Activation of Acetyl- and Butyrylcholinesterase by Enzymatic Removal of Sialic Acid from Intact Neuroblastoma and Astroblastoma Cells in Culture†

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ABSTRACT: Removal of sialic acid from intact mammalian nervous system cells in tissue culture is accompanied by an immediate increase in cellular cholinesterase activity. Treatment of hamster astroblast cells (clonal line NN) and mouse neuroblastoma cells (clonal lines S21, N18, and N115) for brief periods with a low level of *Clostridium perfringens* sialidase, 5×10^{-3} units/ml, removed 1–15 μg of sialic acid per mg of cell protein and brought about a large increase in v_0 and V_{max} of cellular acetylcholinesterase (EC 3.1.1.7). Butyrylcholinesterase (EC 3.1.1.8) activities also increased upon careful enzymatic removal of cellular sialic acid, and cells with characteristically low butyrylcholinesterase activity, e.g., adrenergic clonal line N115 neuroblasts displayed relatively high activity after treatment with sialidase. These findings open the possibility that adaptive regulation of cholinesterases in mammalian cells may be mediated rapidly through changes in their sialic acid content.

Cultured mammalian nervous system cells include acetylcholinesterases (Simantov and Sachs, 1972, 1973) among their important complement of ecto-enzymes (De Pierre and Karnovsky, 1974). Acetylcholinesterases derived from a variety of sources are known to be glycoproteins (Wiedmer et al., 1974); at least those from electric eel (Taylor et al., 1974), mammalian blood (Main et al., 1974), and fish muscle (Brodbeck et al., 1973) contain a substantial amount of bound sialic acid. Removal of sialic acid from fish muscle acetylcholinesterase has been found to enhance its enzymatic activity (Brodbeck et al., 1973). In cultured cells, lowering of sialic acid produces an increase in cell division (Vaheri et al., 1972), and mammalian cells reportedly have a diminished sialic acid level during mitosis (Glick et al., 1971; Rosenberg and Einstein, 1972). There is no available information which relates directly the effects of lowered sialic acid levels to any particular cellular biochemical function. In the present study, we describe changes in activity of acetylcholinesterase and butyrylcholinesterase which take place upon removal of a portion of the sialic acid of intact astroblasts and cholinergic, adrenergic, and inactive neuroblasts in culture. Removal of sialic acid is accompanied by an immediate increase in the acetyl- and butyrylcholinesterase activities of the cells. This effect was studied in terms of the kinetic parameters of acetylcholinesterase.

Experimental Procedure

Materials. Clostridium perfringens sialidase (neuraminidase, EC 3.2.1.18) was obtained from Worthington. Only batches which proved free of hemolytic (Okada et al., 1964), phospholipase C (Ottolenghi, 1969), and protease (Erlanger et al., 1961) activities were used. Dithiobisnitrobenzoic acid was purchased from Sigma. Tetraisopropylphosphoramide was obtained from Koch-Light and 1,5-bis(4-allyldimethylammoniumphenyl)pentane 1,3-dibromide from Burroughs-Wellcome. Acetylthiocholine and butyrylthiocholine were obtained from Sigma and Fluka, respectively. Physostigmine sulfate was obtained from Sigma. All other chemicals were of reagent grade and were used without further purification.

Cells. C1300 mouse neuroblastoma cells, clonal lines N18, N115, and S21, were gifts of Dr. M. Nirenberg, Laboratory of Biochemical Genetics, National Institutes of Health, Bethesda. Hamster astroblasts, clonal line NN, were obtained from North American Biologicals. Approximately 5×10^4 cells were seeded per 25 cm^2 growth surface area in Falcon plastic Petri dishes. The monolayer cultures were grown to approximately 2×10^5 cells in 5 ml of Eagle's medium as modified by Dulbecco, with 10% added fetal calf serum and 50 units of sodium penicillin G and 10 μg of streptomycin sulfate/ml. Cell cultures were incubated under 5% CO_2–95% air, at 37°C. The medium was changed once 3 days after seeding. The cultures were taken for experimental manipulation the next day when they were at the beginning of the stationary phase of growth. For investigation of the effect of continuous growth, the medium was changed twice more at 2-day intervals.

