Biochemical Pharmacology, Vol. 32, No. 5, pp. 911–918, 1983.
Printed in Great Britain.

0006-2952/83/050911-08 $03.00/0

RECOVERY OF ACETYLCHOLINESTERASE FORMS IN QUAIL MUSCLE CULTURES AFTER INTOXICATION WITH DIISOPROPYLFLUOROPHOSPHATE

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(*Received* 21 *June* 1982; *accepted* 23 *August* 1982)

Abstract—Studies of recovery of acetylcholinesterase (AChE, EC 3.1.1.7) after inhibition by organophosphates (OPs) have been hampered by the low number of *in vitro* systems with large collagen-tailed forms of AChE characteristic of motor end plates. Pectoral muscle cultures from Japanese quail with high levels of a large 20S form of AChE were used to study recovery of cells from acute treatment with diisopropylfluorophosphate (DFP), an irreversible AChE inhibitor. Low molecular weight AChE forms were synthesized more rapidly than the large 20S form following a 15-min treatment with 10^{-4} M DFP. Most of the activities of the small forms, but only part of the activity of the 20S form, disappeared within 48 hr after cycloheximide was added to block protein synthesis. To the contrary, virtually all the activity of the 20S AChE that was newly synthesized after DFP treatment was lost within 24 hr after cycloheximide treatment. The results were generally consistent with the idea that the 20S AChE form is assembled inside the cell near to its surface and then is released to bind to its outside.

Cultured muscle and nerve have been useful in studying the development and regulation of molecules of neuromuscular junctions and synapses and the agents that act upon them [1, 2]. This [3–8] and other laboratories [9–13] have found that acetylcholinesterase (EC 3.1.1.7, AChE) activity *in vitro* and *in vivo* rapidly recovers from organophosphates (OPs) like diisopropylfluorophosphate (DFP) and paraoxon. There is evidence that newly synthesized enzyme moves from the inside to the outside of the cell [14], and that small forms of AChE appear first in high activity [4, 8, 9], consistent with the idea that they may assemble into the larger ones. However, few of the studies examined the large collagen-tailed AChE forms [11, 12]. Improvements in extraction procedures [15, 16], and the recent finding that cultures of quail muscle cells contain relatively large amounts of the 20S AChE form of AChE [17–19], made it possible to study recovery of both small and large AChE forms after acute intoxication with DFP. The experiments presented here study the newly synthesized multiple AChE forms and present evidence for differences in the kinetics of the appearance and disappearance of the large 20S and the smaller 7S and 12S AChE molecules.

A preliminary report of part of this research has appeared elsewhere.†

METHODS

Cultures were prepared from 9 day embryos of Japanese quail (*Coturnix coturnix japonica*) from the Department of Avian Sciences, University of California at Davis. Pectoral muscles were dissociated with trypsin, plated onto 35 mm collagen-coated tissue culture dishes, and grown in a medium of 2% homologous embryo extract, 10% horse serum and 88% Eagle's Minimum Essential Medium (MEM) without antibiotics in a CO_2–air atmosphere at 38° [3, 8, 19].

DFP (Sigma Chemicals Co., St. Louis, MO) and paraoxon (analytical grade, a gift of American Cyanamid, Princeton, NJ) were stored in acetone at −20° and diluted with Eagle's MEM immediately before use. Final acetone concentrations were 0.1% (v/v) or less. Cultures were rinsed three times with physiological buffer, incubated for 15 min with the OP at room temperature, rinsed four times with physiological buffer, and returned to the culture medium.

AChE was extracted on ice with 0.4 ml/dish of extraction buffer, generally 0.05 M Tris–HCl, pH 7.3, containing 1% sodium cholate and 0.5 M $MgCl_2$ [19]. The cells were scraped into test tubes and sonicated for one or two 5-sec intervals, incubated on ice for 30 min, and centrifuged at 30,000 *g* for 30 min at 4°.

