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Regulation of Newly Synthesized Acetylcholinesterase in Muscle Cultures Treated with Diisopropylfluorophosphate

(movement/degradation/release/isozymes)

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ABSTRACT **Brief treatment with 0.1 mM diisopropylfluorophosphate inhibited an average of 89% of the acetylcholinesterase (EC 3.1.1.7; acetylcholine hydrolase) activity of cultures of chick embryo muscle. As long as protein synthesis occurred, an average of 78% of the activity returned within 4 hr. Newly synthesized acetylcholinesterase did not stain cytochemically, was rapidly and extensively degraded or released in the presence of 10 μM cycloheximide, and consisted mainly of low-molecular-weight forms. Acetylcholinesterase activity first appeared around the nucleus, about 4 hr after treatment with diisopropylfluorophosphate, and then spread to the rest of the cell about the time release of acetylcholinesterase was detected in the medium. With time, more and more of the enzyme was retained in the cells after treatment with cycloheximide, and the proportions of low-molecular-weight forms decreased and high-molecular-weight forms increased. The results suggest that newly synthesized acetylcholinesterase undergoes an orderly process of binding, movement, and assembly in diisopropylfluorophosphate treated, and probably also in untreated, embryo muscle fibers.**

Localization of acetylcholinesterase (EC 3.1.1.7; acetylcholine hydrolase; AChE) at the neuromuscular junction is the culmination of a complicated path of development. AChE first appears in mononucleated myoblasts before they fuse to form multinucleated myotubes (1, 2). Later, it is found within and at the surface of the differentiating muscle fibers and at the embryonic motor endplates. After birth, most of the AChE activity becomes restricted to the adult motor endplates (3, 4). In the chicken, several molecular forms of AChE occur in embryo twitch muscles that are virtually undetectable in the same muscles after hatching, and large amounts of AChE are released by cultured muscle fibers and appear in plasma from intact embryos (2).

The characteristics of AChE in embryo muscle suggest that newly synthesized enzyme undergoes an orderly process of movement and assembly. To examine the process, chick embryo muscle fibers were grown in culture and treated with diisopropylfluorophosphate (DFP), an irreversible inhibitor of AChE; recovery of enzyme activity was studied.

Pectoral muscles from 11-day-old chick embryos were dissociated with trypsin, and mononucleated cells were plated onto 35-mm plastic petri dishes coated with collagen. The medium contained 10% (v/v) horse serum, screened for cytotoxicity, 2% embryo extract, and 88% Eagle's minimal essential medium with Earle's salts (5). Temperature was 38°, pH was 7.2–7.5, and the atmosphere was a humidified mixture of CO_2 and air. Antibiotics were not used. Cholinesterase activities were determined with acetyl- and butyrylthiocholine esters and with the specific inhibitors 10 μM 284C51 (for AChE) and 0.1 mM iso-OMPA (for nonspecific cholinesterases (6). Previous experiments showed that amounts of nonspecific cholinesterases were low in the cultured cells (2).

Cytochemical localization of AChE was studied by the technique of Karnovsky and Roots (7). Cell cultures were rinsed in saline, fixed 5–10 min in 10% formalin (w/v), stained for 2 hr at room temperature, and examined with light microscopy. AChE activity was determined on homogenized, sonicated cultures by the method of Ellman *et al.* (8). Results are expressed as $\Delta A_{412\ nm}$/min per dish. Multiplication by 0.0735 converts the results to μmoles of substrate hydrolyzed/min per dish. AChE released by the cells was determined by assaying for acetylthiocholine ester hydrolysis of the medium in the presence of iso-OMPA and correcting for the activity of fresh, unincubated medium (2). AChE isozymes were examined by acrylamide gel electrophoresis by described methods (9), with 10% acrylamide gels, and a modification of the staining technique of Koelle and Friedenwald (10).

