

PROPERTIES OF GROWTH-RELATED ACETYLCHOLINESTERASE IN A CELL LINE OF FIBROBLASTIC ORIGIN

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ABSTRACT

We have previously reported the presence and regulation of an acetylcholine-hydrolyzing enzyme in high density suspension cultures of WRL-10A fibroblasts where its activity increases 100-fold when growth is arrested. Substrate specificity, substrate inhibition, and product identification studies indicate that this enzyme is acetylcholinesterase (AChE, EC 3.1.1.7). Treatment of whole cells with 5 mM diazotized sulfanilic acid revealed that most of the AChE is located on the external surface of the cell membrane. It was also found that the enzyme is released in the medium at a rate of 0.5 U/h/mg cell protein and that within a 24-h period the *de novo* synthesized and liberated AChE is equivalent to 90% of the activity associated with the cells. No similar synthesis of AChE was found in six other fibroblastic cell lines examined. These and related findings indicating that acetylcholine is also present in high density populations of WRL-10A cells suggest that this unique phenotype may be used profitably in exploring further the relationship between components of the cholinergic system and non-neuronal cell growth.

Growth-inhibited dense attached cultures of normal euploid mammalian cells and some established cell lines are characterized by a marked depression of total protein synthesis (24, 42, 45), while the synthesis of specialized proteins such as collagen (15, 23, 26, 34), "S100-protein" (33), and certain enzymes (6, 16, 35, 37) is increased.

We have previously reported that density-dependent regulation of growth comparable to that of attached cell populations occurs also in suspension cultures of WRL-10A cells (10, 11, 44), a subline of L-929 mouse fibroblasts (9). In the search for additional criteria for evaluating increased synthesis of specialized proteins in this system, acetylcholinesterase (AChE) was consid-

ered because, besides there being a description of such an increase in neuroblastoma cells by Blume et al. (5), there is suggestive evidence that the activity of this enzyme is inversely related to the rate of cell division in attached cultures of cells of mesenchymal origin as well (14), and that small but quantifiable amounts of AChE are present in L-929 cells (1, 28, 49). In a previous paper we demonstrated that density-dependent metabolic regulation in suspension cultures of WRL-10A cells does indeed extend to the activity of AChE, which was found to be approximately 100-fold greater in high density growth-inhibited populations than in low density exponentially growing cultures (8).

In this report we describe some of the enzymatic properties of the WRL-10A AChE and present evidence that the enzyme is located on the external surface of the cell membrane and that in growth-inhibited populations it is synthesized *de novo* and released into the culture medium. Data will also be presented showing that the expression of growth-related regulation of AChE synthesis is unique to clone WRL-10A and not found in other cells of fibroblastic origin.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

SUSPENSION CULTURES: The derivation of WRL-10A cells from the L-929 mouse fibroblast has been reported elsewhere (9). Other L-cell sublines were L-929 and NCTC 2071 cells obtained from the American Type Culture Collection (No. CCL 1 and No. CCL 1.1, respectively), L-929 cells purchased from Microbiological Associates, Bethesda, Md., and L-M cells which were a generous gift from Dr. K. Higuchi. Suspension cultures of these cell lines were established by using a $3-5 \times 10^5$ cells/ml inoculum obtained either from monolayers or from frozen stock. Both 50- and 200-ml experimental cultures were set up in 125- and 500-ml Erlenmeyer flasks, respectively, equipped with a suspended magnetic stirring bar. All cultures were maintained in Eagle's spinner medium supplemented with 10% horse serum, 100 U penicillin, and 50 μ g/ml gentamicin in a humidity-controlled incubator at 35°C containing 5% CO₂, 95% air.

Low density exponentially growing populations were obtained by maintaining cell density between 3 and 10×10^5 cells/ml through daily renewal of media and dilution to $3-5 \times 10^5$ cells/ml. High density growth-inhibited populations were established by daily renewal of media with no cell dilution. In this manner, cell densities of $6-14 \times 10^6$ cells/ml were obtained, depending on the particular L-cell subline used. Additional methods for the handling of suspension cultures and the determination of cell population kinetics have been detailed previously (10, 11).

