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EFFECTS OF SOME CHOLINESTERASE INHIBITORS ON THE SQUID GIANT AXON— THEIR PERMEABILITY, DETOXICATION AND EFFECTS ON CONDUCTION AND ACETYLCHOLINESTERASE ACTIVITY*

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Abstract—The irreversible cholinesterase inhibitors, *O,O*-diisopropyl S-(2-diisopropylaminoethyl) phosphorothioate (Tetriso), *O*-ethyl Se-(2-diethylaminoethyl) ethylphosphonoselenoate (Selenophos) and *O,O*-diethyl S-(2-ethylthioethyl) phosphorothioate (Isosystox), penetrate in their inhibitory forms into the squid giant axon. Tetriso and Selenophos, when applied for 30 min in concentrations as high as 5×10^{-3}M, did not block axonal conduction. At that concentration, Isosystox caused either a reversible or an irreversible block of conduction depending on the length of time the nerve was exposed to the inhibitor. After pretreatment of the squid axon with cottonmouth moccasin venom, Selenophos (5×10^{-3}M) caused in 25 min a marked and irreversible reduction in the action potential, while Tetriso (5×10^{-3}M) caused a rapid and reversible decrease in the action potential. An extremely sensitive, simple radiometric technique for the assay of cholinesterase activity has been developed and is described in detail. Nevertheless, attempts to measure enzyme activity in squid axons exposed to Selenophos or Tetriso were unsuccessful because small amounts of entrapped inhibitor in the tissue were released upon homogenization. Possible explanations for the high concentrations of inhibitors required to affect conduction are discussed.

A BIOCHEMICAL theory to account for the permeability changes associated with bioelectric activity has been developed by Nachmansohn.[1–3] Support for this theory has come from many sources, including the presence of acetylcholinesterase (AChE) in nerve and muscle fibers and its localization in excitable membranes.[4–7] This enzyme has the kinetic properties[8, 9] necessary for its proposed role of rapidly hydrolyzing acetylcholine (ACh) and thereby restoring the permeability of the excitable membrane to its resting condition. Particular importance was, however, attached to the demonstration that potent and specific cholinesterase inhibitors may block conduction reversibly or irreversibly according to their mode of reaction with the enzyme, thus suggesting a functional interdependence between enzyme and electrical activity.[10–13] The inability of some inhibitors to block conduction and the high concentrations required with others have been shown in several cases to be due either to

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poor penetration of the inhibitors[10, 12] or to their hydrolysis by phosphorylphosphatases in the tissue.[13]

We have recently reported[14] that a potent and irreversible AChE inhibitor, *O,O*-diethyl S-(2-dimethylaminoethyl) phosphorothioate (217AO), had no effect on conduction in the squid giant axon, even though it penetrated into the interior of the axon and was not significantly detoxified by tissue enzymes. This finding raises an interesting question, namely, how to interpret the failure of this and similar compounds to block conduction irreversibly. In order to evaluate possible interpretations critically, it would be desirable to measure the AChE activity in the squid giant axon. Unfortunately, accurate and unequivocal measurements of enzyme activity after exposure of nerves to inhibitors are subject to many difficulties.[14, 15] A major concern in the present study was the possible release of entrapped inhibitor on homogenization of the axons; homogenization is necessary because of the failure of substrate to reach all of the enzyme if intact tissue is used.

We have now extended our studies to include a wider selection of compounds. These are potent irreversible AChE inhibitors having varying degrees of lipid solubility, able to penetrate into the squid giant axon, and not subject to significant enzymatic detoxication. In these studies we have also attempted to measure the remaining AChE activity of axons after exposure to several of these inhibitors. Because of the low AChE activity of this preparation,[16] it is necessary to use a highly sensitive and reliable technique for these measurements. We shall describe the use of such a radiometric technique developed by one of us (L. T. K.).

