Biochemical Pharmacology, Vol. 26, pp. 1955–1960. Pergamon Press, 1977. Printed in Great Britain.

RECOVERY OF ACETYLCHOLINESTERASE IN CULTURED CHICK EMBRYO MUSCLE TREATED WITH PARAOXON

C. MICHAEL CISSON and BARRY W. WILSON
Department of Avian Sciences, University of California, Davis, CA 95616, U.S.A.

(*Received* 18 *October* 1976; *accepted* 4 *February* 1977)

Abstract—Brief treatments with paraoxon (*O,O*-diethyl-*p*-nitrophenyl phosphate) irreversibly inhibited the acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) activity of cultured chick embryo muscle. Enzyme activity recovered as long as protein synthesis occurred, and was most rapid during the first 4 hr after paraoxon treatment. The initial recovery rate was related to the extent of initial inhibition of AChE activity: the more activity inhibited the more rapid the recovery. Differences noted between paraoxon-treated and untreated cultures during recovery included a 192 per cent increase in net AChE activity and an increase of 200 per cent in cell protein levels. AChE activity first appeared around the nuclei after paraoxon treatment, spread through the rest of the cell, and was released into the medium. The results suggest the presence of feedback control of the rapid recovery of AChE activity after organophosphate poisoning.

Previous reports from this laboratory [1] have demonstrated rapid recovery of acetylcholinesterase (AChE, acetylcholine hydrolase, EC 3.1.1.7) activity in cultured chick embryo muscle after brief treatments with di-isopropyl phosphofluoridate (DFP). The behavior of the enzyme activity during recovery indicated that protein synthesis was required and that the enzyme was rapidly synthesized and degraded. In similar studies, Harris *et al.* [2] showed that cholinesterase activity recovered rapidly in soman-treated rabbit bone marrow cells in culture and Lanks *et al.* [3] and Rieger *et al.* [4] demonstrated that AChE also recovered rapidly in cultured neuroblastoma cells treated with soman and DFP respectively. The research reported here examines the recovery of AChE in cultured chick embryo muscle inhibited with various doses of paraoxon (*O,O*-diethyl-*p*-nitrophenyl phosphate). In addition, the effects of paraoxon on total AChE activity and cell protein are also reported.

MATERIALS AND METHODS

Primary muscle cultures of chick pectoral muscle cells were prepared from 11-day-old embryos from a commercial line (Donsing Hatcheries, Rio Linda, CA). The tissue was dissociated with 0.1% (w/v) trypsin. The single cells were inoculated into 35 mm petri dishes coated with collagen at a density of 5×10^5 cells/dish in a medium of 88% (v/v) Eagle's Minimal Essential Medium with Earle's salts, 10% (v/v) horse serum and 2% (v/v) embryo extract. Antibiotics were not used. The medium was changed 24 hr after inoculation and every 2 days thereafter. The cultures were grown at 37° and pH 7.2 to 7.5 in an atmosphere of humidified air and CO_2. Most experiments were performed on 10-day-old cultures. Cultures were routinely observed in the living state with an inverted phase-contrast microscope. There was no evidence of gross cytotoxicity from the organophosphate agents during the experiments.

AChE activity was determined in cells with the spectrophotometric method of Ellman *et al.* [5] using acetylthiocholine iodide (ACTC) as the substrate. Enzyme activity was also determined in the medium because previous work has demonstrated that large amounts of AChE activity are released into the medium by cultured muscle cells [6]. Nonspecific cholinesterase (acylcholine acylhydrolase, EC 3.1.1.8) was selectively inhibited [7] with 0.1 mM iso-OMPA (tetraisopropyl pyrophosphoramide). AChE activity is expressed in terms of μmoles ACTC hydrolyzed/min/dish or as a percentage of the activity of control cultures. Since fibroblasts continued to grow during the life of the cultures, it was unsuitable to express AChE activity, which is contained solely in the myotubes, in terms of the protein content of the cultures. If it were so expressed, the average AChE activity in the untreated cells in one experiment was $2.6 \pm 0.5 \times 10^{-2}$ μmoles/min/μg of protein.

The cytochemical localization of AChE was determined using the staining technique of Karnovsky and Roots [8] as previously described [1]. Multiple molecular forms of AChE were examined with 10% polyacrylamide disc gels [9]. The bands were stained using a modification [10] of the staining technique of Koelle and Friedenwald [11].

