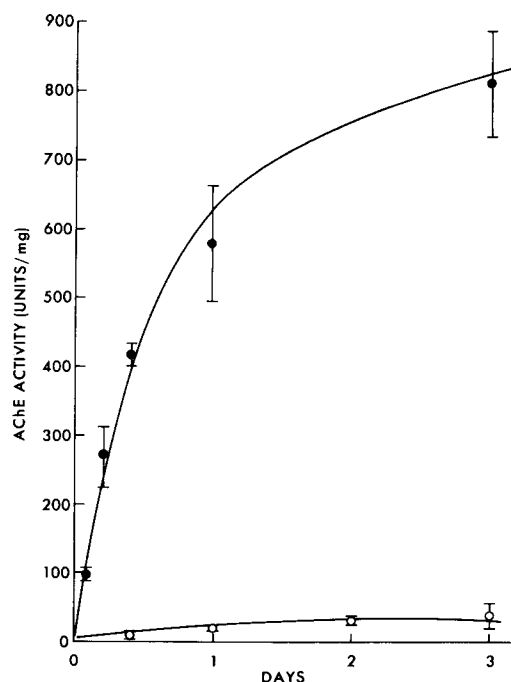


FIGURE 3 Reappearance of AChE activity in neuroblastoma (N18) cultures after exposure to soman. Monolayers containing approximately 2×10^6 cells per 75 cm² dish were either in stationary phase produced by maintenance for 7 days in medium containing 0.1% fetal calf serum (filled circles), or in log phase (open circles). Cultures were exposed to 1×10^{-6} M soman for 1 h, washed once with serum-deficient medium, and incubated for the time indicated as described in Materials and Methods. Averages and extremes of triplicate determinations are indicated. Of the enzyme activity present before inhibition by soman and during the resynthesis period, 95–99% was sensitive to 2×10^{-7} M BW284C51, a specific inhibitor of true acetylcholinesterase (23).

pletely inhibited recovery, so neither reactivation of inhibited enzyme, nor assembly of active enzyme from a pool of inactive precursor was significant. We conclude that reappearance of AChE activity is the result of *de novo* enzyme synthesis.

The actinomycin D effect was more complex, with slight initial inhibition progressing to complete block of recovery later in the experiment. This effect could be produced either by decay of mRNA or by a direct effect of actinomycin D on protein synthesis. The results of cordycepin treatment (described below) indicated that the latter explanation was correct. This argument was further strengthened by the finding that by 5 h after addition of actinomycin D, total protein synthesis,

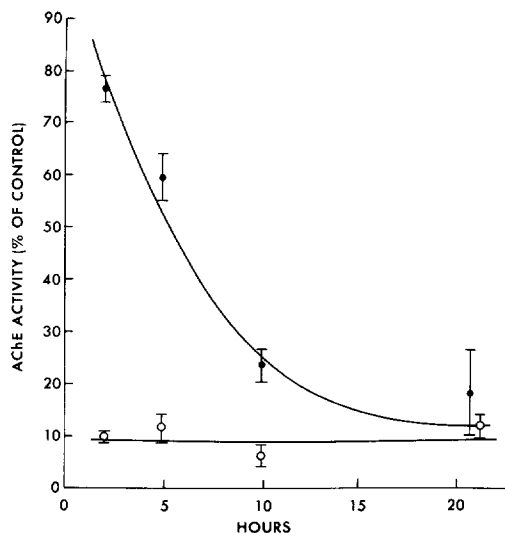


FIGURE 4 Effects of cycloheximide and actinomycin D on synthesis of AChE by neuroblastoma after inhibition by soman. Cultures containing 5×10^6 cells were maintained in serum-deficient medium for 7 days. They were then exposed for 1 h to soman (1×10^{-6} M) in medium containing either $2 \mu\text{g}/\text{ml}$ cycloheximide (open circles), or $2 \mu\text{g}/\text{ml}$ actinomycin D (filled circles). The medium containing either $2 \mu\text{g}/\text{ml}$ cycloheximide (open with fresh medium containing only the inhibitor of protein or RNA synthesis. Incubation was continued for the time indicated. Control cultures were treated identically but were inhibited with soman only. Data points show means and extremes of AChE activity in duplicate cultures expressed as a percentage of control.

as measured by incorporation of [^{14}C]leucine into trichloroacetic acid-insoluble material, was depressed to 20% of its initial value. When the rate of AChE synthesis was corrected for the decrease in total protein synthesis in the presence of actinomycin D, a rate of increase very similar to that in the absence of the antibiotic was obtained. If soman had a selective effect on transcription, the corrected rate of AChE synthesis in the presence of actinomycin D would be much lower than that of the control.