MATERIALS AND METHODS

O,O-Diisopropyl S-(2-diisopropylaminoethyl) phosphorothioate (Tetriso) was synthesized essentially according to Ghosh.[17] The intermediate, 2-diisopropylaminoethyl thiocyanate hydrobromide, was synthesized according to Johnston and Gallagher.[18] 2-Diisopropylaminoethyl bromide hydrobromide (0·2 mole, Columbia Organic Chemicals) was added to 0·22 mole KSCN in 200 ml of warm methanol. The mixture was refluxed for 1 hr, cooled and filtered, and the filter cake was washed with methanol. The product crystallized from the filtrate on cooling; after filtration and drying, it was used without further purification. This product (26·7 g) was added slowly to a stirred solution containing diisopropylhydrogen phosphite and sodium ethylate (0·11 mole each) in 100 ml of absolute ethanol. Due to the extreme toxicity of the products, this and subsequent reactions were carried out in a fume hood. Stirring and warming to about 60° were continued for a further 15 min. The mixture was filtered and the filter cake was washed with a small amount of cold ethanol. To the filtrate was added, with cooling and stirring, 0·1 mole sodium ethylate in 50 ml of absolute ethanol. The mixture was evaporated under pressure and the residue was dissolved in water and extracted with ether for 6 hr. The extract was treated with anhydrous Na_2SO_4 and decolorizing carbon, and filtered. The ether was evaporated and the remaining pale yellow liquid was distilled. On redistillation, the major fraction was collected at 100°–102°/0·01 mm. Phosphorus analysis: calculated for $C_{14}H_{32}O_3NPS$, 9·52%; found, 9·63% and 9·40%. Attempts to make the acid oxalate with anhydrous oxalic acid resulted in a glassy residue which failed to crystallize. Phosphorus analysis, however, gave the following: calculated for $C_{16}H_{32}O_7NPS$, 7·46%; found, 7·35%. *O*-Ethyl Se-(2-diethylaminoethyl) ethylphosphonoselenoate

(Selenophos) was synthesized exactly according to the directions of Åkerfeldt and Fagerlind,[19] to whom we are most grateful for a sample of sodium *O*-ethyl ethylphosphonoselenoate. The 2-diethylaminoethyl chloride was freshly prepared before use.[20] *O,O*-Diethyl S-(2-ethylthioethyl) phosphorothioate (Isosystox) was generously supplied by Dr. R. L. Metcalf, University of California at Riverside.

Dissection of the giant axon of the squid, *Loligo pealii*, extrusion of axoplasm, external recording of electrical activity, and the use of venoms for the partial reduction of permeability barriers have been described previously.[15, 16] Electrical measurements were made on axons which contained adhering small nerve fibers.[21, 22] When venom was used, axons were pretreated for 30 min with 20 μg of lyophilized cottonmouth venom (*Agkistrodon p. piscivorus*) per milliliter of buffered sea water. This concentration and time of application were found to cause less than a 20 per cent decrease in the action potential spike height.

One series of bielectric measurements was made for us by Dr. Toshio Narahashi of Duke University, Durham, N.C., to whom we are indebted. In this experiment intracellular microelectrode recordings were made while the axon was alternately bathed in flowing artificial sea water or sea water containing Tetriso at 10^{-3} M. The inset in Fig. 2 shows a typical recording of the action potential and of the derivative of the action potential, wherein the maximum rates of change of the rising and falling phases are generally considered to reflect Na^+ and K^+ conductances respectively.

We have also previously described[14] the method used to measure penetration of 217AO into, and its detoxication by, the squid axon. The methods employed in the present instance are identical. Because Tetriso is a new compound (although of the 217AO type) and Selenophos has only recently been described,[19] plots of percentage AChE inhibition versus inhibitor concentration for these two compounds are presented

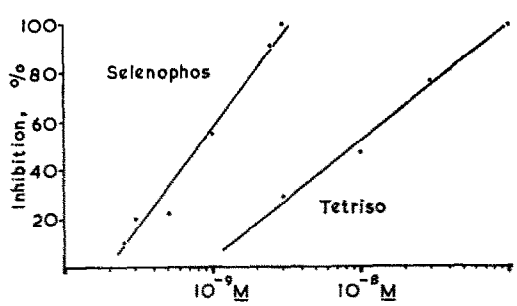

FIG. 1. Inhibition of purified *Electrophorus electricus* acetylcholinesterase by *O,O*-diisopropyl S-(2-diisopropylaminoethyl) phosphorothioate (Tetriso) and by *O*-ethyl Se-(2-diethylaminoethyl) ethylphosphonoselenoate (Selenophos).