Velocity sedimentation profiles were determined in 5–20% sucrose gradients prepared in a buffer of 1.0 M NaCl, 0.5% Triton X-100, 0.20 mM EDTA and 0.05 M Tris–HCl, pH 7.3, using 200 μl samples with catalase (11.4S) and beta-galactosidase (16S) as standards [19, 20]. Gradients were centrifuged for 17 hr at 35,000–38,000 rpm with an SW-40 rotor (Beckman Instruments, Spinco Division, Palo Alto, CA) in an OTD-50 Sorvall ultracentrifuge (Dupont Co., Wilmington, DE) and separated into thirty-six fractions with a fractionater (ISCO Inc., Lincoln, NE). AChE activity was assayed by the radiometric method of Johnson and Russell [21]. Samples were preincubated for 5–20 min with 10^{-4} M iso-OMPA

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† B. W. Wilson, J. E. Bulger, W. R. Randall, G. T. Patterson and P. S. Nieberg, Abstr., *Eighth Ann. Meet. Int. Soc. Neurochem.*, Nottingham, p. 120 (1981).

Table 1. Quail muscle cultures: cell AChE and protein levels*

AChE activity		Protein	Age	
Dish	Protein	per dish	(days)	N
6.93	3.85	1.84	7–21	7
8.98	9.55	0.97	7–13	4
8.77	6.10	1.46	11–13	3
9.23	5.92	1.58	11–13	5
7.89	5.20	1.52	11–13	5
7.45	4.32	1.78	11–14	6
6.47	2.77	2.35	11–14	4
17.78	11.05	1.62	11–13	5
8.73	2.56	3.40	11–13	3
10.10	2.89	3.57	11–13	3
7.29	3.31	2.25	11–12	11
18.05	4.48	4.94	11–13	8
9.81 ± 1.13	5.17 ± 0.78	2.27 ± 0.33	Grand mean ± S.E.M.	

* Average AChE levels for twelve experiments. Values are means of N triplicate samples. Age is defined as the days samples were taken.

Activity is in μmoles/min/dish $\times 10^{-2}$ (Dish) or μmoles/min/mg protein $\times 10^{-2}$ (Protein). Protein is in mg/dish.

(tetraisopropyl pyrophosphoramide) to inhibit non-specific cholinesterase activity and assayed in 0.05 M Tris–HCl, pH 7.5, containing 100 mM NaCl and 50 mM $MgCl_2$. Total enzyme activity of a culture for a given period of time was the sum of the activity released into the medium and the change in cellular enzyme activity during that time.

AChE activity exposed at the surface of the cells was estimated by a modification [19] of the method of Rotundo and Fambrough [10]. Cultures were washed on ice with 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (Hepes)-MEM and covered with 1.5 ml of 1.2 mM [^{3}H]acetylcholine in Hepes-MEM. Aliquots were removed at 2-min intervals for 10 min and added to 150 μl of 1 M monochloroacetic acid, 0.5 M NaOH, and 2 M NaCl. Total cell AChE activity was determined on the same culture by the same procedure in the presence of 1% Triton X-100. The amount of ACh hydrolyzed at each point was determined after correcting for volume changes. The slopes of the lines were used to calculate rates of surface and total cell AChE hydrolyses.

General protein synthesis was determined by measuring the incorporation of [^{3}H]leucine (5 μCi/ml, Amersham, Arlington Heights, IL) into trichloracetic acid (TCA) insoluble material. Protein content was determined according to Lowry *et al.* [22].

Localization of AChE was routinely examined at the light microsope level by fixing the cells in 10% formalin and staining them for enzyme activity according to the method of Karnovsky and Roots [23] using acetylthiocholine as substrate in the presence of 10^{-4} M iso-OMPA [4, 14].

RESULTS

Growth and differentiation of the cultures

Quail cultures grew as previously described [19]; myoblasts fused in 3 days; within 7 days the cultures contained few mononucleated "fibroblasts" and many multinucleated, spontaneously contracting myotubes.

AChE and protein levels of untreated control cultures for twelve of the experiments included in this report are shown in Table 1. AChE activity per dish averaged approximately 10 μmoles/min/dish $\times 10^{-2}$; all but one experiment ranged between approximately 6 and 10 μmoles/min/dish $\times 10^{-2}$. Protein levels averaged approximately 2 mg protein/dish resulting in average AChE levels per protein of 5 μmoles/min/mg protein $\times 10^{-2}$. Variability of the data was slightly less when the data were expressed on a per dish rather than a per protein basis. For example, S.E.M. values were 11% of the mean AChE per dish and 15% of the mean AChE per protein. Quail embryo muscle cultures have very few mononucleated "fibroblasts" compared to cultures of other species [24]. However, examination of vertical sections of the cultures showed that some mononucleated cells were present (W. F. Randall, unpublished observation). For these reasons, the findings were expressed on a per dish rather than a per protein basis, as has been done in the past for chick embryo muscle cultures [4, 6, 8, 19].