DFP was stored in acetone at −20°. Cell cultures were rinsed three times at 38°, and incubated with a saline solution of 0.1 mM DFP for 5–10 min at room temperature. Final concentrations of acetone did not exceed 0.1% (v/v). After treatment, they were rinsed four times with saline and returned to the complete medium at 38°. Cycloheximide was added to the cells at various times after DFP treatment at a final concentration of 10 μM. More than 85% of the incorporation of [^{3}H]leucine was inhibited in less than 20 min. More than 95% of the incorporation was inhibited within 4 hr in another study, under similar conditions (2).

Acetyl- and butyrylthiocholine esters, dithiobisnitrobenzoate, iso-OMPA, cycloheximide, and DFP were purchased from Sigma Chemicals, trypsin from Difco Laboratories, and horse serum and minimal essential medium from GIBCO and North American Biological Co.

In these, as in previous experiments (2), primary muscle cell cultures were maintained for up to 3 weeks in culture. Control and experimental cultures were routinely observed in the living state with an inverted phase contrast microscope. The data presented here were from 14-day-old cultures (except when noted); the cultures showed no gross loss of muscle fibers or cell death during the experiments. The results are presented in terms of enzyme activity per culture dish, since

Abbreviations: AChE, acetylcholinesterase; DFP, diisopropylfluorophosphate.

TABLE 1. *Effect of cycloheximide on the recovery of AChE in DFP-treated cells*

	Cell AChE			AChE release	
	DFP		DFP-CH	DFP	DFP-CH
Exp.	0 hr	24 hr	24 hr	24 hr	24 hr
248	0.037	0.178	0.014	0.780	0
255	0.021	0.129	0.021	0.492	0
262	0.024	0.260	0.021	0.924	0
267	0.018	0.120	0.008	0.424	0

AChE activity is expressed as $\Delta A_{412\ nm}$/min per dish. Cells were treated with DFP at 0 hr and incubated for 24 hr with (*DFP-CH*) and without (*DFP*) 10 mM cycloheximide added immediately after DFP treatment (0-hr values).

the number of fibroblasts in the cultures obviates use of total protein or total DNA as meaningful ways to express the data.

RESULTS

An average of 89.9 ± 5.5% (SD) of the AChE activity of the muscle cultures was inhibited by treatment with 0.1 mM DFP in nine experiments. No AChE activity was detected cytochemically or on acrylamide gels immediately after DFP treatment.

AChE activity rapidly returned in the cells (Fig. 1). Levels reached 60–80% of their initial values within 2 hr. In seven experiments, recovery of AChE activity averaged 78% in 4 hr or less.

The differences between the AChE activities of DFP-treated and control cultures were restricted to the cells; the rate and total amount of AChE released into the medium was little affected by DFP treatment.

AChE activity did not return in the cells when DFP-treated cultures were incubated for up 24 hr in 10 μM cycloheximide (Table 1), indicating that recovery of AChE activity was dependent upon synthesis of new protein.

AChE was not detected cytochemically earlier than 4 hr after DFP treatment, even though the spectrophotometric assays showed that its activity reached high levels in the cells within 2 hr after treatment. When AChE did appear, it was first localized around the nuclei (Fig. 2). Several hours later, it was generally distributed within and at the surface of the muscle fibers.

Further evidence for a delay between synthesis of AChE and its localization in a cytochemically detectable form was provided by experiments in which cycloheximide was added 2 hr after DFP treatment; even though protein synthesis was inhibited, AChE still appeared around the nuclei 2 hr later.

The pattern of AChE isozymes progressively shifted from a preponderance of low to higher molecular weight forms after DFP treatment (Fig. 3). Chick embryo muscle *in ovo* and *in vitro* (11) contains three major isozymes of AChE with molecular weights of 420,000 (band 1), 293,000 (band 2), and 219,000 (band 3). The lightest form (band 3) was often very low in activity in 14-day-old cultures, although it was present in high activity in 8-day-old cultures. Four hours after DFP treatment of 14-day-old cultures, band 3 was high and band 1 was low in activity. With time, the activity of band 3 decreased and that of band 1 increased, coming to resemble the isozyme pattern of untreated controls. In addition to these changes in activity of the major AChE bands,