ATTACHED CULTURES: In addition to WRL-10A cells, WI-38 human embryonic fibroblasts (Microbiological Associates, Bethesda, Md.) and 3T3 Swiss mouse fibroblasts (Flow Laboratories, Rockville, Md.) were grown in attached cultures. The media used were: Eagle's minimum essential medium (MEM) supplemented with 20% horse serum, 100 U penicillin, and 100 μ g/ml streptomycin for WRL-10A cells; Eagle's MEM with 10% fetal bovine serum for the WI-38 cells; and Dulbecco's modified Eagle's medium with 10% calf serum for the 3T3 fibroblasts. Experimental WI-38, 3T3, and WRL-10A cultures were grown in 32-oz prescription bottles with changes of media every 2-3 days

and an initial inoculum of 3, 5, and 10×10^6 cells, respectively. The WI-38 and 3T3 cell lines, known to exhibit density dependent growth inhibition, increased in cell number about three- and five-fold, respectively, upon reaching confluence. In contrast, the WRL-10A cells, which do not stop proliferating once confluence is reached but form multilayers, yielded about a 10-fold increase in cell number.

Enzyme Assays

CHOLINESTERASES: The preparation of cell samples from suspension cultures for AChE and butyrylcholinesterase (BuChE) determinations which includes washing and lysing the cells by sonication was recently described (8). Cells from attached cultures were dislodged with the aid of a rubber policeman and the contents of two to four flasks were pooled, washed, and lysed in a similar fashion. To measure AChE activity on the cell surface of whole cells, the cells were washed twice with Earle's balanced salt solution (EBSS) and once with phosphate-buffered saline (PBS: 50 mM potassium phosphate buffer pH 6.8, 1 mM EDTA, potassium salt, 155 mM NaCl). The cells were then resuspended in PBS and assayed in PBS instead of 0.05 M phosphate buffer. AChE activity on the surface of the whole cells was approximately 87% of the activity in homogenates in which the cells were lysed by sonication. Samples for AChE and BuChE activity in the culture media were obtained by mixing 0.9 ml of the supernate obtained after the first centrifugation of the cell pellet (8) with 0.1 ml of 10X phosphate buffer.

AChE in cell and samples of media was assayed by the method of Wilson et al. (49), using [1-¹⁴C]acetylcholine (ACh) iodide (4.53 mCi/mmol; New England Nuclear, Boston, Mass.) and incubated for 30 min at 37°C. AChE in the above-mentioned samples is operationally defined as enzyme activity that hydrolyzes ACh and is sensitive to 2×10^{-5} M 15-bis-(4 allyldimethylammonium-phenyl) pentan-3-one-dibromide (BW 284C51) and not sensitive to 10^{-4} M tetraisopropyl-pyrophosphoramidate (iso-OMPA). BuChE activity was determined by the same method under identical conditions, using [1-¹⁴C]butyrylcholine (BuCh) iodide (3.57 mCi/mmol; New England Nuclear) as the substrate. BuChE is defined as enzyme activity that hydrolyzes BuCh and is sensitive to iso-OMPA but not BW 284C51 (2, 47). Protein was determined by the method of Lowry et al. (25). One unit of AChE or BuChE activity was defined as 1 nmol of [¹⁴C]acetate or butyrate formed per min; specific activity was expressed as units of activity per milligram protein.

LACTIC DEHYDROGENASE: Lactic dehydrogenase (LDH) activity was determined spectrophotometrically by the method of Wroblewski and LaDue in the 25,000 g supernate of washed cells that were lysed by sonication and centrifuged as reported previously (12).

DIAZOTIZATION: Diazotized sulfanilic acid

(DSA) at a concentration of 50 mM was prepared by treating 32.5 mg of sulfanilic acid (Fisher Scientific Co., Pittsburgh, Pa.) according to the method of Pardee and Watanabe (32). Before treatment with DSA, WRL-10A cells in a volume of 3.5–4.0 ml from a high density growth-inhibited population were centrifuged at 300 g for 15 min, washed three times in EBSS, and resuspended in PBS. Depending on the final concentration of DSA required, an aliquot of the 50 mM DSA was added to the appropriate volume of PBS containing $2.5\text{--}3.2 \times 10^6$ washed cells in a final volume of 1 ml. After incubation at 37°C for 30 min with periodic shaking, the samples for AChE determination were centrifuged, washed as described above, and the whole cells assayed. Samples for LDH determinations were washed three times with EBSS, resuspended in 1 ml EBSS, sonicated, centrifuged and the supernate was assayed for LDH. All procedures were carried out at 5°C except as otherwise indicated. Control determinations contained all components except DSA.