AChE activity was found throughout the myotubes, both within the cells and on their surfaces [19]. Unlike chick embryo myotubes [14], AChE activity was not specially localized around the nuclei. No activity was found in the mononucleated cells.

Table 2 shows the production of AChE of the cultures during the span of an average experiment. Three kinds of AChE activity are shown: the activity of the cells (Cell), the activity that appeared in the medium over successive 2-day periods (Medium), and the accumulated net production of AChE (Total) calculated by adding the increase in AChE in the medium and the change in the activity of the cells that occurred over each period of feeding of the cells. Total AChE levels rose abruptly after fusion, much of it due to AChE activity released into the medium [1, 19].

Table 2. AChE activity of quail muscle cultures*

Day	Cell	Medium	Total
1	0.67		0.7
3	2.06	10.5	12.6
5	5.90	19.2	35.6
7	6.70	13.1	49.5
9	7.60	12.6	63.1
11	9.70	10.7	75.9
13	11.9	13.1	91.2

* Activity is in μmoles/min/dish $\times 10^{-2}$; averages of three samples. Cell: activity of the cells on the dish. Medium: difference in activity in the medium between changes. Total: cumulative activities of cells and media during the experiment. Medium was changed on day 3 and every other day thereafter.

Inhibition of AChE by DFP

The dose–responses of quail cell AChE to DFP and paraoxon are shown in Fig. 1. The I_{50} for DFP was approximately 1.5×10^{-6} M. Similar results were obtained for paraoxon, yielding an I_{50} of 8×10^{-8} M.

Multiple molecular forms and localization of AChE

Three major forms of AChE appear in the cultures

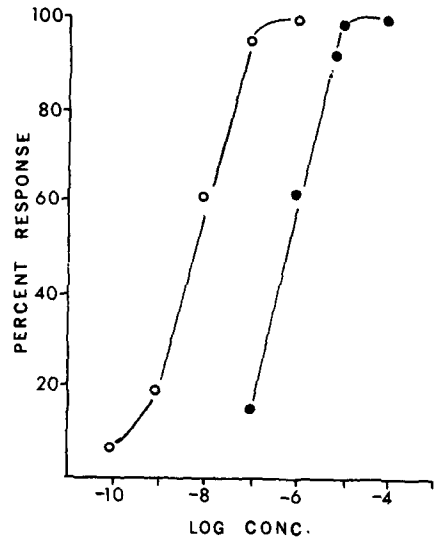

Fig. 1. Inhibition of intact quail muscle cultures by DFP (●) and paraoxon (○). Averages of triplicate cultures are expressed as percent of uninhibited dishes. DFP was from 11-day- and paraoxon from 13-day-old cultures. I_{50} values were: DFP 1.5×10^{-6} M and paraoxon 8×10^{-8} M, calculated from the slopes of probit-regression plots.