in Fig. 1. From this plot we estimate the pI_{50} of Tetriso to be 8·05 and of Selenophos to be 9·1 (reported value of Selenophos with erythrocyte AChE, 9·0 to 9·7);[19] we also estimate the pI_{50} of Isosytox to be 6·0 (reported value for serum ChE, 5·5[23]). Although the inhibition would be more generally expressed by a second-order rate constant,[24] the plots given in Fig. 1 are more immediately useful in the present investigation. To this end, enzyme concentrations and times of incubation with inhibitors were held

constant. The *Electrophorus electricus* AChE preparation used was such that 1 μg of lyophilized material normally hydrolyzed 6–7 μmoles ACh per hr (30°, 0·005 M ACh, pH 7·4). The inhibitor was incubated for 30 min with the enzyme (3 μg lyophilized material/ml) and an aliquot containing 0·6 μg enzyme was removed and mixed with substrate in a total volume of 1·8 ml. The AChE activity was measured manometrically.

For the radiometric measurement of AChE activity, typically, 7 mg of cleanly dissected squid axons was homogenized in 20 μl sea water (buffered to pH 7·8 with 0·001 M Tris). Then 0·4 μmole acetyl-1-^{14}C-*dl*-β-methylcholine (MeCh-^{14}C; supplier, New England Nuclear Corp.) was added in 10 μl of buffered sea water. The homogenizer pestle was washed down with 10 μl of buffered sea water, thus giving a final substrate concentration of 0·01 M. The incubation was carried out in the micro–homogenizer (capacity, 500 μl) at room temperature (22°–25°) usually for 2–5 hr, at which time the homogenizer was placed in dry ice to stop the enzyme reaction. Later, the reaction mixture was diluted to 400 μl with distilled water to reduce the salt concentration of sea water in preparation for chromatographic separation. A column chromatographic procedure was used to separate the labeled unhydrolyzed substrate, which is not held by an anion exchanger, from the labeled product, acetic acid-1-^{14}C, formed by the enzymatic hydrolysis. A 100-μl aliquot from the 400 μl was diluted with an equal volume of distilled water. After allowing the water in the column to run out to a point just above the resin, the 200-μl solution was applied to a microchromatographic column containing 10–15 μl of analytical grade Dowex 1-X8 resin, 100–200 mesh (Bio-Rad). The column was arranged so that its tip was touching the bottom of a scintillation vial containing 0·1 ml of 0·05 N NaOH to prevent any loss of radioactivity. Seven fractions were collected in seven vials, corresponding to the following additions to the column: 200 μl of sample plus 100 μl water used to wash the container; 200 μl water used to wash the container; two more 200-μl portions of water used to wash the column; three more fractions each representing 200 μl of an eluting solution consisting of 0·15 M NaCl and 0·001 N NaOH. To correct for the spontaneous hydrolysis of substrate, the entire procedure was repeated except for the omission of axonal material. Radioactivity was measured by using Bray's solution[25] in a Packard Tri-Carb liquid scintillation counter.

The microcolumn was made from a 0·1-ml serological pipette having an upper end widened for cotton plugging, drawn to a capillary opening at the lower end, and having an overall length of 7 cm. The small bore section of the column had a capacity of approximately 15 μl; the upper cotton plug mouth served as a reservoir for the introduction of resin, sample and solvents. The column support was a fine plug of glass wool adjusted to regulate the flow rate. The column was formed by introducing a drop or two of an aqueous suspension of the resin (from which the very coarse and very fine particles had been removed by decantation on a large scale) into the column previously filled with water. Excess resin was removed by injecting a stream of water from a hypodermic syringe into the top of the column. The liquid above the resin was kept free of entrapped air bubbles to prevent channeling and to maintain proper column flow. The resin was prepared for use by washing with 1 M NaCl, water, 1 N NaOH, water, 1 M sodium acetate, and finally with water to pH 5·5 to 6. The resin was stored in distilled water under refrigeration and was satisfactory several months after preparation.