RESULTS

Properties of WRL-10A AChE

Numerous enzymes catalyze the hydrolysis of ACh including AChE, cholinesterase, and acetyl-esterase. They may be distinguished from each other with respect to substrate specificity, inhibition in the presence of excess substrate, and sensitivity to specific inhibitors as well as by product identification. These properties were considered with respect to the ACh-hydrolyzing enzyme previously reported in high density growth-inhibited WRL-10A cells in suspension cultures (8).

The relative rates of hydrolysis of [^{14}C]ACh and [^{14}C]BuCh were determined in the presence of WRL-10A cell homogenate. It was found that BuCh hydrolysis was 2.8% that of ACh (Table I). ACh hydrolysis by the WRL-10A enzyme exhibited inhibition by excess substrate: the typical bell-shaped activity-substrate concentration curve, a distinguishing characteristic of AChE from various sources (21, 30), indicated a maximum rate of hydrolysis at a substrate concentration of 1 mM, compared to 0.8 mM found by Kumar and Elliott for the AChE of *Bungarus fasciatus* venom (21). Table I shows virtually complete inhibition of the enzyme with BW 284C51, and a comparable inhibition was obtained with 10^{-5} M neostigmine methylsulfate, another well-known potent inhibitor of AChE (3, 5, 30). Finally, in the presence of the WRL-10A cell homogenate and labeled ACh as the substrate, 1 mol of labeled product was produced for each mole of ACh which disap-

TABLE I
Hydrolysis of Acetyl- and Butyrylcholine by Media and Cells from a High Density Growth-Inhibited WRL-10A Cell Culture

Assay conditions	Enzyme activity		
	Unused media	24-h culture media	Cells
ACh	26.4*	60.1*	11.69†
ACh + iso-OMPA	18.1	50.1	10.18
ACh + BW 284C51	7.8	6.98	0.082
BuCh	51.3	54.3	0.33
BuCh + iso-OMPA	4.39	5.37	trace
BuCh + BW 284C51	46.95	48.98	trace

* Units/milliliter.

† Units/milligram protein.

Enzyme assays using ACh and BuCh as substrates are described under Materials and Methods. Cells and media were obtained from the same culture shown in Fig. 2. BW 284C51 and iso-OMPA final concentrations were 2×10^{-5} and 10^{-4} M, respectively.

peared. This product was identified as sodium acetate in two chromatographic systems using the double label technique of Wilson et al. (49).

Cellular Localization of WRL-10A AChE

Most acetylcholinesterases investigated thus far from neural (29, 30) or other sources (19, 46) are known to be associated with the membrane of the cell. Other enzymes, e.g. those related to nucleotide metabolism, also localized on the cell surface, are known to be active on substrates outside the cell, i.e. directed toward the external cellular milieu, and have recently been referred to as ectoenzymes (7). One of the techniques for detecting this type of enzyme is to expose intact cells to a reagent or chemical that does not penetrate the cell membrane but might inhibit the ectoenzyme in question (7, 32). Serving as a control for such a determination would be an intracellular enzyme such as LDH whose activity is not likely to be affected by the diazonium salt, provided it did not penetrate the cell membrane.