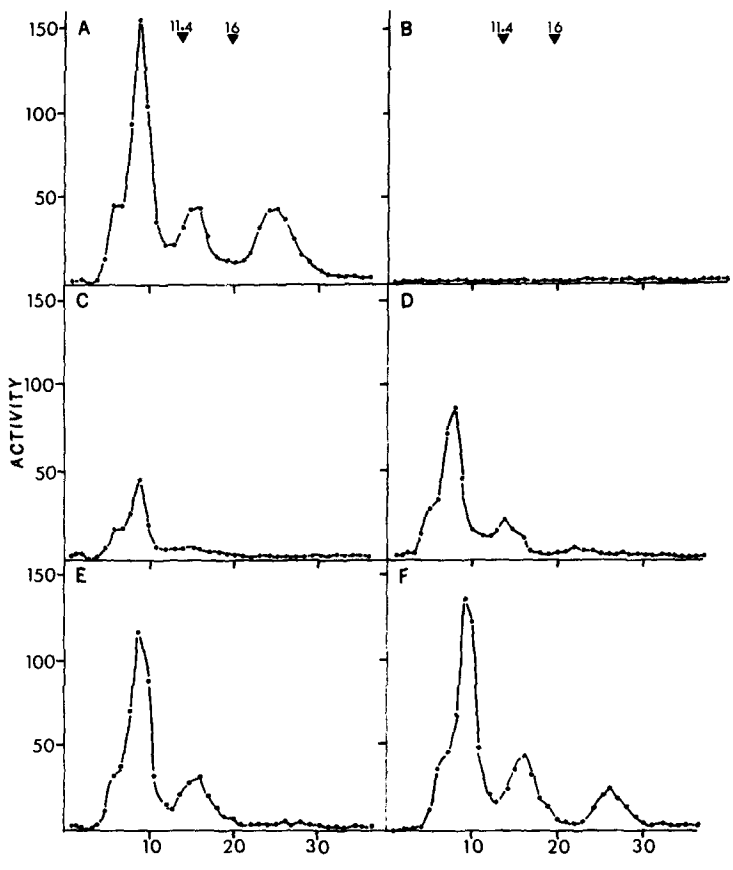

Fig. 2. Activity of multiple molecular forms of AChE in quail muscle cultures after sucrose density gradient centrifugation. Twelve-day-old cultures. (A) Untreated 0 time controls, DFP-treated cells: (B) 0 time, (C) 2 hr, (D) 4 hr, (E) 8 hr, and (F) 24 hr after treatment with 10^{-4} M DFP for 15 min. Activities are in μmoles/min $\times 10^{-4}$. Arrows are catalase (11.4S) and beta-galactosidase (16S) standards.

Table 3. Recovery of AChE after DFP treatment*

	Hours	Cell	Medium	Prod
Untreated	0	7.2		
	24	7.2	5.9	5.9
	48	7.7	15.3	15.8
	72	8.0	19.7	20.5
DFP	0	0.1		
	24	5.5	5.2	10.6
	48	6.3	14.9	21.1
	72	7.2	20.9	28.0

* Twelve-day culture. Values are in μmoles/min/dish × 10^{-2}; averages of triplicate samples. DFP: cells were treated at 0 time for 15 min with 10^{-4} M DFP. Cell: activity of cells on dishes. Medium: sum of activity released to the medium since 0 hr. Prod: the total activity produced since 0 hr.

[19] with peaks of migrations at approximately 7, 12 and 20S, and a pronounced shoulder at 5S (Fig. 2A). Approximately 39% (38.5 ± 19.5) of the activity was found in the region of the 5-7S peaks, 22% (21.6 ± 2.9) in the region of the 12S form, and 33% (32.6 ± 6.7) in the region of the high molecular weight 20S form. (These values were derived from three experiments by arbitrarily dividing the gradients into three regions, separating the 5-7S, 12S and 20S peaks, and calculating their relative percentages.)

Recovery after DFP treatment

The return of AChE activity after a brief acute poisoning of the cells with DFP is shown in Table 3. Although AChE activity recovered rapidly in the cells, it often did not reach the levels of the untreated controls for up to 72 hr. Regardless of when the AChE activity released into the medium was considered, total AChE activity produced by the DFP-treated cells invariably exceeded that of the untreated cells. For example, in three experiments AChE levels 24 and 48 hr after DFP treatment exceeded control values by an average of 210% (range: 134–438, five values). AChE activity did not return after DFP treatment when protein synthesis was inhibited by the addition of 10^{-5} M cycloheximide, as was reported previously for chicken cells [4].

I_{50} values for DFP were similar for the several AChE forms when the 7, 12 and 20S peaks were treated separately with DFP. (Values were 7S: 3.4×10^{-6} M, 12S: 2.7×10^{-6} M and 20S: 4.4×10^{-6} M.)

The initial rates of recovery of AChE activity after DFP treatment were linear and proportional to the dose of DFP applied and to the extent of the inhibition similar to our findings with chick muscle cultures [6]. The more the activity was inhibited, the more rapid was its rate of recovery (Fig. 3).