RESULTS

Table 1 shows the penetration of Tetriso, Selenophos and Isosystox into the squid giant axon. The results, which are in general agreement with those obtained with 217AO,[14] show that these compounds approach or somewhat exceed equivalent distribution in 1 hr or less. Table 2 shows that there is a negligible amount of enzymatic detoxication of either Tetriso or Selenophos by the squid axon. Inasmuch as Table 1 shows the penetration of Tetriso and Selenophos at 10^{-3} M in their active

TABLE 1. PENETRATION OF ORGANOPHOSPHORUS AChE INHIBITORS INTO THE INTERIOR OF THE SQUID GIANT AXON*

Compound	Exposure time (min)	Penetration (% of equivalent distribution)
Tetriso	10	47 ± 30
$(C_3H_7O)_2P(O)SCH_2CH_2N(C_3H_7)_2$	60	151 ± 2
Selenophos		
C_2H_5O	10	55 ± 7
$>P(O)SeCH_2CH_2N(C_2H_5)_2$	60	110 ± 10
C_2H_5		
Isosystox	10	34 ± 1
$(C_2H_5O)_2P(O)SCH_2CH_2SC_2H_5$	60	74 ± 1

* Compounds were dissolved in sea water buffered with 10^{-3}M Tris, pH 7·8. Temp., 18–20°. All inhibitors were applied at 10^{-3}M.

TABLE 2. DETOXICATION OF ORGANOPHOSPHORUS AChE INHIBITORS*

Compound	Conc. (M)	Medium	Incubation time (hr)	Detoxication (%)
Tetriso	10^{-5}	axonal	2	14 ± 4
		aqueous	2	15 ± 8
		axonal	16	27 ± 20
		aqueous	16	29 ± 19
Selenophos	10^{-5}	axonal	2	0 ± 0
		aqueous	2	5 ± 5
		axonal	20	0 ± 0
		aqueous	20	0 ± 0
Isosystox	3×10^{-5}	axonal	2	53 ± 8
		aqueous	2	0 ± 21
		axonal	18	86 ± 1
		aqueous	18	58 ± 8

* In addition to the organophosphorus compound, the incubation mixture contained 100 mg squid axon homogenized in a final volume of 1·0 ml sea water, 10^{-3}M Tris, pH 7·8, room temp. Axonal material was omitted from aqueous controls.

inhibitory form and in view of the results of Table 2 at 10^{-5} M, it appears that, for these two compounds, detoxication is probably a negligible factor in the subsequent discussion. Isosystox, on the other hand, appears to be detoxified both enzymatically and non-enzymatically, as had been reported previously under other conditions.[26] The somewhat lower penetration of the inhibitory form of the very lipid-soluble Isosystox (Table 1) may be due to this detoxication, which was also found with

DFP,[13] or may be due to an inhibition of its penetration by the detoxication products accumulating in the axonal envelope. This would be similar to the competition for entry observed with other pairs of compounds.[27]

Table 3 shows the effects of Tetriso, Selenophos and Isosystox on the externally recorded action potential of the squid giant axon. In general, there appear to be weak effects, except at the higher concentration of Isosystox, and after venom pretreatment. Table 3 also records the effects of an Isosystox hydrolysate which caused a reversible block of conduction. Tetriso at 10^{-3} M has no apparent effect on the externally recorded action potential of the squid giant axon, although 5×10^{-3} M

TABLE 3. EFFECTS OF ORGANOPHOSPHORUS AChE INHIBITORS ON ACTION POTENTIAL OF THE SQUID GIANT AXON*

Compound	Conc. (M)	No. of expts.	Decrease of action potential (%)	Time (min)	Reversal (%)
Tetriso	10^{-3}	2	0 ± 0	60	
	5×10^{-3}	2	30 ± 0	30	0
	5×10^{-3}(V)	4	60 ± 4	10	70–100
Selenophos	10^{-3}	2	0 ± 0	60	
	10^{-3}(V)	2	25 ± 5	30	60
	5×10^{-3}	2	0 ± 0	30	
	5×10^{-3}(V)	5	60 ± 18	20–30	0
Isosystox	10^{-3}	2	0 ± 0	60	
	5×10^{-3}	4	100 ± 0	5	100
	5×10^{-3}	1	100	30†	80
	5×10^{-3}	1	100	60‡	0
Hydrolyzed Isosystox	5×10^{-3}	4	100 ± 0	10–20	100