Creatine kinase (CK, EC 2.7.3.2) activity of cells and medium was determined using the spectrophotometric technique of Hess *et al.* [12]. Cell protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin as standard.

The cells were scraped from their dishes, homogenized, and sonicated in buffered saline prior to assay. CK activity was determined on fresh tissue, AChE activity on tissue frozen for up to 1 week, and protein on tissue frozen for up to 2 weeks.

Paraoxon and DFP were stored in acetone at − 20°. Cultures were rinsed three times with buffered

saline at 37°, and incubated for 10–15 min at room temperature in a saline solution with various concentrations of paraoxon or DFP. The final concentration of acetone was 0.1% (v/v) or less. After treatment, cultures were rinsed four times with saline and incubated at 37° in medium in which the horse serum and embryo extract were treated at least 3 days in advance with 10^{-5} and 10^{-7} M DFP respectively. This DFP treatment reduced cholinesterase levels by 95 per cent in horse serum and 100 per cent in embryo extract. Cycloheximide was added to cultures at a final concentration of 10 μM immediately after treatment with paraoxon. More than 85 per cent of the incorporation of [^{3}H]leucine was inhibited in less than 20 min, and more than 95 per cent of the incorporation was inhibited within 4 hr in previous studies of the cells under similar conditions [1, 6].

Paraoxon was a gift from American Cyanamid, Princeton, NJ. DFP was purchased from CalBiochem, San Diego, CA. Minimal Essential Medium and horse serum was obtained from Gibco, Grand Island, NY. Horse serum was also obtained from KC Biological, Lenexa, KS. Acrylamide was obtained from Biorad Laboratories, Richmond, CA. All other chemical agents were obtained from Sigma Chemical Co., St. Louis, MO.

Statistically significant differences in AChE activity and cell protein were determined using appropriate applications of the Student's t-test [14].

RESULTS

Paraoxon, in concentrations ranging from 10^{-4} to 10^{-12} M, inhibited AChE activity in cultured chick embryo pectoral muscle. Figure 1 shows the log dose–effect relationship of paraoxon, and the relative potency of this compound compared to DFP, another organophosphate inhibitor of AChE activity. The I_{50} for paraoxon was 1.0×10^{-9} M compared to 5.0×10^{-7} M for DFP. Neither paraoxon nor DFP produced complete inhibition of AChE activity and the maximal response (95 per cent inhibition) of each

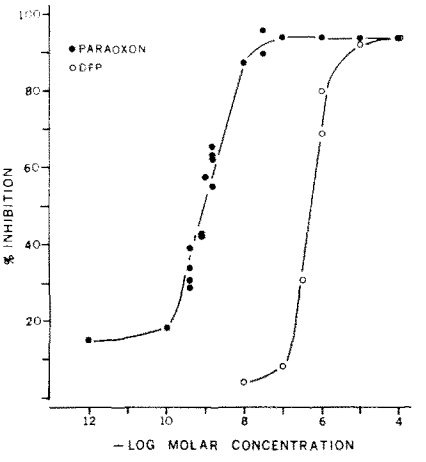

Fig. 1. Log dose–response relationship of AChE inhibited by paraoxon and DFP. Cultures were treated for 10 min with paraoxon (●) or DFP (○). AChE activity is expressed as the percentage of inhibition of untreated control cultures activity. Each point is the average of two dishes.

agent appeared to be the same in this range of doses. Treating cells with high concentrations of paraoxon ($> 10^{-7}$ M) delayed recovery of AChE activity for up to 2 hr, and addition of homogenates from these paraoxon-treated cell cultures to homogenates of untreated cell cultures resulted in detectable inhibition of enzyme activity, indicating a residual amount of the agent remained for some time within the cells.

Cultures treated with 3×10^{-8} M paraoxon rapidly recovered their AChE activity (Fig. 2). For example, AChE was reduced by an average of 92.8 per cent in two experiments, and 86.8 per cent of the activity recovered within 4 hr. Release of AChE activity into the medium was not detectable until most of the enzyme activity had recovered in the cells (Table 1). Cultures treated with paraoxon and then incubated with 10 μM cycloheximide did not recover their AChE activity. For example, in one experiment, the activity of these cultures averaged $2.4 \pm 0.2 \times$

