Toxicity of DFP and Related Compounds to Squids in Relation to Cholinesterase Inhibition and Detoxifying Enzyme Levels

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The metabolism and detoxication of organophosphorus anticholinesterase agents, first reported in 1946 (12), was reviewed in 1963 (13). The practical implications of the research then and since are often related to the search for pesticides which are moderately unstable under the normal influence of soil and weather, and which are detoxified more readily by man and other animals of value than by pests. However, there has often been a lack of fundamental interest in the enzymes involved, which sometimes appear to have no known natural substrate or physiological role or which appear likely to be some other already characterized enzyme.

On the other hand the relation of these agents to AChE and of this latter enzyme to nerve function is well understood although often subject to different interpretations. In an attempt to explore the question of the possible involvement of AChE in nerve conduction, the organophosphate, DFP, was applied to single giant axons of the squid, Loligo pealei (2, 5). The results, while not answering the original question, indicated the presence of an enzyme in squid nerve capable of hydrolyzing DFP (5). Since then this work has been continued (9, 10), leading to the general conclusion that there is a single identifiable enzyme which is termed "DFPase", that this particular DFPase is restricted to the cephalopods and, indeed, to their nerves, and that the concomitant occurrence of this DFPase and isethionate ($HOCH_2CH_2SO_3^-$), the major anion of cephalopod nerve, suggests a natural substrate and physiological function for the squid nerve type DFPase (10).

While many organisms have been found to contain organophosphate-metabolizing enzymes (13), the presence of a specific pesticide-destroying or nerve-gas-destroying enzyme in the

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Abbreviations and trivial names: ACh, AChE, ChE, ChAc, acetylcholine, acetylcholinesterase, cholinesterase, choline-acetylase (choline acetyltransferase); DFP, diisopropylphosphoroflouridate; 217A0, O,O-diethyl S-(2-dimethylaminoethyl) phosphorothioate; Paraoxon, diethyl p-nitrophenylphosphate

Bulletin of Environmental Contamination & Toxicology, Vol. 13, No. 2 ©1975 by Springer-Verlag New York Inc.

nerve of a single class, namely the cephalopods, has suggested to us a rather simple correlation between the detoxication of a given agent and its toxicity to the whole animal. Furthermore, the problem of the disposal of stockpiles of the so-called nerve gases in the ocean, the immense cephalopod population of the oceans, and its utilization as a food both in marine food chains and for human consumption, suggest that information concerning the toxicity of anticholinesterase agents to squid may be of both fundamental and practical importance.

MATERIALS AND METHODS

Squids (Loligo pealei) were collected at the Marine Biological Laboratory, Woods Hole, Massachusetts. They were of either sex, 15-20 cm in length, and 50-100 g in weight. The animals were used within 24 hrs after removal from the ocean, often within 4 hrs. They were held in a large concrete aquarium tank, were removed as needed, and wrapped gently in a sea-water-wetted paper towel. A flap of the paper towel in the vicinity of the eye was torn away and the squid was administered the compound being tested by hypodermic injection into the optic orbit using a 25-27 gauge needle and a volume of 0.05 to 0.3 ml. Each squid so injected was then floated free of the paper towel into a smaller individual aquarium tank. All tanks were supplied with running sea-water.

Because of the special nature of squids some steps were taken which would not generally apply to small laboratory animals such as mice. Squids are delicate animals which survive poorly in captivity, seldom lasting more than 48 hrs. Also, the signs of anticholinesterase poisoning usually occur precipitately, soon after injection, and terminate in death or recovery quite rapidly, differing of course from species to species and compound to compound, but in our experience often permitting a conclusion as to outcome within an hour after injection. Therefore, final observations were made 5 hrs after injection. Also, because of the manner in which squids suck in and eject water as well as because of their delicate nature it would be difficult to weigh them before injection. This was done after the experiment either on well-drained animals which had not survived, or survivors which were drained to a comparable degree.

Compounds were obtained from commercial sources, except for Paraoxon which was obtained from Dr. Robert A. Neal of Vanderbilt University, Nashville, Tennessee. Compound 217A0 was freed of its oxalate as described previously (6). All compounds were dissolved in artificial sea-water for injection.