When washed intact cells from the high density growth-inhibited WRL-10A cultures were treated with such a nonpenetrating reagent, the diazonium salt of sulfanilic acid (Fig. 1, lower curve), as the concentration of the diazonium salt was increased the AChE activity declined, with approximately 30% of the activity remaining at 5 mM. Control

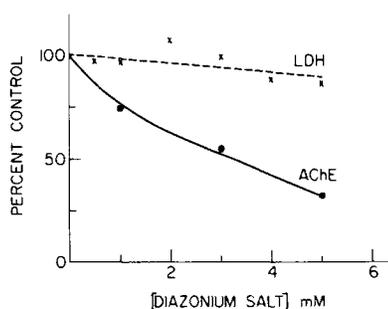


FIGURE 1 Effect of the diazonium salt of sulfanilic acid on the enzymes of intact cells. Washed WRL-10A cells from high density growth-inhibited populations were resuspended in PBS containing varying concentrations of the diazonium salt (indicated on the abscissa) and incubated at 37°C. After 30 min the reaction was terminated by washing the cells. Intact cells were assayed for AChE while others were sonicated and centrifuged, and the supernate was assayed for LDH (cf. Materials and Methods). Control determinations were performed on samples treated in the same fashion, except that DSA was omitted.

cells were treated with varying concentrations of diazonium salt in the same fashion, then washed free of the reagent, sonicated, and centrifuged, and the supernate was assayed for LDH. The upper curve of Fig. 1 shows that under the same conditions that inhibit AChE activity, LDH activity remained virtually unchanged. That this was due to the intracellular localization of LDH and not to an inherent resistance of this enzyme to the DSA reagent was demonstrated by the fact that, in sonicated cells, LDH activity was 87% inhibited at 1 mM and completely abolished at 3 and 5 mM DSA. These results indicate that the major portion of AChE activity in WRL-10A cells is accessible to the DSA and therefore must be associated with the external surface of the cell membrane.

Release of AChE into the Media

The localization of the enzyme on the cell membrane, in conjunction with the release of AChE into the surrounding media, reported recently for cultured embryonic muscle cells (47, 48), suggested the possibility that WRL-10A cells may also release AChE into their media. Properties of enzyme activity in the media with respect to substrates and inhibitors are compared with the cellular AChE activity in Table I. It can be seen that with ACh as the substrate, although the BuChE in the horse serum imparts an initial ACh-hydrolyzing activity to the media, the ACh-

hydrolyzing activity of the media after 24 h of contact with the high density cell population has increased by more than two-fold. The possibility that this increased activity was due to dissociable activators or inhibitors was excluded by mixing and dilution experiments which gave the predicted additive results. In the presence of iso-OMPA, hydrolysis of ACh in the unused media decreased by 31% due to inhibition of BuChE activity in the horse serum. It is apparent that the increment of activity exhibited by the 24-h culture media is not BuChE because it was not affected by iso-OMPA. With BW 284C51, a relatively specific AChE inhibitor, 70% of the ACh hydrolysis in the unused media was inhibited. The same inhibitor completely abolished the increment of activity in the 24-h culture media. Thus, the increased activity obtained in the 24-h culture media was found to be sensitive to BW 284C51 but insensitive to iso-OMPA, properties characteristic of the cellular enzyme. In contrast to the increment of the activity of the 24-h culture media with ACh as the substrate, there was no significant change observed in the hydrolysis of BuCh by the same 24-h culture media. The behavior of the BuChE activity in the unused media was identical with that in the 24-h culture media, i.e. over 90% was inhibited by iso-OMPA while less than 10% was sensitive to BW 284C51. These results indicate that the increment of cholinesterase activity in the media after 24 h of exposure to a high density WRL-10A population is due to AChE released by the cells into the media. No similar increase in ACh-hydrolyzing activity in the media after 24 h was detectable with low density exponentially growing populations.

Fig. 2 shows the kinetics of the medium AChE, cellular AChE, and cellular protein in a high density growth-inhibited WRL-10A population during the 24-h medium renewal cycle of these cultures (cf. Materials and Methods). It can be seen that with respect to the release of the enzyme from the cells, there is an initial rate of 0.94 U/mg cell protein/h during the first 8 h followed by a lower rate of 0.28 U from 8 to 24 h, the time weighted average rate being 0.5 U/mg cell protein/h. These values represent minimal rates of enzyme synthesis since AChE degradation and turnover under these conditions are not known. After 24 h, the accumulated medium AChE reached approximately 90% of the AChE activity associated with the cells. The cellular AChE during this time interval shows a slight increase in