Treatment of Cells with Clostridium perfringens Sialidase. After careful removal of the growth medium and rinsing of the cells three times with an excess of glucose–salt solution composed of 130 m*M* NaCl, 5.5 m*M* glucose, 20 m*M* Tris-HCl, and 0.2 m*M* $MgCl_2$ (pH 7) the fully attached monolayer cell cultures were treated at 37°C with 1.5 ml of this same glucose–salt solution containing 5×10^{-3} units (7 μg) per ml of hemolysin-, phospholipase C, and protease-free *Clostridium perfringens* sialidase. The maximum time length of incubation was 10 min. At the end of the incubation, the glucose–salt solution was removed and analyzed for enzymatically liberated sialic acid by the method of Warren (1959), and for detectable cytoplasmic lactic dehydrogenase activity (Wroblewski and LaDue, 1955) as an index of cell intactness. Growth medium was immediately restored for 4 min at 37°C. This step was introduced to remove sialidase adhering to the cells (McQuid-

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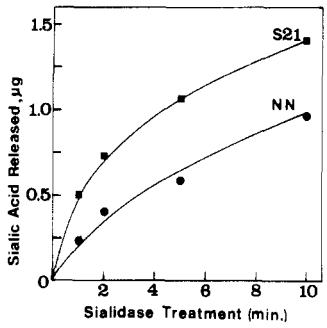

FIGURE 1: Progress curves for the enzymatic removal of sialic acid from clonal line S21 cholinergic neuroblasts and clonal line NN astroblasts by *Clostridium perfringens* sialidase. Four individual attached monolayer cultures in 25-cm^2 plastic Petri dishes were treated at 37°C with 1.5 ml of a solution composed of 130 m*M* NaCl, 5.5 m*M* glucose, 0.2 m*M* $MgCl_2$, and 20 m*M* Tris-HCl (pH 7) and containing 5×10^{-3} units/ml of sialidase. The extracellular fluid was poured off, lyophilized, and redissolved in 0.5 ml of water, and liberated sialic acid was measured by a micromodification of the thiobarbituric acid method of Warren (1959). (●) NN astroblasts; (■) S21 neuroblasts.

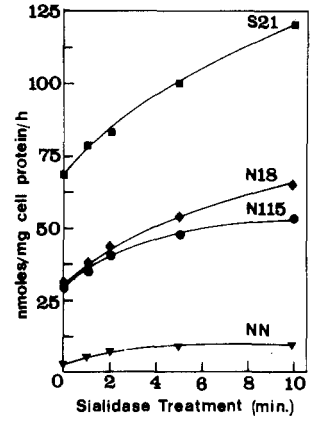

FIGURE 2: Activation of acetylcholinesterase of intact clonal lines NN, N115, N18, and S21 nervous system cells upon enzymatic removal of sialic acid. Sialic acid was removed enzymatically as described for Figure 1. Acetylcholinesterase activity was determined by incubation at 37°C for 30 min with 0.5 m*M* acetylthiocholine as substrate in a glucose-salt solution composed of 130 m*M* NaCl, 0.1 m*M* $MgCl_2$, 5.5 m*M* glucose, and 20 m*M* Tris-HCl (pH 7.8) containing 10^{-5} *M* tetraisopropylphosphoramide to inhibit nonspecific esterase activity. Enzymatically released thiocholine was measured by a modification of the method of Ellman et al. (1961). Acetylcholinesterase activity of the cells is expressed as nanomoles of acetylthiocholine hydrolyzed per milligram of total cell protein per hour. (▼) NN astroblasts; (●) N115 neuroblasts; (♦) N18 neuroblasts; (■) S21 neuroblasts.

dy and Lillien, 1973). Control cell samples were subjected to the same protocol, except that sialidase was omitted.

Assay for Cellular Acetyl- and Butyrylcholinesterases. The growth medium was removed from the cells and discarded. The attached cell monolayers were gently washed three times with a buffered pH 7.8 glucose-salt solution composed of 130 m*M* NaCl, 0.1 m*M* $MgCl_2$, 5.5 m*M* glucose, and 20 m*M* Tris-HCl, plus an inhibitor of nonspecific cholinesterases, tetraisopropylphosphoramide, at a concentration of 10^{-5} *M*, when acetylcholinesterase activity was to be measured, or plus 1,5-bis(4-allyldimethylammoniumphenyl)pentane 1,3-dibromide, an inhibitor of acetylcholinesterase, at a concentration of 5×10^{-6} *M*, when butyrylcholinesterase was to be measured. The cells were then incubated at 37°C for 10–30 min, depending upon activity, with 0.5 m*M* acetylthiocholine for assay of acetylcholinesterase activity, or with 0.5 m*M* butyrylthiocholine