* Compounds were dissolved in 0·001 M, Tris-buffered sea water, and the pH was readjusted to 7·8. Hydrolyzed Isosystox was prepared by allowing a solution of the compound to stand for several days at pH 11, and then readjusting the pH. Experiments in which axons were pretreated for 30 min with 20 μg of cottonmouth venom/ml of sea water are indicated by (V). Reversibility of action potential decrease checked for 20 min. Temp., 18–20°. Results are indicated as mean ± standard error.

† Isosystox left on axon for 25 min after block, which occurred in 5 min.

‡ Isosystox left on axon for 55 min after block, which occurred in 5 min.

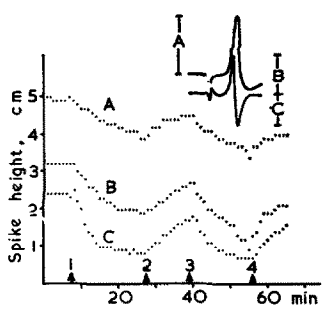

FIG. 2. Effects of 10^{-3}M Tetriso on conduction by the squid giant axon. Results were obtained by microelectrode impalement and internal recording. Inset shows typical action potential and derivative of the action potential. A, action potential spike height, 20 mV/cm; B and C, maximum rates of rise and fall, respectively, of the action potential, 200 V/sec/cm. Axon bathed in sea water; at point marked 1 above time scale, bathing commenced in 10^{-3}M Tetriso in sea water; at 2, return to sea water only; 3 and 4, same as 1 and 2.

had a definite effect. However, Fig. 2 shows that the more sensitive technique of internal recording does reveal a decrease of about 20 per cent in action potential spike height, and a decrease in the maximum rates of rise and fall of the action potential produced by 10^{-3} M Tetriso. These changes commenced immediately upon external perfusion of the axon with Tetriso and were immediately reversible upon return to normal sea water. After pretreatment of the squid axon with a low concentration of cottonmouth venom, the effects of Tetriso and Selenophos were markedly enhanced, Selenophos causing, for example, a 60 per cent irreversible decrease in the action potential (Table 3). Isosystox (5×10^{-3} M) produced, even without venom treatment, a reversible or irreversible block of conduction depending on the length of time the axon was exposed to the inhibitor.

In addition to the results presented in Table 3, it was also found that Tetriso at $2{\cdot}5 \times 10^{-3}$ M or Selenophos at 10^{-3} M had no effects in 30 min on the propagated action potential spike height of the spider crab (*Labinia emarginata*) (4 experiments) or lobster (*Homarus americanus*) meropodite nerves (4 experiments).

Attempts were made to measure axonal AChE after treatment with inhibitors

TABLE 4. RADIOCHEMICAL DETERMINATION OF ACHE ACTIVITY BY MEANS OF CHROMATOGRAPHIC SEPARATION OF SUBSTRATE AND PRODUCTS*

Fraction	Counts/min	
	Enzymatic	Nonenzymatic
1	68,833	83,291
2	5434	2059
3	324	43
4	75	6
5	6132	880
6	2331	3
7	68	0
Total, 1 through 7	83,197	86,282
Total, 5, 6 and 7	8531	883

* Calculation:

$$\frac{0{\cdot}4(8531/83{,}197) - 0{\cdot}4(883/86{,}282)}{5 \times 0{\cdot}007} = 1{\cdot}06\ \mu\text{moles}$$

acetyl-*dl*-β-methylcholine hydrolyzed/g of homogenized axon/hr. Incubation mixture contained 0·4 μmole acetyl-1-^{14}C-*dl*-β-methylcholine bromide and 7·00 mg of cleanly dissected homogenized squid axons in a final volume of 40 μl sea water buffered to pH 7·8 with 0·001 M Tris. After 5 hr, incubation at room temperature, a portion of the reaction mixture was applied to an ion-exchange column as described in Methods. For nonenzymatic hydrolysis, the enzyme source was omitted.