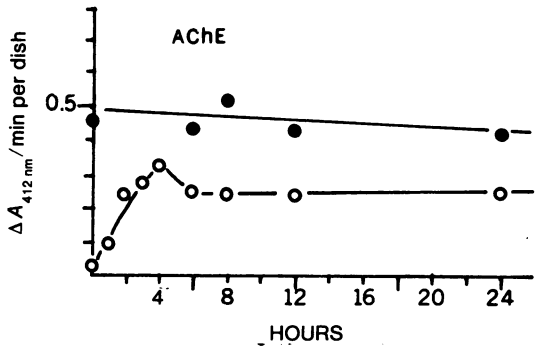

FIG. 1. Recovery of cellular AChE activity in DFP-treated cells. DFP (0.1 mm) was added to 14-day-old muscle cultures at 0 hr and washed out 10 min later. (About 90% of the AChE activity was inhibited in the experiment shown here.) ●, AChE activity of untreated cells; O, AChE activity of DFP-treated cells. AChE activity is acetylthiocholine ester hydrolysis that is insensitive to 0.1 mM iso-OMPA expressed as ΔA/min per dish.

DFP-treated cultures often exhibited two additional bands of activity between bands 1 and 2, particularly during the first few hours after DFP treatment.

Another difference between the AChE of untreated cultures and those recovering from DFP treatment was the retention of AChE activity in the presence of cycloheximide (Table 2 and Fig. 4). Untreated cells retained 41–44% of their AChE activity when proteins synthesis was inhibited. However, when cycloheximide was added within 6 hr after DFP treatment, only 13% of the AChE synthesized was retained by the cells. When cycloheximide was added 12 and 24 hr after DFP treatment, 32–37% of the AChE in the cells was retained. In other words, the "older" the enzyme, the more it was retained in the absence of protein synthesis. The initial rates of loss of AChE were similar for untreated and DFP-treated cells; AChE concentration decreased rapidly and then remained relatively unchanged for up to 48 hr.

Protein determinations indicated that the decreases in AChE activity were not due to gross toxicity of cycloheximide and breakdown of the cells. For example, in one experiment total cell protein per dish of untreated controls was 1.49 mg per dish at 0 time and decreased (as was often the case with cells of this age) to 1.17 mg/dish in 24 hr. Total cell protein of cultures treated with DFP, with DFP and cycloheximide, and with cycloheximide only were 1.34, 1.25, and 1.37 mg per dish, respectively, after 24 hr.

The relative contributions of degradation and of release of AChE to the rapid loss in AChE activity after cycloheximide treatment was studied by examining the AChE activity of the medium in which the cells were incubated. When untreated and DFP-treated cells were incubated with complete medium, release of AChE usually accounted for no more than 15% of the loss of AChE activity of the cells in the presence of cycloheximide. Thus, most of the decrease in AChE of the cells shown in Table 2 and Fig. 4 can be attributed to enzyme degradation.

However, several experiments in which the cells were incubated in minimal essential medium without serum or embryo extract (in hopes of increasing the sensitivity of the assays), showed considerable release of AChE into the medium in the presence of cycloheximide. For example, in one experiment with cells that had been treated with DFP, 24 hr after cycloheximide addition 74% of the AChE lost by the cells

