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Penetration of an Organophosphorous Compound into Squid Axon and Its Effects on Metabolism and Function

Abstract. *The tertiary analogue of phospholine, namely,* $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, *is a potent, irreversible inhibitor of cholinesterase which, when externally applied to the squid giant axon, readily penetrates in its inhibitory form into the axoplasm. However, even a* 10^{-2} *molar solution of this compound does not block axonal conduction unless the axon is first treated with a low concentration of venom from the cottonmouth moccasin. The question of the activity of acetylcholinesterase in these axons is considered, and the possibility of subcellular permeability barriers for individual components of the excitable membrane is discussed.*

The finding (*1*) of a high activity of an enzyme [phosphorylphosphatase (*2*)] that hydrolyzes diisopropylphosphorofluoridate (DFP) in parts of the squid giant axon has provided an explanation for the high external concentration of this compound required to block conduction. We have now used the dimethylaminoethylthio ester of diethylphosphoric acid, a powerful irreversible inhibitor of cholinesterase capable of crossing permeability barriers (*3*) and, in contrast to DFP, not subject to rapid enzymatic hydrolysis and detoxication. Although this and similar compounds are probably detoxified by microsomal enzymes (*4*), the rates of the detoxication are vastly slower than those for the enzymatic hydrolysis of DFP, sarin, tabun, and others of this acid anhydride group (*5*).

Dissection of the giant axon of the squid *Loligo pealii*, external recording of electrical activity, extrusion of axoplasm, and related techniques have been described previously (*6*). In deference to previous publications (*3*), we will refer to $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, the tertiary analogue of phospholine, as 217AO (acid oxalate). The 217AO was dissolved in water; an amount, equivalent to that of 217AO, of calcium chloride in solution was added, and the *p*H was adjusted to about 7.5 with sodium hydroxide. The calcium oxalate was removed by centrifugation, and the supernatant was used after it was diluted to the desired concentration, tonicity, and buffer composition. For the determination of penetration, axoplasm was extruded from squid axons that had been bathed in a buffered sea-water solution of 217AO. This axoplasm, suitably diluted, was mixed with a sample of acetylcholinesterase (AChE) of known activity, and after 30 minutes the remaining AChE activity was measured manometrically. By comparison with a curve of the inhibition of AChE plotted as a function of known concentrations of 217AO, arithmetical corrections having been made for dilutions, the 217AQ concentration in the axoplasm, and hence its penetration, was determined. Using AChE from *Electrophorus electricus*, we estimated the pI_{50} of 217AO (negative log of the concentration of 217AO needed for 50 percent inhibition of AChE) to be 7.9. [The reported value for erythrocyte AChE is 7.9 (*7*).]

Table 1 shows that 217AO penetrates readily into the interior of the squid giant axon and even exceeds equivalent distribution in 1 to 2 hours. The methods and the calculations involved are exactly comparable to those reported previously, except that the ability to inhibit AChE was measured instead of C^{14} (*6*). Nachmansohn and associates (*8*) and Brzin *et al.* (*9*) examined the penetration of physostigmine, DFP, and paraoxon into the squid giant axon. However, paraoxon, which appeared to penetrate only a few percent, was probably mostly detoxified (*10*); the DFP was most certainly detoxified (*1*). Physostigmine, which obviously penetrated, is, like 217AO, rather poorly soluble in lipids. In addition, the inhibition of AChE caused by physostigmine is reversed fairly rapidly on removal of the inhibitor. We have attempted to avoid some of these difficulties by using 217AO, an irreversible and not readily detoxified inhibitor.

Table 2 shows the effects of 217AO on the electrical activity of the squid giant axon. In addition, although the effect of venom on the penetration of 217AO was not a part of this investigation, we did study the enhanced effects of 217AO on the action potential of squid axon after the axon had been treated with a concentration of cottonmouth moccasin venom (*Agkistrodon p. piscivorus*) which, of itself, had little or no effect on the action potential (*6*) (Table 2).

Inasmuch as Table 1 shows the penetration of 217AO in its active, inhibitory form, it may be concluded that detoxication is an insignificant factor in these experiments. However, the studies of penetration were performed with $10^{-3}M$ 217AO. Lest an enzyme system for detoxication be saturated at a far lower concentration and the percentage of detoxication, therefore, appear unusually small, we determined

Table 1. Penetration of $10^{-3}M$ 217AO, $(C_2H_5O)_2P(O)SCH_2CH_2N(CH_3)_2$, from a buffered sea-water medium into the axoplasm of squid giant axon. Temperature, 18°C; *p*H 7.8; results are shown as mean ± standard error.

Exposure time (min)	Experiments (No.)	Penetration (%)
5	2	49 ± 12
60	6	133 ± 21
120	4	147 ± 7

Table 2. Effect of 217AO on electrical activity of squid axons with and without previous treatment with venom. Those treated were incubated for 30 minutes in sea water containing 15 μg of cottonmouth venom per milliliter before exposure to 217AO. Results are shown as mean ± standard error.