While we could not rigorously eliminate the possibility that soman had a selective effect on translation of the AChE messenger, we could show that exposure had no effect on total protein synthesis and that repeated exposures did not alter the final level of AChE activity attained in cultures maintained in the absence of serum.

Cordycepin is a more useful inhibitor of mRNA metabolism in mammalian cells than actinomycin D because it is both less toxic and more specific,

with no effect on the stability of mRNA synthesized before treatment (17). If differentiated neuroblastoma cultures were continuously exposed to cordycepin ($20 \mu\text{g}/\text{ml}$) after treatment with soman, AChE activity reappeared with kinetics very similar to those shown in Fig. 3 for uninhibited differentiated cultures. The extent of AChE synthesis in the presence of cordycepin relative to that in its absence declined only slowly over the period of observation, indicating that synthesis of the enzyme could continue at about 80% of its initial rate after metabolism of mRNA had been inhibited for 48 h. This is further evidence that the resynthesis of AChE after soman inhibition does not require new mRNA and reflects the rate of enzyme synthesis at the time of inhibition. In contrast to its modest effect on AChE synthesis in differentiated cells, cordycepin had a dramatic effect on the induction process itself, as demonstrated in Fig. 5. Addition of the antibiotic con-

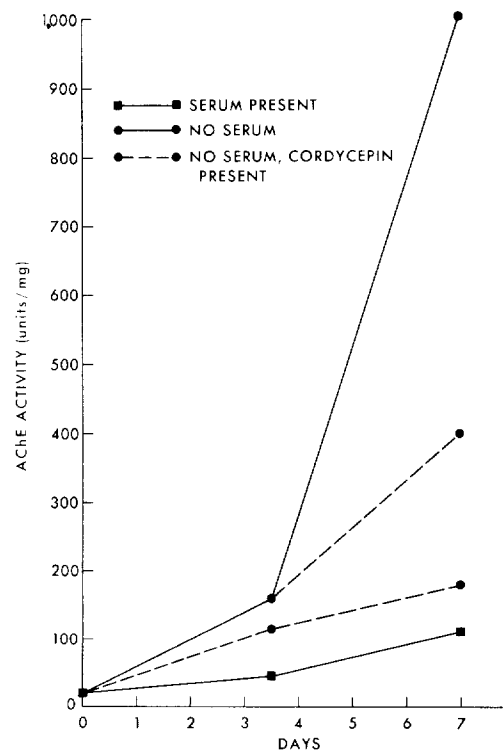


FIGURE 5 Effect of cordycepin on induction of AChE by serum deprivation. Cultures containing 2×10^6 log-phase cells were placed in serum-deficient medium, and cordycepin ($20 \mu\text{g}/\text{ml}$) was added either concomitantly or after 3 days of incubation. Dashed lines indicate periods of incubation in the presence of cordycepin.

comitant with serum deprivation effectively inhibited AChE induction. Although the length of neurites seemed somewhat reduced, most cells had definite processes after 7 days of exposure to the inhibitor and the onset of process extension was not dramatically affected. If cultures were exposed to cordycepin when the increase in rate of AChE synthesis was beginning (after 3.5 days of serum deprivation), enzyme synthesis continued but only at the rate existing at the time of inhibition and no further induction occurred.

Rate of AChE Synthesis After Induction by Serum Deprivation

The effects of serum removal on the rate of AChE synthesis are shown in Fig. 2 B. Separate experiments (Fig. 3) established that the rates of synthesis remained nearly linear over the 9-h period allowed for resynthesis. After an initial lag of 2–3 days, both the level and the rate of synthesis of AChE increased rapidly and leveled off after approximately 10 days of growth in the serum-deficient medium. It will be noted that for the first 5 days the rates of AChE production roughly paralleled the increase in the total amount synthesized as calculated from the rate measurements. This observation suggests negligible turnover of enzyme protein in the early stages of induction.