and to compare the results to AChE activity found in untreated axons. Typical results obtained by the radiometric determination of AChE activity are shown in Table 4. The radioactivity appearing in samples 1 to 4 is due to unhydrolyzed substrate, in this case acetyl-1-^{14}C-*dl*-β-methylcholine; that appearing in samples 5 to 7 is due to the hydrolysis product, acetate-1-^{14}C. From the sum of our results, we calculate that the squid giant axon, when free of all adhering small nerve fibers and not treated

with inhibitors, can hydrolyze 1·01 ± 0·27 (mean ± standard error; 4 experiments) μmole acetyl-*dl*-β-methylcholine per gram of homogenized fresh tissue per hour. The rate of spontaneous hydrolysis in the same units was 0·0006 ± 0·0001 (5 experiments). Although the specific stereoisomer was not available in radioactive form, the racemic compound is nevertheless a more specific substrate than is ACh for AChE.[28, 29] A series of experiments was then carried out in which giant axons containing adhering small nerve fibers were exposed for 1 hr to Selenophos or Tetriso under conditions identical to those used for electrical activity measurements. The axons were then carefully dissected free of all adhering small nerve fibers and washed for 3 hr in running sea water prior to homogenization and measurement of enzymatic activity. No hydrolysis of *dl*-acetyl-^{14}C-β-methylcholine was detectable after exposure to 10^{-3} M Selenophos (4 experiments) or 5×10^{-4} M Tetriso (1 experiment). After 10^{-6} M Tetriso, 0·395 μmole of substrate was hydrolyzed per gram of fresh tissue per hour. In order to ensure the reliability of these values, it was necessary to determine whether unreacted inhibitor might have remained entrapped in the intact axons, only to be released on subsequent homogenization. Axons were therefore bathed in 10^{-3} M Tetriso or Selenophos for 1 hr, washed in running sea water for 3 hr, and homogenized in the presence of purified AChE. By referral to standard curves of inhibition (Fig. 1), it was possible to calculate the amount of inhibitor still present in the axon at the time of homogenization. Because of the extreme potency of the inhibitors and the high concentrations applied, it could be accurately estimated that 99·995 to 99·997 per cent of the Tetriso or Selenophos was washed out of the axons. The amount of inhibitors remaining in these axons, 0·003 to 0·005 per cent of equivalent distribution, would be more than sufficient to inhibit all of the AChE in a squid axon on homogenization. The washing out of inhibitors appeared to resemble an exponential decay so that longer washing would reduce the inhibitor content only slightly, whereas there would be an increased possibility of enzyme reactivation. Attempts to measure axonal AChE after treatment with inhibitors must, therefore, be considered unsuccessful.*

DISCUSSION

The potent irreversible AChE inhibitors used in this and in a previous study[14] penetrate through the membrane and into the axoplasm of the squid giant axon in their active inhibitory form at concentrations 10^3–10^5 times those required to cause complete inhibition of AChE in solution. The rather high concentrations of Tetriso and Isosystox required to affect conduction, and the negligible effect of Selenophos, in the absence of venom pretreatment, cannot therefore be due to a generally poor penetration of the major permeability barriers or to inactivation of the inhibitors by tissue enzymes, as was previously found with other cholinesterase inhibitors.[10, 12, 13] Similarly, it had been found that the reversible AChE inhibitor, physostigmine, penetrated to about 10^{-3} M before conduction was blocked, in spite of a K_i of about 10^{-7} M.[30] The inhibitors used in the present study also did not block conduction in spider crab or lobster axons when applied at 10^{-3} M for 1 hr, even though block of conduction by ACh in the latter preparation[31, 32] may indicate

* One of us (F. C. G. H.) reported finding no AChE activity remaining in squid axons exposed to these inhibitors. These results were presented at the Marine Biological Laboratory, Woods Hole, Mass. in July 1967 before measurement (by the same author) of the levels of entrapped inhibitors which could have caused the observed inhibition.