Several experiments were performed in which sucrose density gradients were run with cells that were recovering from DFP treatment. The data shown in Fig. 2 indicate that low molecular weight forms recovered more rapidly than did the larger forms, particularly the 20S form. When double the number of samples were collected in the regions of the 5S through 12S forms, the extent of initial recovery of the 5S form, usually seen as a shoulder on the 7S peak, and the 7S form was the same (data not shown).

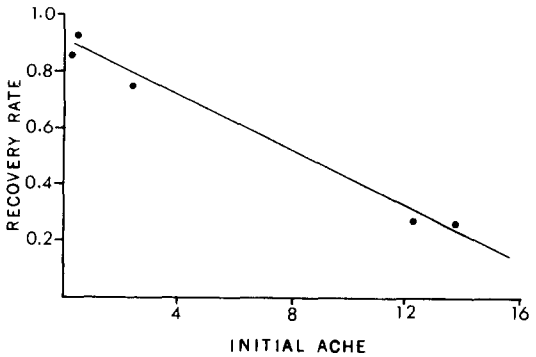

Fig. 3. Extent of inhibition and rate of recovery of AChE activity in μmoles/min/dish × 10^{-2} for 11-day-old cells. Values are slopes of AChE activity/hr derived from triplicate samples removed at various times after DFP treatment. Regression line: recovery rate = 0.89 − 0.047 initial AChE; correlation coefficient = 0.990.

Dividing the gradients into small, medium and large regions for the experiment shown in Fig. 2, and integrating the areas under the gradients during recovery from DFP treatment, yielded the recovery curves for the regions of the enzyme forms shown in Fig. 4. Increases in AChE hydrolysis were found in regions of all the forms as early as a few hours after DFP treatment. Recovery was initially linear, and dependent on the size range of the molecules. The smaller the forms, the more rapid the recovery rate. Recovery of the smaller forms became nonlinear after 8 hr, while that in the region of the 20S form continued at a low but linear rate for at least 24 hr.

Action of cycloheximide

Cycloheximide was added to stop protein synthesis at various times after DFP treatment, and the AChE levels in cells and media were followed for up to 72 hr to examine the degradation of AChE activity. AChE activity was degraded faster and to a larger extent after DFP treatment, but, in most cases, the loss of activity was slower and less than was reported previously [25] for chicken muscle cultures (Table 4).

Regardless of the effect on total AChE activity, small forms were decreased more in activity than was the large 20S form after cycloheximide treatment (Table 5).

In addition, the activity of the 20S form that appeared during recovery of the cells from DFP was almost entirely lost after cycloheximide treatment as if it were more susceptible to degradation than the enzyme in untreated cells. For example, when cycloheximide was added to cultures 24 hr after DFP treatment, the region of the 20S form of the DFP-treated control cells increased 143% and that of the cycloheximide-treated cells decreased to 26% 1 day later.