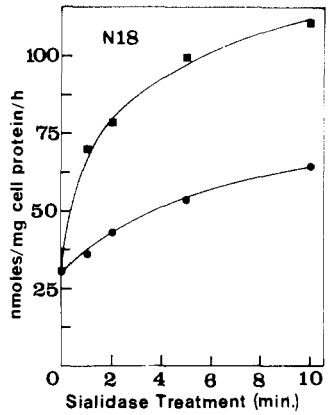

FIGURE 3: Comparison of progress curves for the activation of acetylcholinesterase of N18 neuroblasts by *Clostridium perfringens* sialidase at two levels. (●) 5×10^{-3} units of sialidase/ml.; (■) 10^{-2} unit/ml. Conditions for enzymatic treatment of the cells are the same as for Figure 1.

for butyrylcholinesterase activity, in 1.5 ml of this latter pH 7.8 glucose-salt solution containing the appropriate inhibitor. The enzymatic reaction was stopped by addition of physostigmine sulfate to give a final concentration of 1.5 m*M*, and, at the same time, reaction for the spectrophotometric analysis of enzymatically liberated thiocholine (Ellman et al., 1961) was started by addition of dithionitrobenzoic acid to give a final concentration of 10^{-4} *M*. Appropriate controls were run simultaneously to correct for the low levels of nonenzymatic hydrolysis of substrate and background due to sulfhydryl compounds of cellular origin. Incubation media once more were analyzed for cytoplasmic lactic dehydrogenase to ensure that no breakage of the outer cell membranes had occurred during the incubation. Cell samples were analyzed for protein by the method of Lowry et al. (1951). Cholinesterase activity was calculated in terms of nanomoles of substrate hydrolyzed per milligram of cell protein per hour.

Results

Removal of Sialic Acid. A controlled progressive removal of sialic acid from the intact, attached cells was achieved under the mild conditions described under Experimental Procedure. Examples of progress curves for the release of cellular sialic acid by *Clostridium perfringens* sialidase are shown for clonal line S21 cholinergic mouse neuroblasts and for clonal line NN hamster astroblasts in Figure 1. Results with the other cell lines tested were similar to those shown. At the end of the 10-min incubation period, very roughly half of the enzymatically available sialic acid had been released from the intact cells.

Activation of Acetylcholinesterase. Activation of acetylcholinesterase in all of the cell lines tested occurred simultaneously with the enzymatic removal of sialic acid, as shown in Figure 2. Acetylcholinesterase activity of control NN astroblasts normally is quite low, but nevertheless this activity approximately doubled after mild treatment of the cells with sialidase for 10 min. Activity of cholinergic S21 neuroblasts normally is high. Substantial augmentation of activity rapidly was brought about by partial removal of sialic acid from these cells. No measurable activity was released from the cells. Drastic conditions of treatment generally were avoided in order to ensure intactness of the cells. However, activation of acetylcholinesterase could be increased greatly by treatment of the cells with higher concentrations