weaker permeability barriers. At the highest concentrations which it was possible to use (because of limited solubility), Isosystox and Tetriso did have marked effects on conduction of the squid giant axon. Provided these compounds were left on the axon for a sufficient length of time, the effects were irreversible. Over 20 years ago, the effects of organophosphates on axonal conduction were shown to be either reversible or irreversible depending on concentration and duration of exposure to inhibitors.[33, 34] These reversible effects may be due either to reaction with a membrane component other than AChE, possibly with the postulated ACh receptor,[1–3] or to reactivation of inhibited enzyme by nucleophilic compounds which may be normal constituents of the tissue. With longer exposure to the inhibitors "aging" of the enzyme–inhibitor complex might make reactivation more difficult.[35]

It would be of great interest to determine the AChE activity of axons after exposure to inhibitors. All previous attempts to relate quantitatively electrical and AChE activity have been inadequate for reasons previously discussed.[15] In our present studies, several conditions appeared favorable for exploring the relationship between electrical and AChE activity: the availability of a single fiber nerve preparation; potent irreversible AChE inhibitors, which were likely to penetrate and which are not readily detoxified; and an improved radiometric technique for measuring the decrease of AChE in a preparation which normally has relatively low activity.[16] The new method for the determination of AChE appears to be an improvement over the existing radiochemical assays[36, 37] in that the determination may be made after only a small fraction of the substrate is hydrolyzed, it is not necessary to volatilize any of the reaction products, and it is possible to determine simultaneously both the unhydrolyzed substrate and the radioactive hydrolysis product. Despite these promising features, our attempts to measure AChE under the conditions described must be considered unsuccessful due to the release of extremely small amounts of entrapped inhibitor, amounts which were more than sufficient, however, to inhibit all of the AChE on homogenization.

Nevertheless, the results in this and in a previous publication[14] raise the question why, under the conditions of our studies, potent inhibitors of AChE affect electrical activity, even after venom pretreatment, only at rather high concentrations. Extreme caution must be exercised in extrapolating directly from the properties of purified enzyme in solution to those of enzymes in more highly organized states in which they are usually found in cellular membranes.[38–41] For example, charges surrounding the subunits containing the AChE system may prevent compounds from reaching and interacting with the enzyme.[38] It may be speculated that such subcellular permeability barriers prevent the organophosphorus inhibitors from reaching all of the axonal AChE in the course of their penetration into the axoplasm. It has been estimated by many investigators that only a small fraction of the axonal membrane is involved in the actual events of conduction (see e.g. reference 42); the inhibitors may have penetrated elsewhere. In support of this speculation is our finding that venom pretreatment, which reduces permeability barriers,[22, 27, 43, 44] increased the effects of cholinesterase inhibitors on conduction in this and in previous studies.[14, 45, 46] Other studies have also shown that the concentration of a cholinesterase inhibitor required to affect conduction may be drastically modified by permeability barriers.[47] In those studies, physostigmine was about a thousand times as potent on a single node of Ranvier from the sciatic nerve as it was on the entire sciatic nerve bundle. The evidence

accumulated in favor of a biochemical theory of nerve conduction, based in part on the essentiality of AChE,[1-3] is also compatible with the speculated existence of uninhibited membranal AChE after exposure to inhibitors. According to this theory, ACh, after its interaction with a membranal receptor, thereby leading to the generation of an action potential, would have to be rapidly removed in order to allow another impulse to travel along the axon. AChE has the appropriate localization and properties required for the rapid hydrolysis of axonal ACh.[1-9] It has also been shown that block of conduction by anticholinesterases can often be reversed by a specific reactivator of AChE.[45, 48] On the other hand, within the framework of the biochemical theory of nerve conduction it may be speculated that, after cholinesterase inhibition, endogenous ACh could be rapidly removed after its interaction with the receptor by some means other than, or in addition to, enzymatic hydrolysis—perhaps rapid diffusion over a short distance or binding to other proteins or subcellular components near the receptor.

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