Surface activity

The DFP recovery and cycloheximide experiments

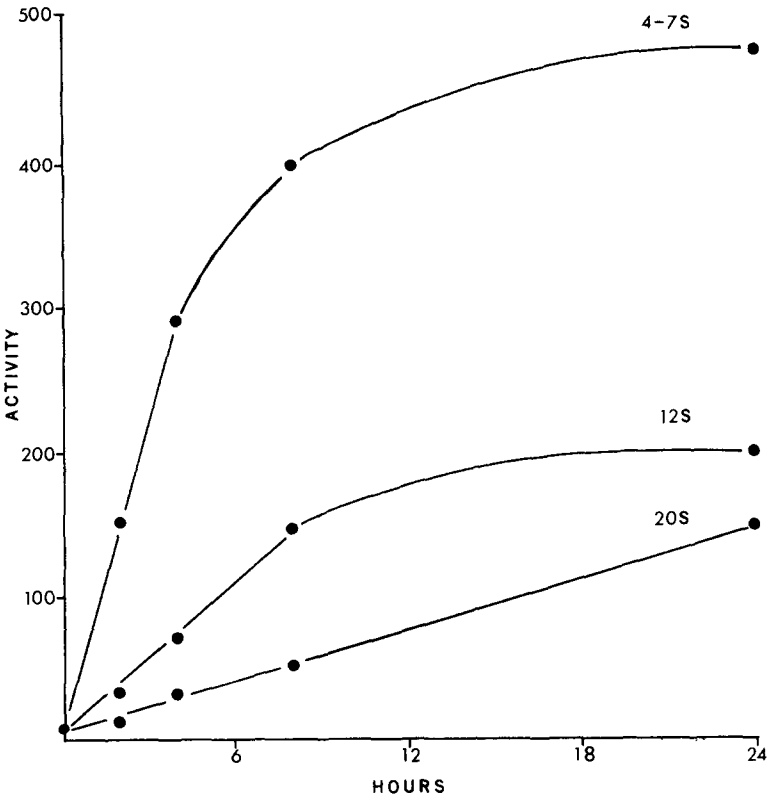

Fig. 4. Recovery of AChE activity after DFP treatment. AChE activities (μmoles/min $\times 10^{-4}$) for fractions containing the 4-7S (fractions 1–12), 12S (13–20) and 20S (21–36) are plotted versus time after treatment with 10^{-4} M DFP for 15 min. Same experiment as shown in Fig. 3.

suggested that AChE activity on the cell surface would recover more slowly than the total AChE after DFP treatment and be less affected by cycloheximide treatment. These predictions were confirmed by the results of experiments shown in Figs. 5 and 6. Surface AChE recovered more slowly than total cell AChE after DFP treatment (Fig. 5), whereas AChE decreased little in activity under conditions where total cell AChE (and by inference internal AChE levels) was progressively much reduced in the presence of cycloheximide (Fig. 6).

Table 4. Cycloheximide and AChE of cultured muscle*

	Hours	Cell	Medium	Total
Control	0	7.2	0	7.2
	24	7.2	5.9	13.1
	30	7.3/4.9	1.9/1.2	16.2/12.0
	48	7.7/5.6	9.3/1.6	23.0/13.1
	72	8.0/5.5	13.7/1.9	27.7/13.3
DFP	0	0.1	0	0.1
	24	5.5	5.2	10.7
	30	4.7/1.8	1.8/0.9	11.7/7.9
	48	6.3/1.2	9.7/1.7	21.2/8.1
	72	7.2/1.0	15.7/1.9	28.1/8.1

* Twelve-day-old cells; same as in Table 2. Values are in μmoles/min/dish $\times 10^{-2}$; averages of triplicate dishes. DFP: cells were treated with 10^{-4} M DFP for 15 min at 0 time. Medium was changed and 10^{-5} M cycloheximide was added at 24 hr. Key: (xx/yy) xx are values from untreated dishes, and yy are values from cycloheximide-treated cells.

DISCUSSION

Quail muscle cultures

Highly differentiated primary cell cultures provide systems to study the actions of chemicals that would be difficult to examine in intact animals. Muscle cultures have been especially useful in studying drugs that regulate the levels and forms of tissue-specific molecules like myosin [26], acetylcholine receptor [2], and AChE [1]. Much of the research has been carried out on cells from rats, mice, chickens, and

Table 5. Action of cycloheximide on AChE forms*

Hour	A	B	C
5			
Control	64.8	27.7	43.1
Cyclohex	28.7	21.0	43.4
24			
Control	33.2	22.1	40.6
Cyclohex	4.0	8.4	21.4
48			
Control	52.5	29.8	38.5
Cyclohex	2.4	8.0	15.9

* Cycloheximide (2×10^{-5} M) was added at 0 hr to a 10-day-old culture. AChE activity was determined after sucrose gradient sedimentation and shown for low, medium and large molecular size regions, each with a single peak. (A) wells 1–11; (B) 12–19; and (C) 20–36. Values are in μmoles/min $\times 10^{-3}$.