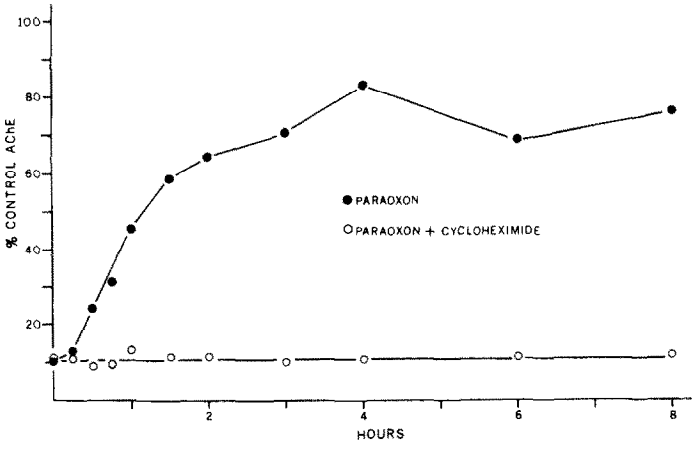

Fig. 2. Recovery of cellular AChE activity in paraoxon-treated muscle cultures in the absence or presence of cycloheximide. Cells were incubated for 10 min in 3×10^{-8} M paraoxon, rinsed at time 0 hr, and sampled after incubation in DFP-treated medium (●) or DFP-treated medium with 10 μM cycloheximide (○). Activities are expressed as a percentage of the activity of initial control cells. Each point is the average of three dishes in one experiment.

Table 1. AChE activity of cultured muscle after paraoxon treatment*

Hr after paraoxon treatment	AChE activity†	
	Cells	Δ Medium‡
0	0.24	0.00
1	1.03	0.00
2	1.47	0.15
4	1.91	0.74
8	1.62	1.84

* Paraoxon concentration = 3×10^{-8} M.
† Activity is expressed as $\times 10^{-2}$ μmoles/min/dish.
‡ Medium activity at time t minus activity at time 0 hr

10^{-3} μmoles ACTC hydrolyzed/min/dish compared to control culture values of $2.40 \pm 0.42 \times 10^{-2}$ μmoles (Fig. 2).

AChE activity also recovered in cultures treated with concentrations of paraoxon that did not completely inhibit the enzyme (Fig. 3). In four experiments, cultures treated with 4×10^{-10} M paraoxon averaged 61.5 per cent of the initial AChE activity, and cultures treated with 1.6×10^{-9} M paraoxon averaged 38.6 per cent of the initial AChE activity. Under these conditions, the rate of recovery of enzyme activity was directly dependent on the level of inhibition, i.e. the greater the initial inhibition, the faster the recovery. AChE activity also appeared in the medium of cultures treated with 1.6×10^{-9} M paraoxon before those treated with 4×10^{-10} M paraoxon. However, at higher concentrations of paraoxon, for example 3×10^{-8} M, the rate of initial recovery of AChE activity was slower than the recovery of enzyme activity after treatment with 1.6×10^{-9} M paraoxon (Table 2).

Total AChE production in paraoxon-treated cultures was greater than that of control cultures. In six experiments, AChE production in paraoxon-treated cultures was double that of control cultures 8 hr after treatment (Table 3). Total cell protein also increased an average of 205 per cent more than control cultures 8 hr after paraoxon treatment in three experiments (Table 4).

Neither CK activity of the cells nor the proportion of CK found in the medium was greatly affected by paraoxon treatment. For example, in one experiment, there was no inhibition of CK activity immediately following paraoxon treatment, and after 24 hr of recovery, CK activity in paraoxon-treated cells was

Table 2. Rate of recovery of AChE activity in muscle cultures treated with paraoxon

Con ($\times 10^{-10}$ M)	Recovery rate*	No. of experiments
4†	25.8 ± 8.9	4
8†	30.2 ± 3.3	2
16†	57.7 ± 12.8	4
300	49.0 ± 5.3	2

* AChE activity 2 hr after treatment minus AChE activity immediately following treatment expressed as a percentage of the initial activity of control cultures. Values are expressed as mean ± standard error.
† Linear regression is $r = 0.82$; $y = 2.73 \times +13.28$. Statistically significant difference between r and 0: $P < 0.01$ [14].