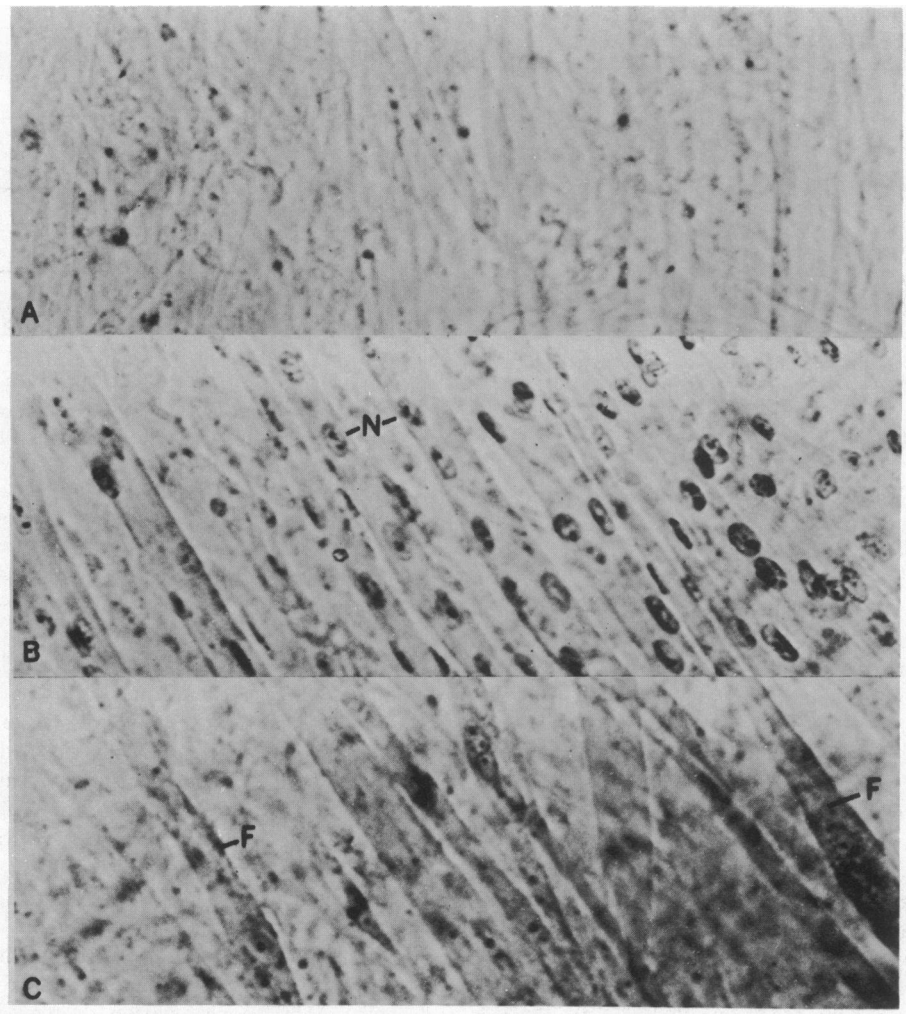

FIG. 2. Localization of AChE in DFP-treated cells. Photomicrographs of 14-day-old muscle cultures, stained for AChE by the method of Karnovsky and Roots (7) after formalin fixation. AChE stains darkly. (*A*) After treatment for 10 min with 0.1 mM DFP. (*B*) 4 hr after DFP treatment; *N* are nuclei. (*C*) 24 hr after DFP treatment; *F* are muscle fibers. Magnification ×360.

could be accounted for in the medium and only 26% of it could be attributed to degradation.

Activities of AChE did not return to their initial values in cells within 24 hr after DFP treatment (Fig. 1). Final activities of cell AChE averaged 74% (six experiments) of untreated controls. Gross cell death was not observed nor were the protein contents altered in these cultures.

DISCUSSION

AChE rapidly returned in primary cultures of chick embryo muscle after treatment with DFP. Activity was almost 80% of its initial level within 4 hr after organophosphate treatment. The results indicate AChE recovery was due to synthesis of new protein rather than reactivation of preexisting forms. In other studies (12), cholinesterase activity often returned slowly over a period of days when organophosphates were given to intact animals. In some cases, activity partially recovered in a few hours (13, 14), but it was not always clear whether this was due to reactivation or to synthesis of new enzyme. However, in one study, Davis and Agranoff (15) showed that protein synthesis was required for rapid return of a low-molecular-weight isozyme in DFP-treated rat retina.