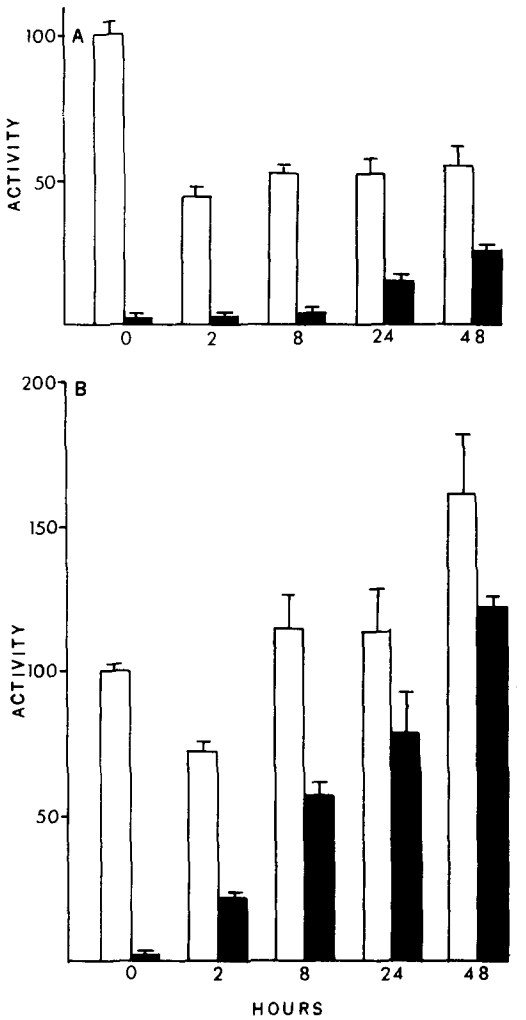

Fig. 5. Surface (A) and total (B) AChE after DFP treatment. Eleven-day-old cells. Activities are percents of 0 time untreated values. Open columns: untreated cells; dark columns; treated with 10^{-4} M DFP for 15 min at 0 time. Triplicate samples; bars at 1 S.E.M.

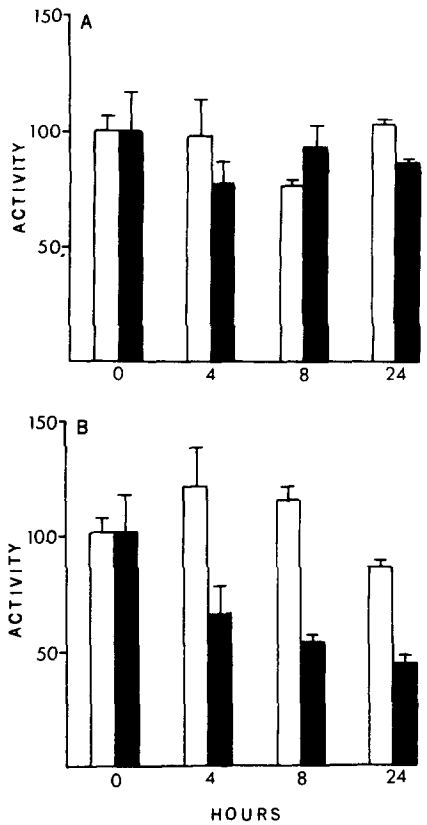

Fig. 6. Surface and total cell AChE after cycloheximide treatment. Twelve-day-old cells. Cells were continuously incubated with 10^{-5} M cycloheximide from 0 to 24 hr. Activities are percents of 0 time untreated values. (A) Untreated; (B) cycloheximide-treated. Open columns: surface AChE; dark columns: total cell AChE activities. Triplicate samples; bars at 1 S.E.M.

sometimes humans. The finding of Emmerling *et al.* [17] that quail muscle cultures contain high levels of a 20S collagen-tailed form of AChE [18] suggested that quail muscle would be a good system to examine the actions of organophosphate insecticides on AChE and would provide a way to test hypotheses derived from results from chick embryo muscle cultures [5, 6, 9, 14, 25].

Recovery of muscle cultures from organophosphorus agents

Previous work from this [4, 6, 8, 25] and other laboratories [10–12] has shown that acute poisoning with organophosphorus agents is followed by rapid recovery of new AChE activity and that small forms return faster than larger ones. AChE activity in chick muscle cultures is first detected around the nucleus, then throughout the rest of the cell, until finally it is released into the medium [14]. AChE production is increased during the first 24 hr after OP poisoning the more enzyme inhibited, the more rapid is its recovery [6].