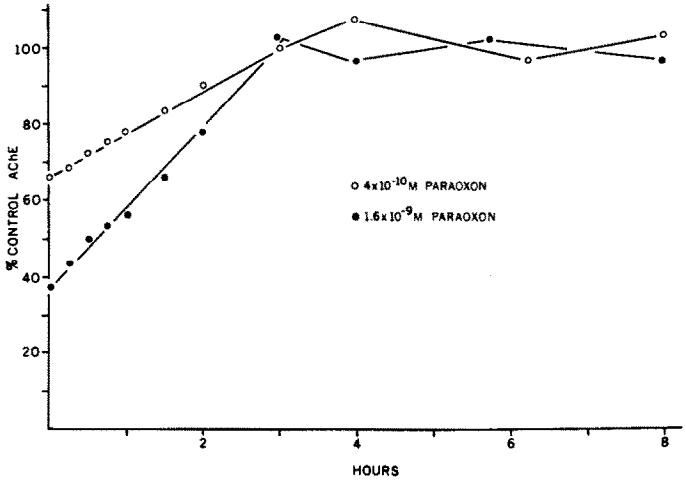

Fig. 3. Recovery of cellular AChE activity from partial inhibition by paraoxon in cultured muscle. Cells were incubated for 10 min in 4×10^{-10} M (○) or 1.6×10^{-9} M (●) paraoxon, rinsed at time 0 hr, and incubated in DFP-treated medium until sampled. Activities are expressed as a percentage of the activity of initial control cultures. Each point is the average of two dishes in one experiment.

Table 3. AChE production during recovery from paraoxon treatment, 0–8 hr

Experiment	AChE activity*			% Control
	Δ Cells†	Δ Medium†	Total production	
(1) Control‡	0.51	3.75	4.26	
3×10^{-8} M	1.40	1.84	3.24	76.1
(2) Control	0.07	3.53	3.60	
3×10^{-8} M	3.01	1.91	4.92	136.7
(3) Control	0.07	1.47	1.54	
4×10^{-10} M	1.25	1.76	3.01	195.5
16×10^{-10} M	0.74	3.31	4.05	263.0
(4) Control	−0.15	2.94	2.79	
4×10^{-10} M	0.81	5.29	6.10	218.6
16×10^{-10} M	1.40	5.95	7.35	263.4
Mean ± standard error 192.2 ± 74.0§				

* Activity is expressed as $\times 10^{-2}$ μmoles/min/dish.
† AChE activity 8 hr after treatment minus activity immediately after treatment.
‡ Values are averages of three dishes in Expts. 1 and 2 and are averages of two dishes in Expts. 3 and 4.
§ Statistically significant difference between paraoxon-treated and untreated cultures, $P < 0.02$ [14].

6.69 ± 2.86 μmoles $NADPH_2$ formed/min/dish compared to 6.83 ± 2.46 in untreated cells. The amount of CK activity in the medium averaged 5.2 ± 1.9 per cent the activity found in the cells, regardless of the treatment given the cultures.

The changes in AChE localization and isozyme patterns after paraoxon treatment resembled those found previously for DFP [1]. However, using low concentrations of paraoxon (10^{-9} to 10^{-10} M), AChE activity appeared around the nuclei 30 min after treatment and was generally spread throughout the fibers within 4 hr. During the first 4 hr of recovery, a low molecular weight form of AChE exhibited a transient increase in activity. By 8 hr, the activity of this isozyme had decreased, and the isozyme pattern of the treated cultures was the same as the untreated cultures.

DISCUSSION

Paraoxon is a potent organophosphate inhibitor of AChE and other cholinesterases *in vitro* and *in vivo* [15]. The I_{50} value obtained for the inhibition of AChE activity of intact muscle cultures was comparable to those that have been described for other systems. For example, the I_{50} for paraoxon was 7.4×10^{-9} M for the AChE of rat cardiac muscle [16]. The reason for the small (less than 10 per cent) amount of AChE activity that remained in cells treated with higher concentrations ($> 10^{-7}$ M) of paraoxon is unclear. Rieger *et al.* [4] observed similar results in cultured neuroblastoma cells treated with DFP.

The recovery of AChE activity from paraoxon in cultured chick muscle resembles previous observations with DFP [1], an irreversible inhibitor of cholinesterases [17]. Enzyme activity first appeared around the nuclei, moved through the cell, and was released into the medium when the cellular activity reached its maximal value about 4 hr after paraoxon treatment. Ultrastructural localization of AChE after DFP treatment using electron microscopy confirms these light microscope observations [18]. The recovery of enzyme activity after paraoxon treatment was rapid and resembled similar observations *in vivo*. For