In the experiments reported here, newly synthesized AChE underwent a number of changes during recovery of muscle cultures from DFP. They involved its cytochemical localization, isozyme composition, and susceptibility to degradation or release into the medium. One simple interpretation of the results is that newly synthesized AChE is relatively soluble, of low molecular weight, and readily degraded or released. With time, the newly synthesized AChE binds to cell constituents, moves through the cell, and assembles into higher molecular weight forms. The order in which these events occur is unknown, and the mechanisms that bring them about may be very complex. Questions such as: What molecular forms of AChE contribute to the cytochemical staining, and is the AChE that remains after cycloheximide treatment bound to the outside or the inside of the cell, need to be answered before the mechanism of AChE mobilization can be understood. AChE has been detected in the nuclear envelope, the Golgi, sarcoplasmic reticulum, and transverse tubules of muscle *in situ* (16).

The electrophoresis studies reported here and the findings of Davis and Agranoff (15) suggest that high-molecular-weight AChE forms are assembled from enzymatically active, lower molecular weight precursors. Dudai *et al.* (17) recently reported AChE forms from *Electrophorus* with molecular weights of more than 10^6 that appear to be composed of aggregates of smaller molecules. The ultimate size of bound

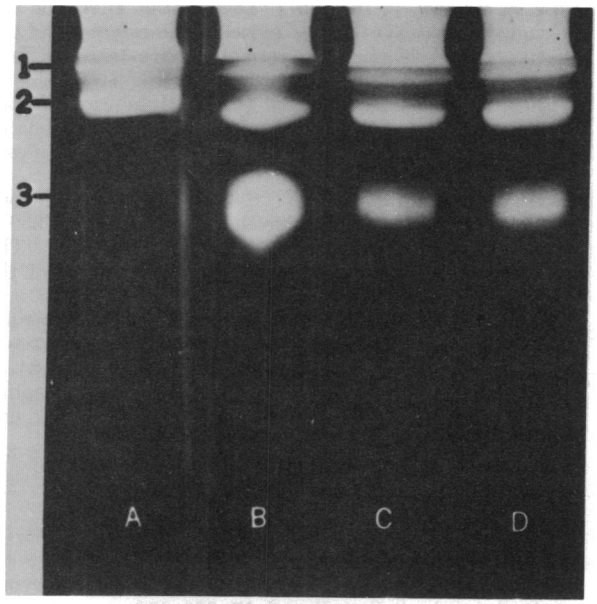

FIG. 3. AChE isozymes of DFP-treated cells. Ten percent acrylamide gels of homogenates of 14-day-old muscle cultures. (*A*) Untreated control; (*B*) 4 hr after treatment with 0.1 mM DFP; (*C*) 8 hr after DFP; (*D*) 24 hr after DFP. *Light bands 1, 2,* and *3* are major regions of AChE activity. *Band 3* is present in low activity in *gel A*. Note *bands* between *1* and *2*, particularly in *gel B*. Light color of the spacer gel is not due to enzyme activity. Migration is from the *top* (cathode) to the *bottom* (anode) of the figure.

AChE forms in embryo muscle cultures is not known; AChE molecules that migrate onto the acrylamide running gels may not represent all the forms present in the cells.

The results demonstrated that AChE is rapidly synthesized, and a portion of it is rapidly degraded by cultured embryo muscle. Small changes in such rates may have dramatic effects upon AChE levels, such as in the so-called "induction" of AChE by acetylcholine and acetylcholine analogs (18–20). Studies reported elsewhere (21) show that acetyl-β-methylcholine and choline itself increased AChE

TABLE 2. *Retention of AChE after cycloheximide addition*

Cycloheximide addition, hr after DFP treatment	Sampling time, ΔT	% Retention of cell AChE
	DFP-treated cells	
2	22	13
4	20	12.5
6	18	13
8	16	26
12	24	32
24	24	35
	48	37
	Non-DFP-treated cells	
0	24	44.2
	48	41.2

Cycloheximide (10 mM) was added at times indicated after treatment with DFP. The results are averages of five experiments, expressed as percentages of the AChE in the cells when cycloheximide was added.