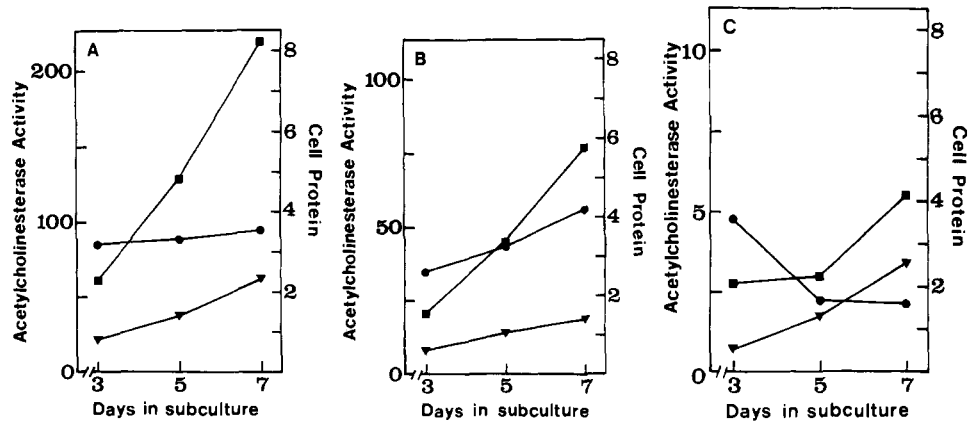

FIGURE 4: Effect of continuous culture on acetylcholinesterase activities of intact nervous system cells. Cells were grown in Dulbecco's modification of Eagle's medium in 25-cm^2 plastic Petric dishes at 37°C under 95% air-5% CO_2. Medium was changed every second day. Acetylcholinesterase activity was determined as described for Figure 2. (■) Total acetylcholinesterase activity per dish; (▼) total cell protein; (●) specific activity of acetylcholinesterase (total activity/total cell protein). (A) Clonal line S21, cholinergic, neuroblasts; (B) clonal line N18, inactive, neuroblasts; (C) clonal line NN astroblasts.

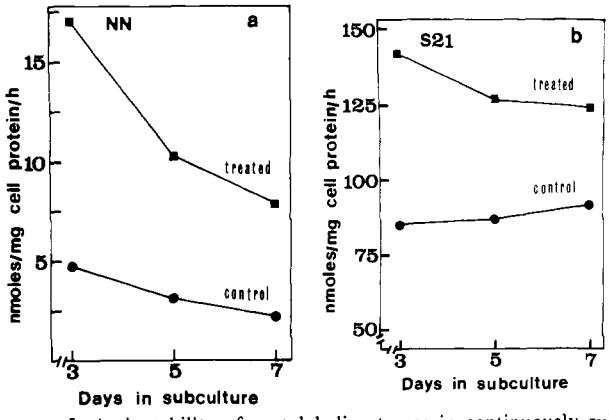

FIGURE 5: Activatability of acetylcholinesterase in continuously cultured nervous system cells by treatment with sialidase. Conditions are as described for Figure 1. Sialidase treatment was for 5 min. Cells were grown as described for Figure 4. Acetylcholinesterase activity was determined as described for Figure 2. (a) NN astroblasts, (b) S21 neuroblasts.

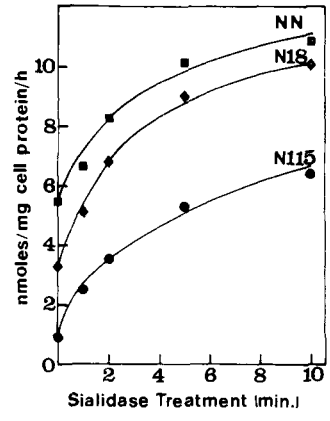

FIGURE 6: Activation of butyrylcholinesterase of nervous system cells in culture upon enzymatic removal of cellular sialic acid. Conditions for treatment with sialidase are the same as described for Figure 1. Butyrylcholinesterase activity was determined in the manner described for acetylcholinesterase in Figure 2, except that 5×10^{-6} *M* 1,5-bis(4-allylmethylammoniumphenyl)pentane 1,3-dibromide was included in the incubation medium as an inhibitor of acetylcholinesterase instead of tetraisopropylphosphoramide, and 0.5 m*M* butyrylthiocholine substituted for acetylthiocholine as substrate. Butyrylcholinesterase activity is expressed as nanomoles of butyrylthiocholine hydrolyzed per milligram of cell protein per hour.

of sialidase. For example, as shown in Figure 3, an approximately fivefold increase in initial activation rate of acetylcholinesterase of N18 cells was brought about by doubling the sialidase concentration for treatment of the cells from 5 $\times 10^{-3}$ to 10^{-2} unit/ml.

Kinetic Parameters of Activated Acetylcholinesterase. Activation of acetylcholinesterase by enzymatic removal of cellular sialic acid was entirely related to an increase in V_{max}. No substantial change in apparent K_m was observed. Acetylcholinesterase activities of the control cells and the sialidase-treated cells obeyed Michaelis-Menten kinetics. V_{max} for S_{21} cells rose, upon sialidase treatment, from 54.8 to 120 nmol per mg of cell protein per hr while apparent K_m remained constant at 1.2×10^{-4} *M*, and, for N115 cells, V_{max} rose from 38.4 to 53.3 while apparent K_m diminished slightly, from 1.73×10^{-4} to 1.51×10^{-4}. K_m and V_{max} were calculated according to the program of Wilkinson (1961). It is clear from these data that removal of cellular sialic acid causes an increase in available acetylcholinesterase in the cells. These increases in V_{max} do not represent the full level of activatability, but rather that achieved after only 5 min of treatment with a low concentration of sialidase.