Table 4. Cell protein of muscle cultures treated with paraoxon

Condition	Cell protein (mg/dish)			% Control
	$t = 8$ hr	$t = 0$ hr	Production*	
Control†	1.83	1.30	0.53	
4×10^{-10} M†	2.29	1.12	1.17	220.8
16×10^{-10} M†	2.28	1.44	0.84	158.5
Control‡	1.01	0.86	0.15	
3×10^{-8} M‡	1.24	0.88	0.36	240.0
Mean ± standard error 206.4 ± 42.6§				

* Cell protein 8 hr after treatment minus cell protein immediately after treatment.
† Values are averages of two dishes.
‡ Values are averages of three dishes.
§ Statistically significant difference between paraoxon-treated and untreated cells, $P < 0.05$ [14].

example, rats treated with paraoxon recover 49 per cent of the AChE activity in the gastrocnemius muscle within 12 hr [19].

The inhibition of recovery of AChE activity by cycloheximide suggests that protein synthesis was required for a return of AChE activity. Similar results have been observed with cultured muscle cells treated with DFP [1], cultured bone marrow cells treated with soman [2], cultured neuroblastoma treated with soman [3] and DFP [4], and rat retina treated with DFP *in vivo* [20]. Such results are consistent with the view that synthesis of new enzyme and not reactivation of pre-existing forms accounts for the rapid recovery of AChE in organophosphate-treated cells. However, RNA synthesis was required for the recovery of cholinesterase in cultured rabbit bone marrow cells [2], but not for the recovery of AChE in cultured neuroblastoma [4] or cultured muscle [21]. Whether there are differences in the turnover of messenger RNA molecules between these cultured cells is not known.

The results show that there is a transient increase in the activity of the lowest molecular weight form of AChE during recovery from paraoxon treatment. However, the activity of this form begins to decrease 4 hr after paraoxon treatment, while the activity of the two higher molecular weight forms appears to be increasing. Similar observations in rat retina *in vivo* [20], cultured muscle [1], and cultured neuroblastoma [4] treated with DFP suggest that the recovery of AChE activity is characterized by the rapid synthesis of lower molecular weight forms, which in turn are assembled into higher molecular weight forms.

Several lines of evidence suggest that the lack of a total recovery of AChE activity to initial values during the 8-hr intervals of the experiments was not due to a gross cytotoxicity of the anticholinesterase agents. There was no indication of the development of cytotoxicity for up to 72 hr after brief treatments with paraoxon. CK, an enzyme known to be released from diseased or injured muscle [22], did not increase in the medium, and there was no loss in cell protein.

The data show that muscle cells subjected to acute poisoning with paraoxon have a net production of AChE activity in excess of untreated cells when the amount of AChE activity released into the medium is taken into account. Similar results have been observed in DFP-treated muscle cells (C. R. Walker and B. W. Wilson, unpublished data). It is as if more AChE were produced and less were retained by the cells after treatment with organophosphorus agents. Whether such cells have fewer binding sites for AChE is not known, nor has the fate of the phosphorylated enzyme been studied. Regardless, the data indicate that there has been an alteration in the balance of the synthesis and degradation of AChE after paraoxon treatment. Indeed, the results suggest that total protein production of the cells may have been enhanced. Previously, Welsch and Dettbarn [23] observed that pretreatment of isolated lobster nerve fibers with paraoxon increased protein synthesis. Other workers have reported similar findings in rat brain and rat liver treated with 217 AO and soman respectively [24, 25]. Such results suggest that paraoxon and other organophosphorus compounds may affect protein metabolism in target cells. However, since our data showed that cell CK activity was not affected by paraoxon treatment, it is possible that such an effect is selective rather than general. The bases for such effects of organophosphates on enzyme activity and protein levels are unknown. Paraoxon also inhibits chymotrypsin activity [26] and might, therefore, be suspected of inhibiting other proteolytic enzymes. Whether paraoxon significantly decreases protein degradation or increases protein synthesis by some other mechanism remains to be determined.

Although rapid rates of recovery of AChE activity have been noted previously in cells that have been treated with organophosphates, this is the first report of rates of recovery of AChE activity that depended upon the extent of inhibition of the enzyme. Such results cannot be explained by retention of unreacted organophosphate at high dosages; if this were true, recovery should have been slower rather than faster at higher concentrations of inhibitor. The fact that recovery of AChE activity was inhibited in the presence of cycloheximide indicates that hydrolytic reactivation of previously synthesized AChE (see Ref. 27–29) did not play a role in the differences in the rates of recovery. It is possible, however, that changes in the rates of synthesis and/or degradation of protein, such as those discussed above, could explain such a phenomenon. These changes could provide the basis for some type of feedback control mechanism, where the earliest recovery of AChE activity is the most rapid, due to an immediate increase in protein synthesis (see Ref. 23–25) and/or decrease in protein degradation due to the inhibition of other proteolytic enzymes (see Ref. 26 and 30). Such a feedback control mechanism may also explain the so-called "biphasic recovery" of AChE activity in DFP-treated rat brain that was observed by Chippendale *et al.* [31].