To determine whether physostigmine is detoxified or similarly metabolized by squid nerve, experiments were performed

exactly analogous to those performed for other anticholinesterases (6, 7). In brief, physostigmine was incubated for 2-5 hrs in a buffered homogenate of squid nerve (axons, ganglia) with concentrations of physostigmine and tissue adjusted so that, had DFP been the agent so treated, it would virtually all have been hydrolyzed within a few minutes. At the end of the incubation period, aliquots of the physostigmine-tissue homogenate were tested for their ability to inhibit a test solution of commercially obtained AChE and were compared to physostigmine solutions which had been incubated with suspensions of boiled tissue to allow for possible non-enzymatic decomposition and non-specific absorption.

ChE activity was determined colorimetrically (3). Squids were decapitated 30 min after injection of the inhibitor, and the optic ganglia from the side opposite the injection were homogenized in a motor driven homogenizer surrounded with ice. A typical incubation contained, in final concentration, ACh at 5×10^{-3} M, and tissue at 20 mg per ml. The aqueous medium used throughout was sea-water containing Tris at 0.1 M, pH 7.6. The final incubation volume was 5.0 ml. The reaction flask was gently agitated at room temperature and aliquots were taken for the actual assay (3) at hourly intervals for 5 hrs. Control optic ganglia were taken from squids injected with a corresponding volume of buffered sea-water.

RESULTS

Squids injected with inhibitor often became extremely active, displayed a rapidly pulsating change in pigmentation, discharged ink, and sometimes made leaps of a meter or more (the tanks were thereafter covered to prevent leaps out onto the laboratory floor). Squids receiving doses in the lower end of the toxic range would usually remain highly pigmented, and would sometimes attach themselves to the aquarium wall or floor and "walk" on their tentacles. After a period of agitation (10-20 min) they would quite suddenly return to normal swimming and color-altering behavior, but remain rather more pigmented thereafter. At the higher doses, those animals due to recover would attach themselves firmly to the aquarium wall or floor, often turn white, and then after a period of 10-30 min quite suddenly regain color, regain fin motion, detach themselves, swim violently and in a disoriented manner for perhaps a minute, and thereafter return to a normal behavior but again remain rather more pigmented than normal. Animals which would eventually die would behave similarly except that they would remain white, slowly detach from the tank side or bottom and after about 30 min float away. At the highest doses the signs occur more quickly and often did not allow for a period of violent action. The difference in

TABLE 1

Toxicity of ChE Inhibition to Squids in Relation to Remaining ChE Activity

Inhibitor	Number dead / Number Injected	Dosage mg/kg	ChE Activity In Homogenized Optic Ganglia %
Control	0/10	---	100
217A0	0/6	1.0	30.8
	3/5	2.0	12.3
	4/4	3.0	8.2
	4/4	4.0	4.9
Paraoxon	0/5	0.5	60.7
	3/8	1.0	36.0
	4/4	1.5	22.0
	4/4	2.0	14.2
DFP	0/6	20.0	53.9
	0/7	30.0	39.8
	3/6	45.0	----
	1/6	60.0	24.9
	2/3	75.0	7.3
Physostigmine	0/2	2.2	----
	0/2	3.6	----
	0/2	4.7	----
	2/2	6.3	----
	3/3	11.0	----

toxic signs elicited by DFP, Paraoxon, 217A0, and physostigmine were not clear cut. Although the animals appeared to react more violently to DFP, and less violently in the order shown above, the individual variations were nearly as large as the compound-to-compound variations. Squids injected with sea-water (controls) would also often show increased pigmentation, and sometimes discharge ink, but this behavior is sometimes seen in squids which are merely handled.

The toxicity of DFP, of 217A0, of Paraoxon, and of physostigmine with respect to squids is shown in Table 1. The inhibition of squid optic ganglia ChE as an indication of the toxic action of these compounds is also shown in Table 1. On the average a lethal dose of DFP or of 217A0 caused a 90% or greater reduction in the enzyme activity, and a lethal dose of Paraoxon caused a 75% reduction, compared to untreated animals. It should be noted however, (as will be emphasized shortly) that a lethal dose of DFP is some 30 times larger (μmoles/kg) than that of either 217A0 or Paraoxon. ChE determinations were not made on physostigmine-treated squids because of the reversibility of this inhibition.

The possibility of physostigmine detoxication by squid nerve was examined with the results shown in Table 2. The rate of detoxication of physostigmine estimated from the results, about 3 μmoles per g squid head ganglion per hr, is less than 1% of that for DFP (8) incubated under comparable conditions and is, therefore, similar to the low or negligible rates already reported for squid nerve with respect to 217A0 and Paraoxon (6, 10).

DISCUSSION

The approximate LD_{50}'s of DFP, of 217A0, of Paraoxon, and of physostigmine on injection into squids are summarized in Table 3, as well as comparable data so far as possible, from the literature.