Effect of Continuous Culture on Activity and Activatability of Acetylcholinesterase. Total activity of acetylcholinesterase per cell culture increased with cell growth. Measured against the increase in total cell protein, specific activity increased for the neuroblast cell lines, but declined continuously for the NN astroblast cells, as shown in the examples given in Figure 4A-C. Although it still remained substantial even after 7 days of subculture, activatability of acetylcholinesterase by removal of cellular sialic acid continuously declined, as shown for NN and S21 cells in Figures 5a and b.

Activation of Butyrylcholinesterase. Butyrylcholinesterase activity also increased upon removal of sialic acid from the cells. The progress curves, as shown in Figure 6, generally resembled those for acetylcholinesterase. Normally, the butyrylcholinesterase activity of neuroblast cells was relatively lower than that of astroblast cells but could be brought to levels equal to or greater than that of the normal level of astroblast cells by partial removal of cellular sialic acid. A 500% increase in butyrylcholinesterase was mea-

sured after 10 min of sialidase treatment of adrenergic N115 cells.

Discussion

It has been known for some time that activity of acetylcholinesterase in cultured neuronal cells is susceptible to regulation. The mechanisms for such regulation have remained obscure. Deprivation of serum in the growth medium has been shown to induce a slow quasi-exponential increase in acetylcholinesterase activity of mouse neuroblastoma cells (Blume et al., 1970). Restoration of serum causes a reestablishment of growth, which ceases upon its removal, and diminution in acetylcholinesterase activity accompanies renewed growth, after a lag period. High acetylcholinesterase activity has been related to low saturation density and diminished attachability of neuroblastoma cells; it has been suggested that differences in cells with high and low acetylcholinesterase activity may be due to structural differences in their surface membranes which contain most of the cellular acetylcholinesterase (Simantov and Sachs, 1972). It further has been suggested that a common gene regulates the amount of both acetylcholinesterase and the acetylcholine receptor during cell growth (Simantov and Sachs, 1973).

It is clear from the results obtained in the present study that acetylcholinesterases and butyrylcholinesterases may be immediately activated in intact cells simply by enzymatic removal of a portion of their cellular sialic acid. We have found this effect for astroblastoma cells as well as for neuroblastoma cells, which points to the possible generality of this phenomenon. While there have been no prior reports on direct activation of acetylcholinesterase in intact nervous system cells in cultures, studies have been made on activation of isolated membrane-bound acetylcholinesterase from mammalian brain (Crone, 1973). The reported activation of isolated plaice muscle acetylcholinesterase by enzymatic removal of sialic acid (Brodbeck et al., 1973) is suggestive of the possibility that removal of sialic acid may represent a circumscribed cellular mechanism for directly enhancing cholinesterase activities without the nonphysiological necessity for wide variations in ionic strength of the medium (Changeux, 1966). Among the salient possibilities arising from the findings reported here are that (a) cells potentially may possess high acetylcholinesterase and butyrylcholinesterase activities, but these activities are not expressed until a diminution in cellular sialic acid has occurred, and (b) cells with apparently negligible acetyl- or butyrylcholinesterase activities under normal circumstances may be converted to cells of relatively high activity by partial removal of their sialic acid. Under the conditions devised for removal of cellular sialic acid for the present experiments, the cells remained fully attached, and cytoplasmic lactic dehydrogenase activity was not measurable in the extracellular medium. We therefore make the presumption that the activation of cholinesterases results directly from the partial removal of sialic acid from the fully intact cells.

Activation of acetylcholinesterase is related to an increase in V_{max}. This finding may indicate that inactive enzyme molecules are rendered active by an allosteric effect, or by relief of a total steric block, upon removal of sialic acid, or else, that deacetylation of an acetylated enzyme intermediate during the hydrolytic reaction mechanism (Roufogalis and Thomas, 1969), i.e., the rate of release of product, is accelerated in the absence of glycosidically bound sialic acid. For the present, we cannot distinguish between these possibilities.

Although mammalian acetylcholinesterases isolated thus far have proven to be glycoproteins, when examined in this regard, we have no information yet on the nature of the cholinesterases of nervous system cells in culture. The findings reported in this study show that these esterases may be activated readily by removal of cellular sialic acid. This effect should be taken into account in considering possible mechanisms for the regulation of cellular cholinesterase activities.

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