Similarities and differences between quail and chick cultures

The results presented here for quail muscle cultures generally confirm previous findings: I_{50} values for DFP and paraoxon in the quail were similar to those in chicken [6]; AChE activity recovered rapidly after DFP poisoning, with rates that were proportional to the enzyme inhibited; more enzyme activity was produced by previously inhibited, than by untreated, controls; and small forms reappeared more rapidly than large ones. However, there were significant differences in the responses of quail and chicken muscle cultures to organophosphates. The most striking ones were the relatively high levels of AChE in quail cells [19], the relatively slow recovery of cell AChE levels after DFP treatment, and the reduced effect of inhibition of protein synthesis on the loss of AChE activity in quail, compared to chick embryo, muscle cultures.

The first studies on the recovery of AChE isozymes concluded that the small forms appear before, and recover more rapidly than, the larger ones [8, 12].

forms may have reappeared soon after DFP treatment but that the small forms recovered more rapidly and with different kinetics than the large 20S form. Interestingly, all forms had similar I_{50} values with regard to both DFP and paraoxon, indicating that both inhibitors affected the same site on each macromolecular form.

Mechanisms of AChE regulation

The experiments provide more circumstantial evidence for the existence of a recovery system for AChE following DFP poisoning that results in a rapid recovery of more enzyme than would be predicted from the untreated controls. One possibility is that the phosphorylated enzyme itself stimulates synthesis of more AChE.

In general, the data are consistent with a model of AChE mobilization and localization in which small (5 and 7S) AChE forms are synthesized within the cell, move to the cell surface, and are assembled into larger (12S) and collagen-tailed (20S) forms. For example, more of the 5-7S and 12S forms were degraded after cycloheximide treatment than was the 20S form. Approximately half the 20S form was lost after cycloheximide treatment, as if part was within and part outside of the cells. The finding that most of the 20S AChE form that appeared after DFP treatment was lost after cycloheximide treatment may indicate that the enzyme was either inside of the cells or attached to surface elements that rapidly re-entered the cell by endocytosis and were ultimately degraded. The fact that we [19] and Emmerling and Rotundo* have found that all the AChE forms are partly resistant to echothiophate (a relatively impermeable AChE form) supports the idea that all forms are present within the cells. It is difficult to define clearly in biochemical terms what is meant by the "cell surface" or to specify whether a molecule is inside the cell, within a membrane as an "integral" protein, or on the cell surface, particularly when the fate of this molecule is to be synthesized one place and transported to another, and when there is evidence for both hydrophobic and nonhydrophobic forms of the enzyme, at least with respect to the globular non-collagen-tailed (i.e. 5S, 7S, 11S) forms of mammals [1].

The nature of the binding of AChE to the extracellular matrix is not clear [1]. Indeed, some of the AChE may not have been extracted. The treatments used were designed to solubilize cell membranes; there is little reason to expect *a priori* that detergents like Triton X-100 should extract protein from a matrix of collagens, fibronectin and glycosaminoglycans.

There have been several reports that nerves and neural extracts are responsible for the appearance of high molecular weight AChE forms in rat [12, 27] and chick embryo muscle cultures [28, 29]. A recent report from this laboratory [19] and an abstract of Emmerling and Rotundo* suggest that at least part of these findings may be due to the presence of an inhibitor of the expression of AChE in the serum of the culture media. The 20S form was readily detectable in chicken cultures, and all AChE activities were greatly increased in quail cultures following removal of the horse serum in the medium [19]. The presence of all forms in quail muscle cultured under optimal growth conditions is an added reason for using them to study OP actions and AChE regulation.

Quail muscle cultures have more AChE than chicken and rat muscle cultures. One reason is probably the large number of myotubes in the quail muscle cultures. Even so, each quail myotube stained strongly for AChE. An idea deserving of further study is that there is more of, or a more mature basal lamina in, quail muscle cultures, providing conditions for extracellular assembly and binding of 20S AChE and stimulating synthesis of the enzyme or one of its critical subunits.

Acknowledgements—The authors acknowledge the assistance of Mr. Kyle Holmes and Ms. Janelle Sonada, the advice of Dr. J. E. Bulger, particularly in the surface activity assays, and the constructive criticisms of Mr. G. T. Patterson, Mr. W. R. Randall and Dr. M. G. McNamee. Supported in part by NIH ES-00202 and the Muscular Dystrophy Association, and sponsored by the Agricultural Experiment Station of the University of California and the Northern California Occupational Health Center.

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