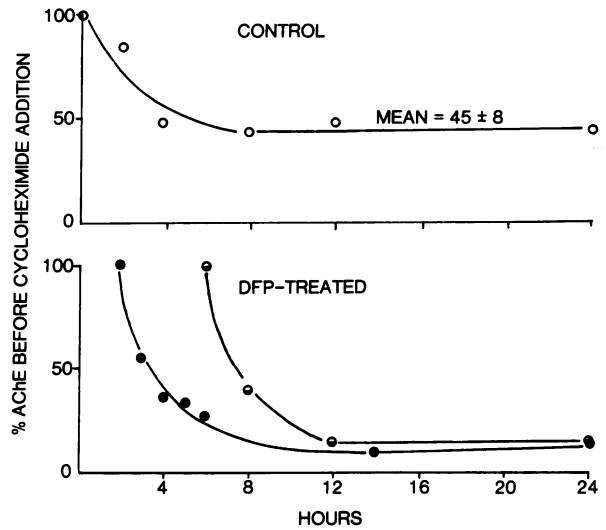

FIG. 4. Degradation of cellular AChE after addition of cycloheximide. (*Top*) Muscle cultures were incubated in 10 μM cycloheximide starting at 0 time, and their AChE activities were determined up to 24 hr later. (*Bottom*). Similar cultures were treated for 10 min with 0.1 mM DFP and incubated in 10 μM cycloheximide after 2 (●) and 6 (◒) hours. Values are averages of five experiments. Percentages are calculated from $\Delta A_{412\ nm}$/min per dish.

production and release by 40% as early as 4 hr after addition of the chemicals to DFP-treated cultures. Although they attributed the action of acetyl-β-methylcholine to an induction, data of Oh and Johnson (20) indicate that the compound prevented loss of AChE activity in puromycin-treated muscle cultures, suggesting the compound may have protected the enzyme from degradation or release.

The results suggest DFP was more than a passive participant in the experiments. The lack of return of AChE to its initial cellular levels and the enhancement of its production in DFP-treated cells deserve further study, particularly with regard to the fate of DFP-inhibited enzyme and its possible effects on the behavior of newly synthesized forms.

AChE is not the only component of the neuromuscular junction that undergoes a complicated history during muscle development. There are many similarities in the behaviors of AChE and acetylcholine receptor. Both occur in myoblasts, increase during fusion, and become localized at the neuromuscular junction later in development. However, AChE is released in quantity and acetylcholine receptor probably is not (22). Possible relationships between AChE and acetylcholine receptor deserve investigation. Simantov and Sachs (23) recently compared AChE and acetylcholine receptor in lines of *neuroblastoma*, selected for low levels of AChE. They proposed that AChE and acetylcholine receptor are synthesized by separate genes and controlled by a common regulatory gene. However, total production of AChE was not determined nor was the possibility considered that the low levels of AChE had fewer binding sites for the enzyme, and released larger amounts of it into the medium.

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1. Fluck, R. A. & Strohman, R. C. (1973) "Acetylcholinesterase activity in developing skeletal muscles *in vitro*," *Develop. Biol.* **33**, 417–428.
2. Wilson, B. W., Nieberg, P. S., Walker, C. R., Linkhart, T. A. & Fry, D. M. (1973) "Production and release of acetyl-