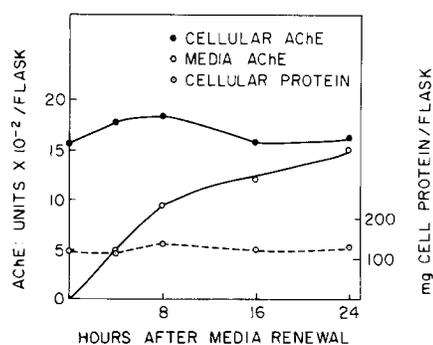


FIGURE 2 AChE activity, in the cells and in the media, and cellular protein in a high density growth-inhibited WRL-10A culture. At the indicated times after medium renewal in a 50-ml culture maintained in a 125-ml flask, 1-ml samples were obtained, centrifuged, and the supernate medium was assayed for AChE; the cell pellet was washed, resuspended in buffer, and lysed by sonication before assay for AChE and protein. The 0-h cellular protein and AChE were obtained just before medium renewal since at least 2 h are required for proper dispersion of the cells (11). AChE activity of media was corrected for the initial AChE activity of unused media (Table I).

the first 8 h followed by a return to the initial level by 16 h, this level being maintained to the end of the 24-h period. During this time, cellular protein was maintained at a nearly constant level, reflecting the constancy of the cell density which was found to range from 15.5 to 15.8×10^6 cells/ml. In agreement with previous findings (11), this indicates that during the 24-h medium renewal cycle no significant cell breakdown occurs in these cultures.

Distribution of AChE in Other Sublines of L-929 Cells

To determine whether the regulation of AChE in high density growth-inhibited suspension cultures is a general characteristic of L-929 mouse fibroblasts or an unusual feature manifested only in clone WRL-10A, a number of L-929 cell sublines were cultured under the same conditions and assayed for AChE activity. From Table II it can be seen that in the low density exponentially growing populations of all five sublines tested, including the WRL-10A, only trace amounts of AChE activity were detected; the maximum specific activity obtained was 0.053 in the NCTC 929(L) cells, which is near the lower limit of sensitivity of the assay. In the high density growth-inhibited populations, sim-

ilar trace amounts of AChE activity were found in all of the sublines except the WRL-10A cells which exhibited a 100- to 200-fold increase of the specific activity of the enzyme, depending on the final cell density obtained.

Distribution of AChE Activity in Other Fibroblastic Lines

The growth-related regulation of AChE in WRL-10A fibroblasts raised the question of whether similar regulation of AChE occurs in other cells of fibroblastic origin, such as the 3T3 mouse and WI-38 human embryonic fibroblasts, when the growth is arrested in attached cultures upon reaching confluence. We found that under these conditions 3T3 Swiss mouse and WI-38 human fibroblasts exhibited only trace or minimal AChE activities, while WRL-10A cells, the growth of which is only minimally inhibited in attached cultures (11), showed a small AChE increase (approximately 1 U/mg protein), as one might expect.

TABLE II
AChE Activity in Suspension Cultures of L-929 Mouse Fibroblast Sublines

Sublines of L-929 fibroblasts	Low density populations ($4-8 \times 10^6$ cells/ml)	High density populations ($6-14 \times 10^6$ cells/ml)
L-929, MA*	0.010	0.010
NCTC 929 (L)†	0.053	0.020
NCTC 2071‡	0.008	0.015
L-M	0.037	0.049
WRL-10A	0.050	6.0-14.0

* Microbiological Associates.

† American Type Culture Collection.

AChE specific activity determinations and culture conditions are described under Materials and Methods. Samples from low density exponentially growing populations were obtained from cultures after exponential growth for at least 3 days. Samples from the high density populations were obtained at different times over a period of 10-40 days after establishment of the high density populations (11). Data represent the average of three determinations. Values for the AChE activity of high density WRL-10A cell populations range from those obtained previously (8), where maximum population density did not exceed 10×10^6 cells/ml, to the more recent ones where the use of a suspended magnetic stirring bar yielded cell populations up to 14×10^6 cells/ml.