Acknowledgements—This study was supported by United States Public Health Service Grants ES 00202 and NS 10957. The authors gratefully acknowledge the technical assistance of Ms. P. S. Nieberg and the advice and interest of Dr. C. R. Walker in this project

REFERENCES

1. B. W. Wilson and C. R. Walker, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3194 (1974).
2. L. W. Harris, V. F. Garry, Jr. and R. D. Moore, *Biochem. Pharmac.* **23**, 2155 (1974).
3. K. W. Lanks, J. M. Dorwin and B. Papirmeister, *J. Cell Biol.* **63**, 824 (1974).
4. F. Rieger, A. Faivre-Bauman, P. Benda and M. Vigny, *J. Neurochem.* **27**, 1059 (1976).
5. G. L. Ellman, K. D. Courtney, V. Andres, Jr. and R. M. Featherstone, *Biochem. Pharmac.* **7**, 88 (1961).
6. B. W. Wilson, P. S. Nieberg, C. R. Walker, T. A. Linkhart and D. M. Fry, *Devl. Biol.* **33**, 285 (1973).
7. B. W. Wilson, T. A. Linkhart, C. R. Walker and P. S. Nieberg, *J. neurol. Sci.* **18**, 333 (1973).
8. M. J. Karnovsky and L. Roots, *J. Histochem. Cytochem.* **12**, 219 (1964).
9. B. J. Davis, *Ann. N.Y. Acad. Sci.* **121**, 404 (1964).
10. E. Maynard, *J. exp. Zool.* **161**, 319 (1966).
11. G. B. Koelle and J. S. Friedenwald, *Proc. Soc. exp. Biol. Med.* **70**, 617 (1949).
12. J. W. Hess, K. J. Murdock and G. J. W. Natho, *Am. J. clin. Path.* **50**, 89 (1968).
13. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).

14. H. L. Alder and E. B. Roessler, *Introduction to Probability and Statistics*, p. 143. Freeman, San Francisco (1968).
15. W. N. Aldridge, *Biochem. J.* **46,** 451 (1950).
16. A. N. Davison, *Biochem. J.* **54,** 583 (1953).
17. A. Mazur and O. Bodansky, *J. biol. Chem.* **163,** 261 (1946).
18. T. K. Golder, P. S. Nieberg and B. W. Wilson, *J. Cell Biol.* **70,** 221a (1976).
19. L. Wecker and W. D. Dettbarn, *Exp. Neurol.* **51,** 281 (1976).
20. G. A. Davis and B. W. Agranoff, *Nature, Lond.* **220,** 277 (1968).
21. C. R. Walker and B. W. Wilson, *Neuroscience* **1,** 501 (1976).
22. S. Okinaga, H. Kumagai, S. Ebashi, H. Sugita, H. Momoi, Y. Tokura and Y. Fujie, *Archs. Neurol., Chicago* **4,** 520 (1961).
23. F. Welsch and W. D. Dettbarn, *Comp. Biochem. Physiol.* **38B,** 393 (1971).
24. D. H. Clouet and H. Waelsch, *J. Neurochem.* **10,** 51 (1963).
25. W. Domschke, G. F. Domagk, S. Domschke and W. D. Erdmann, *Arch. Tox.* **26,** 76 (1970).
26. B. S. Hartley and B. A. Kilby, *Biochem. J.* **50,** 672 (1952).
27. W. N. Aldridge, *Biochem. J.* **54,** 442 (1953).
28. A. N. Davison, *Biochem. J.* **60,** 339 (1955).
29. F. Welsch and W. D. Dettbarn, *Biochem. Pharmac.* **21,** 1039 (1972).
30. E. F. Jansen, M. D. F. Nutting, R. Jang and A. K. Balls, *J. biol. Chem.* **179,** 189 (1949).
31. T. J. Chippendale, C. W. Cottman, M. D. Kozar and G. S. Lynch, *Brain Res.* **81,** 485 (1974).