cholinesterase by cultured chick embryo muscle," *Develop. Biol.* **33**, 285–299.
3. Mumenthaler, M. & Engel, W. K. (1961) "Cytological localization of cholinesterase in developing chick embryo skeletal muscle," *Acta Anat.* **47**, 274–299.
4. Filogamo, G. & Gabella, G. (1967) "The development of neuro-muscular correlations in vertebrates," *Arch. Biol. (Liège)* **78**, 9–27.
5. O'Neill, M. C. & Stockdale, F. E. (1972) "A kinetic analysis of myogenesis *in vitro*," *J. Cell Biol.* **52**, 52–65.
6. Wilson, B. W., Linkhart, T. A., Walker, C. R. & Nieberg, P. S. (1973) "Tissue cholinesterase in plasma of chick embryos and dystrophic chickens," *J. Neurol. Sci.* **18**, 333–350.
7. Karnovsky, M. J. & Roots, L. (1964) "A 'direct-coloring' thiocholine method for cholinesterase," *J. Histochem. Cytochem.* **12**, 219–221.
8. Ellman, G. L., Courtney, D. D., Andres, V. & Featherstone, R. M. (1961) "A new and rapid colorimetric determination of acetylcholinesterase activity," *Biochem. Pharmacol.* **1**, 88–95.
9. Wilson, B. W., Kaplan, M. A., Merhoff, W. C. & Mori, S. S. (1970) "Innervation and the regulation of acetylcholinesterase activity during the development of normal and dystrophic chick muscle," *J. Exp. Zool.* **174**, 39–54.
10. Koelle, G. B. & Friedenwald, J. S. (1949) "A histochemical method for localizing cholinesterase activity," *Proc. Soc. Exp. Biol. Med.* **70**, 617–622.
11. Wilson, B. W., Mettler, M. A. & Asmundson, R. A. (1969) "Acetylcholinesterases and non-specific esterases in developing avian tissues—distribution and molecular weight of esterases in normal and dystrophic embryos and chicks," *J. Exp. Zool.* **172**, 49–58.
12. Filogamo, G. & Gabella, G. (1966) "Cholinesterase behavior in the denervated and reinnervated muscles," *Acta Anat.* **63**, 199–214.
13. Rose, S. & Glow, P. H. (1967) "Denervation effects on the presumed *de novo* synthesis of muscle cholinesterase and the effects of acetylcholine on retinal cholinesterase," *Exp. Neurol.* **18**, 267–275.
14. Welsch, F. & Dettbarn, W. D. (1972) "Inhibition of cholinesterase of rat diaphragm muscle by organophosphates and spontaneous recovery of enzyme activity *in vitro*," *Biochem. Pharmacol.* **21**, 1039–1049.
15. Davis, G. A. & Agranoff, B. W. (1968) "Metabolic behaviour of isozymes of acetylcholinesterase," *Nature* **220**, 277–280.
16. Tennyson, V. M., Brzin, M. & Slotwiner, P. (1971) "The appearance of acetylcholinesterase in the myotome of the embryonic rabbit," *J. Cell Biol.* **51**, 703–721.
17. Dudai, M., Herzberg, M. & Silman, I. (1973) "Molecular structures of acetylcholinesterase from electric organ tissue of the electric eel," *Proc. Nat. Acad. Sci. USA* **70**, 2473–2476.
18. Goodwin, B. C. & Sizer, I. W. (1965) "Effects of spinal cord and substrate on acetylcholinesterase in chick embryonic skeletal muscle," *Develop. Biol.* **11**, 136–153.
19. Turbow, M. M. & Burkhalter, A. (1968) "Acetylcholinesterase activity in the chick embryo spinal cords," *Develop. Biol.* **17**, 233–244.
20. Oh, T. H. & Johnson, D. D. (1972) "Effects of acetyl-β-methylcholine on development of acetylcholinesterase and butyrylcholinesterase activities in cultured chick embryonic skeletal muscles," *Exp. Neurol.* **37**, 360–370.
21. Wilson, B. W. & Walker, C. R. (1974) "Exploratory concepts in muscular dystrophy (II)," ed. Milhorat, M. D. (Excerpta Medica, Amsterdam, The Netherlands), in press.
22. Hartzell, H. C. & Fambrough, D. M. (1973) "Acetylcholine receptor production and incorporation into membranes of developing muscle fibers," *Develop. Biol.* **30**, 153–165.
23. Simantov, R. & Sachs, L. (1973) "Regulation of acetylcholine receptors in relation to acetylcholinesterase in neuroblastoma cells," *Proc. Nat. Acad. Sci. USA* **70**, 2902–2905.