DISCUSSION

The experimental results presented here demonstrate that the ACh-hydrolyzing enzyme from WRL-10A mouse fibroblasts is inhibited by high substrate concentrations, is sensitive to two selective AChE inhibitors, hydrolyzes ACh at a much more rapid rate than butyrylcholine (Table I), and with ACh as the substrate, 1 mol of acetate was produced for each mole consumed. On the basis of these criteria, the same as those used to identify the AChE in cultured neuroblastoma C-1300 cells (5, 49), the WRL-10A enzyme appears to be AChE (EC 3.1.1.7). More definitive characterization will require analysis of purified enzyme fractions.

In initial studies with suspension cultures containing $6-10 \times 10^6$ cells/ml, the average cellular AChE specific activity obtained was about 6 U/mg protein (8). Recently, in place of a magnetic stirring bar resting on the floor of the culture flask (10), the use of a suspended bar has consistently yielded higher cell densities of $10-14 \times 10^6$ cells/ml with a concomitant increase of the AChE specific activity up to 14 U/mg protein (Table II). This suggests a possible effect of cell density on AChE activity similar to that reported for phenylalanine hydroxylase in hepatoma cells (27). In any event, the level of AChE activity obtained in the present work is similar to the AChE specific activity of freshly explanted nongrowing bone marrow cells found by Harris et al. (18) to be 10 U/mg protein. The specific activity reported in the high density growth-inhibited neuroblastoma C-1300 ranges from 150 to 450 U/mg protein, or 10- to 30-fold greater than the activity in high density growth-inhibited WRL-10A cells (5, 22). While AChE activity in growing and differentiating cultured chick embryo muscle cells has been reported to be as high as that of neuroblastoma cells, the simultaneous proliferation of fibroblasts in the same cultures renders doubtful specific activity comparisons with other cell types (47).

In regard to the cellular localization of the enzyme, the demonstration that approximately 70% of the AChE activity of WRL-10A cells is accessible to the DSA reagent (Fig. 1) which does not penetrate the cell membrane as shown by the failure to affect intracellular LDH activity, indicates conclusively that the WRL-10A enzyme is predominantly located on the external surface of the cell membrane. The remaining 30% of the activity, not accessible to the DSA, may possibly represent newly synthesized intracellular AChE, as

in the case of chick embryo muscle AChE (48). The finding that the major portion of AChE in WRL-10A cells is associated with the outer surface of the cell membrane is in good agreement with the well-known localization of this enzyme in the membranes of neural cells *in situ* (30, 40) and of erythrocytes (19) as well as in neuroblastoma cells cultured *in vitro*. In regard to the latter, Simantov and Sachs (39) using immunological methods reported that the AChE of cultured neuroblastoma C-1300 cells was located on the external surface of the cell membrane. Later, on the basis of an assay utilizing whole cells and ACh iodide as substrate, they found that AChE activity on the surface of intact cells was about 90% of the activity in cell extracts (40). However, since the substrate as well as products formed on enzymatic hydrolysis could penetrate the cell membrane, it is not actually certain what portion of the total AChE activity is located on the external surface of the neuroblastoma cell membrane.

Table I shows that the WRL-10A enzyme was released into the culture media. The properties of an increase in ACh hydrolysis but not BuCh hydrolysis in media exposed to the cells, as well as the behavior of this increment of activity toward two specific cholinesterase inhibitors, are comparable to the properties of AChE released by cultured chick embryo muscle cells into the culture media described by Wilson et al. (47). In WRL-10A cell cultures, the ratio of medium to cellular AChE was approximately one (Fig. 2), while in the chick muscle cells it was found that during the first 2 wk after initiation of the cell cultures, when maximum cellular AChE levels were obtained, AChE was continuously released into the media for a 24-h period, the calculated average ratio of medium to cellular AChE was about three. This higher ratio may reflect a lower AChE membrane-binding capacity (48) in these short-term cultures of embryonic muscle cells in contrast to the long-term, growth-inhibited, stable populations of WRL-10A cells. The liberation of enzymes from intact cells has been demonstrated also in ascites tumor cells which were found to release numerous cytoplasmic enzymes, including virtually all of those involved in glycolysis (20, 50).