Concentration of 217AO (mole/liter)	Experiments (No.)	Decrease of action potential (%)	Exposure time (min)
No previous treatment			
10^{-3}	8	6 ± 3	60
10^{-2}	4	15 ± 9	60
Previous treatment with venom			
10^{-3}	3	35 ± 8	30
10^{-2}	4	100 ± 0	5–30

Table 3. Detoxication of 217AO in the presence and absence of squid axon homogenate. Incubation mixture contained 90 to 100 mg of axonal material per milliliter and 10^{-5} mole of 217AO per liter at *p*H 7 to 7.5, room temperature, and aerobic conditions; the incubation time was 24 hours. Results are shown as mean ± standard error of the results of the four experiments in each group.

Concentration of 217AO after 24-hr incubation (mole/liter)	Detoxication (%)
In presence of axonal homogenate	
1.02×10^{-5}	20 ± 7
0.74×10^{-5}	
0.68×10^{-5}	
0.74×10^{-5}	
No axonal material; aqueous medium only	
0.95×10^{-5}	10 ± 5
1.02×10^{-5}	
0.81×10^{-5}	
0.83×10^{-5}	

the possible enzymatic detoxication of 217AO by incubating the agent ($10^{-5}M$) with a homogenate of whole squid axon for 24 hours. The results indicate that in the 1 to 2 hour period of the penetration and electrical studies, when 10^{-2} to $10^{-3}M$ 217AO and single axons (15 to 20 mg) were used, the detoxication of 217AO either enzymatically or nonenzymatically was negligible (Table 3).

These results appear to indicate that the propagation of an action potential is not blocked in the presence of an inhibitor of AChE capable of crossing permeability barriers. The fact that the inhibitor is applied externally and is found internally in its active form at a concentration some 10^4 times greater than that required to produce 100 percent inhibition of a solution of AChE may seem to raise the question of the essentiality of AChE for the permeability changes associated with electrical activity (*11*). Similarly, at the time at which conduction was blocked, concentrations of physostigmine were found in the axoplasm of the squid giant axon which were about 10^3 times greater than those required to inhibit AChE in solution (*8*). Even more relevant is the question of the amount of uninhibited AChE in the axon under these conditions. Attempts to determine AChE activity in squid axons after their treatment with high concentrations of 217AO are made difficult by the small fractions remaining to be measured of an initially low amount of enzyme activity, by the low substrate penetration if intact material is used, and by the release of small amounts of entrapped inhibitor if homogenization is attempted. Recent work (*12*) indicates that treatment with detergent or venom may be combined with the use of acetylthiocholine and electron microscopy, although even in a thin frozen section 1000 Å thick, or less, AChE does not react with the substrate except after treatment of the section with detergent. Whether or not the question is now answerable, AChE does seem to be intimately associated with the conducting membranes of many excitable tissues (*12, 13*). Furthermore, there is biochemical evidence that AChE is essential for bioelectric activity (*8, 11, 14*). The organization of the conducting membranes may be as complex as that of other membranes, such as those of mitochondria (*15*). Individual permeability barriers may exist for these subcellular organized elements. There is evidence that only a fraction of the area of the axonal membrane is involved in the actual events of conduction (*16*); the 217AO may have penetrated elsewhere. On the basis of recent studies (*17*) one may suppose that charges surrounding the subunits containing the AChE system prevent compounds from reaching and reacting with the enzyme. In addition, the rather low solubility of 217AO in lipids (oil-water partition coefficient ≈ 0.1) (*3*), while obviously not preventing the penetration of this compound into the axon, may prevent its reaching all parts of the membrane components. Thus, in axons first treated with venom (Table 2), the 217AO reaches the subcellular apparatus with the resultant block of conduction.

The ability of 217AO to penetrate into the interior of an axon without blocking electrical activity appears at present to be an unexplained observation. The use of homologs of 217AO may prove to be a new and interesting approach to a chemical exploration of membrane substructure.

F. C. G. HOSKIN
PHILIP ROSENBERG
Departments of Biochemistry and *Neurology, College of Physicians and Surgeons, Columbia University, New York*

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Synaptic Vesicles of Inhibitory and Excitatory Terminals in the Cerebellum

Abstract. *Populations of synaptic vesicles within cerebellar terminals considered excitatory or inhibitory on the basis of physiological evidence differ with respect to size and shape. Size rather than shape appears to be the main morphological difference between these populations. Elongation of vesicles is dependent on fixation with aldehyde fixatives, and both size and elongation change with age mainly during maturation.*

Several investigators (*1*) have reported nerve terminals containing elongated synaptic vesicles in nervous tissue fixed in aldehydes. Uchizono (*2*) has suggested that excitatory and inhibitory terminals contain synaptic vesicles that differ in shape. A similar suggestion has been made recently by Bodian (*3*). We have analyzed the shape and size of vesicles within terminals of basket, Golgi, and Purkinje cells