There are fundamental reasons why it is difficult to compare the AChE-inhibiting properties of the organophosphates with those of a carbamate such as physostigmine. For the former group, a rate constant is the appropriate term, whereas for physostigmine and the other members of this group, a concentration producing a constant (steady state) level of inhibition is more appropriate. Nevertheless, for relatively pure AChE and for reasonable inhibition times, of the order of 30 min, some appreciation of the enzyme inhibitor potency can be gained from a consideration of the I_{50}'s, i.e., the inhibitor concentrations causing 50% inhibition of a given preparation of AChE. These are, for DFP, 217A0, Paraoxon, and physostigmine, approximately 10^{-6} M, 10^{-8} M, 10^{-7} M, and 10^{-7} M, respectively. However, no account has been taken of

TABLE 2. Physostigmine Detoxication by Homogenized Squid Optic Ganglia

Concentration of tissue, mg/ml	Initial Concentration of Physostigmine, M	Incubation time, hrs	Final Concentration of Physostigmine, M	Rate of Detoxication, μmoles/g/hr
50	10^{-3}	2	6.7×10^{-4}	3.3
50	10^{-3}	5	3.3×10^{-4}	2.7

TABLE 3. Summary of Toxicities of Four Anticholinesterases to Squid

Compound	Approximate LD_{50} mg/kg		Approximate LD_{50} μmoles/kg	
	Squids	Mice	Squids	Mice
DFP	>60	4 (4)	>325	21.7
217A0	1-2	0.4 (4)	3-6	1.2
Paraoxon	1	0.6-0.8 (4)	3.6	2.2-2.9
Physostigmine*	5	0.5-1.2 (1)	15.4	1.6-3.7

*Molecular weight = physostigmine·$\frac{1}{2}H_2SO_4$

species and organ specificities, as, for example, between bovine erythrocytes, Electrophorus electric tissue, etc. Furthermore, while it appears that 217A0 is not enzymatically detoxified at rates comparable to those of DFP detoxication in either squid or mammals, Paraoxon is detoxified by mammalian enzymes (13) but not by the squid (10). Nevertheless, 217A0 and Paraoxon are about equally toxic to squid or mouse, even when allowance is made for the difference in molecular weight. DFP is about a tenth as toxic to mice as either 217A0 or Paraoxon, probably reflecting both the mammalian DFPase activity and the intrinsically poorer AChE-inhibiting properties of DFP. The most marked deviation from the other data is the very low toxicity of DFP toward squids, about 65 mg/kg (350 μmoles/kg) or perhaps even somewhat higher. These results are compatible with the lower AChE inhibiting power of DFP, and the higher level of squid-nerve-type DFPase found in these animals (9). Assuming that virtually all of the injected DFP results in the release of flouride, whether by the phosphorylation and inhibition of enzyme or by enzymatic hydrolysis, from the dosage level of about 50 mg DFP per kg squid (272 μmoles/kg) upward, the toxicity of flouride itself will play an increasing role, judging from its toxicity to a variety of species.

While the results confirm the difficulties in trying to relate whole-animal toxicities to in vitro observations on the kinetic properties of enzymes, one conclusion seems clear in the present instance. DFP is more than an order of magnitude less toxic to squids than to mammals, and less toxic to squids than are 217A0, Paraoxon, or physostigmine. This appears to be a reflection of the high level of DFPase in the squid nervous system. From the special nature of squid-nerve-type DFPase as contrasted to mammalian type DFPase (9, 10), it may be speculated that the nerve gases Tabun, Sarin, and Soman (14) would be somewhat more toxic to squids than was DFP, but the extreme hazards involved in handling these compounds in a manner compatible with LD_{50} determinations was a factor in our not performing these experiments.

So far as the role of ChE in squid nerve function is concerned two aspects have received considerable attention. The view of a general role for the ACh system in nerve conduction (14) remains as unresolved as ever despite the application of these potent AChE inhibitors to the squid giant axon (15). With regard to synaptic transmission, although the transmitter agent at some synapses remains unknown (11), it has been amply shown that squid ganglia contain large quantities of ACh, ChAc, and AChE (16, 17). From the signs of toxicity which we have observed in the present experiments it appears that there are cholinergic synapses in the squid, probably both in the ganglia and at peripheral sites.

ACKNOWLEDGEMENTS

This research was supported by grants ES-00619 and NS-09090 from the U.S. Public Health Service.

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