The pattern of AChE release in these populations, with a higher rate during the first 8 h of the 24-h medium renewal cycle (Fig. 2), is consistent with other previously observed heightened metabolic activities immediately after renewal of the media. These include an increase of respiratory

activity (44) and of the synthesis of DNA (11) and protein (10). This variation of cellular activities undoubtedly mirrors the daily recurrent dual effects of medium renewal: on the one hand, the interference of mechanical manipulations and temperature changes with the normal metabolic activity of the cells and, on the other, the increased availability of oxygen (44), glucose (12), glutamine (44), and other nutrients upon resuspension of the cells in fresh media.

With renewal of the culture media on a regular 24-h basis and no evidence that the duration of our high density WRL-10A cell cultures is limited (11), the daily accumulation of AChE in their media indicates continuous *de novo* synthesis of the enzyme. Since the rate of AChE synthesis required to maintain the steady-state cellular level of the enzyme has not been determined in these experiments, the calculated rate of release of 0.5 U/h/mg protein, from Fig. 2, represents a minimal rate of AChE synthesis. Of particular interest is the report by Harris et al. (18) that net AChE synthesis by freshly explanted bone marrow cells ranged from 0.1 to 0.2 U/h/mg protein or about one-half the minimal synthetic rate of WRL-10A cells. The AChE synthesis required to maintain the steady-state enzyme level in high density growth-inhibited neuroblastoma cells was approximately 25 U/h/mg protein or two orders of magnitude higher than the minimal rate of AChE synthesis in our system (22).

Work currently in progress in our laboratory on the synthesis of AChE in WRL-10A cells could provide a more meaningful comparison of AChE synthetic rates in different cell types, especially if the possibility of AChE release into the media of neuroblastoma cultures is considered. The latter could provide an alternative explanation for the leveling of cellular AChE in the presence of progressively increasing synthetic rates in neuroblastoma, attributed by Lanks et al. (22) to increased turnover.

The synthesis of AChE in WRL-10A cells is inversely related to growth: only trace amounts of AChE are present in low density cultures growing exponentially while high density growth-inhibited populations exhibit specific activities of the enzyme ranging from 6 to 14 U/mg of protein (8) (Table II). Several instances of growth-related regulation of enzymes in cultured cells are known, such as β -glucuronidase in human diploid fibroblasts (6), phenylalanine hydroxylase in rat hepa-

toma cells (16), and esterase activity in PK 65 cells (37). Although little is known concerning the molecular mechanisms involved, it appears that post-transcriptional regulation may be common in these systems (43). In regard to AChE synthesis, the phenomenon of growth-related regulation has previously been demonstrated only in neural and muscle cells in vivo and in vitro (5, 14). The phenotype of WRL-10A cells thus appears to be unique among fibroblastic cell lines as illustrated by the absence of growth-related regulation of AChE in other L-929 sublines (Table II) as well as in growth-inhibited 3T3 and WI-38 fibroblasts.

While this manuscript was in preparation, growth-related synthesis of components of the cholinergic system was reported in bacteria, where the ACh content of *Lactobacillus plantarum* is low during the log phase but where relatively large amounts are synthesized and released during the stationary phase (41), and in the human placenta which exhibits peak concentrations of ACh, AChE, and choline acetyltransferase in the mid-gestational period when fetal growth is most active (17, 38). In addition, the activity of several plant growth retardants has been shown to be directly related to their effectiveness as AChE inhibitors (36), and, in a number of different mammalian tissues, ACh was shown to raise the level of cyclic GMP which in turn has been considered capable of triggering cell division (13). Finally, it has been proposed that the hyperpolarizing response of L-929 mouse fibroblasts to ACh, which is associated with increased membrane permeability to K^+ , may reflect some significant growth control mechanism (31).

To explore further the interrelations between the cholinergic system and cellular growth suggested by these reports as well as by the findings described in this and in a previous paper (8), we are currently investigating the phenotype of WRL-10A cells for the possible expression of additional cholinergic components under a variety of experimental conditions. Preliminary results indicate that acetylcholine is also synthesized by high density growth-inhibited populations of these cells (4); a detailed report of these findings will appear elsewhere.

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