After this period, while the rate of synthesis went on to double and remain at this high level up to the 17th day, the level of enzyme activity increased only about 20%. This result is consistent with a higher rate of turnover of enzyme in the later stages of induction. It can also be seen from Fig. 2 B that when the synthesis of AChE reaches a plateau after 10–15 days, the rate is about 50 times greater than before induction. This result agrees well with the initial rates obtained from Fig. 3. A decrease in the rate of synthesis was noted after 3 wk of maintenance in serum-deficient medium.

DISCUSSION

The object of this study was to ascertain whether the increase in AChE specific activity which accompanies morphological differentiation of neuroblastoma cells in serum-deficient medium is the result of an increased rate of enzyme synthesis. In order to approach this question, we have shown that the reappearance of AChE activity after irreversible inhibition is a valid measure of the rate of enzyme synthesis. Data obtained with this technique indicate that the increase in the rate of

de novo synthesis can completely account for the observed activity level in induced neuroblastoma cells.

More detailed examination of our data allows us to conclude that in the early phases of induction nearly all of the AChE which was synthesized accumulated in the cells, and that turnover of the enzyme became significant only late in the induction process. At that time, the rate of synthesis continued to increase after the level of AChE had ceased to rise (Fig. 2 A, B). This behavior would be expected if AChE in the cell membrane were to turn over more slowly than the AChE free in the cytoplasm, and if membrane sites were to become saturated after the early phases of induction. This hypothesis is being tested by histochemical localization of the AChE produced throughout the induction process.

The effects of cordycepin on both the synthesis and induction of AChE suggest the presence of a long-lived mRNA for AChE. Furthermore, the accumulation of such stable AChE mRNA over the course of several days appears to be responsible for the high rate of AChE synthesis characteristic of fully induced neuroblastoma. Increased intracellular content of cyclic AMP has been implicated in differentiation induced by prostaglandins (18), but the role of this intermediate in regulating the synthesis or accumulation of AChE messenger is not yet clear.

We have not examined the possibility that the extent of induction is the same in all cells, so recruitment of cells into the induced population, in addition to accumulation of mRNA within all cells, may also be involved. Inhibition of induction by cordycepin suggests that the long lag between serum removal and increase in rate of AChE synthesis is probably not due to a delay in translation of already synthesized messenger, but rather to a delay in mRNA transcription. This suggests that serum removal is probably not the proximate inducing event, but rather that it acts only indirectly, initiating or triggering a time-consuming series of intermediate steps.

The rate of AChE synthesis which we observed in induced neuroblastoma cells appears to be much greater than that reported in other systems. Bone marrow cells are capable of synthesizing AChE under the conditions of cell culture at a rate of 0.2 units/h⁻¹/mg⁻¹ (19). While pure erythroid precursors may exhibit a higher rate of synthesis *in vivo*, the reported rate corresponds to that which we have observed for uninduced neuroblastoma

cells. Similar comparisons of our data with those obtained in whole animals is difficult because specific rates of AChE synthesis *in vivo* are not available. However, the time required to recover 0.5 of the initial enzyme activity before inhibition can be estimated in both kinds of preparations. Comparisons based on this $t_{0.5}$ should be valid, especially if initial activities are similar.

Austin and James (20) report initial AChE activities in total rat brain similar to those seen in uninduced neuroblastoma and a $t_{0.5}$ for recovery of 15 days. Shorter recovery times are suggested for guinea pig retina (21) and monkey brain (22), but insufficient data were available to calculate a $t_{0.5}$. A more recent investigation indicates that the $t_{0.5}$ for recovery of AChE activity in total rat brain after soman inhibition is on the order of 4–5 days (T. L. Yaksh, M. Filbert, L. Harris, and H. Yamamura, unpublished observations). Since these investigators also observed faster recovery in selected brain regions, it is possible that certain groups of cells in the brain are capable of synthesizing AChE as fast as are differentiated neuroblastoma cells.

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