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## Diisopropylphosphorofluoridate and Tabun: Enzymatic Hydrolysis and Nerve Function

Abstract. *Squid nerve contains an enzyme that hydrolyzes the nerve gas Tabun at about one-tenth the rate it hydrolyzes diisopropylphosphorofluoridate (DFP), and at about one-third to one-fourth the rate it hydrolyzes Sarin and Soman. Tabun is a more potent inhibitor of acetylcholinesterase than is DFP, is both lipid- and water-soluble, and penetrates readily into the squid giant axon in its inhibitory form. The failure of Tabun to block or markedly decrease the conducted action potential in the squid axon makes it likely that the blocking of conduction caused by DFP is probably not due to inhibition of acetylcholinesterase. Substrate specificity with regard to organophosphate metabolism by squid enzyme has possible implications for the disposal and detoxication of nerve gases in the ocean.*

The use of potent, relatively specific, and essentially irreversible inhibitors of acetylcholinesterase for the study of nerve function is subject to several ambiguities (*1*). For example, some inhibitors, while capable of penetrating into axons in their inhibitory form (*2*) have oil-water partition coefficients of about 0.1 (*3*). It has been suggested (*4*) that such compounds may be incapable of penetrating into all of the substructure of excitable membranes, thereby adding to the uncertainty in assigning reasons for unexpected (especially lack of) pharmacological responses. Some inhibitors are enzymatically detoxified. Thus the finding (*5*) of a high level of diisopropylphosphorofluoridate-hydrolyzing enzyme in the giant axon of the squid *Loligo pealii* has been considered as a possible explanation for the high concentration of DFP required to block conduction relative to the concentration which inhibits a solution of acetylcholinesterase. The squid head ganglion is an even richer source of the DFP-hydrolyzing enzyme than the giant axon (*6*) and is easier to obtain in quantity. During the collection of ganglia in preparation for a purification of the DFP-hydrolyzing enzyme, experiments have been performed which bear on enzyme specificity, on nerve function, and, to a more limited extent, on a matter of recent public concern, namely, the disposal of toxic compounds in the ocean.

In view of the more rapid hydrolysis of the nerve gas (so-called) ethyl *N,N*-dimethylphosphoramidocyanidate (Tabun; GA) than of DFP by various mammalian serums (*7*) and of similar data published for isopropyl methylphosphonofluoridate (Sarin; GB) and for 3,3-dimethyl-2-butyl methylphosphonofluoridate (Soman; GD), these compounds were tested as possible alternate substrates for the DFP-hydrolyzing enzyme. The DFP was obtained commercially. Tabun was synthesized according to Holmstedt (*8*); Sarin and Soman, essentially according to Reesor *et al.* (*9*). Partition of Tabun and DFP between olive oil and water was determined by measuring the inhibitor concentrations in the aqueous phase, with a standard solution of *Electrophorus* acetylcholinesterase as the test agent. Penetration of Tabun into squid giant axons was measured as described for other compounds (*2*). Enzymatic hydrolysis of the organophosphorus agents and of acetylcholine was measured titrimetrically or manometrically, the choice usually depending on considerations of sensitivity and safety. Corrections for nonenzymatic hydrolysis were made in the usual way. Dissection of axons, external recording of electrical activity, and extrusion of axoplasm were performed as previously described (*2*).

Table 1 shows that squid nerve, and especially head ganglion, hydrolyzes DFP about nine times more rapidly than it hydrolyzes Tabun, whereas there is a sixfold difference in the opposite direction for rat serum. This latter order has also been reported for rabbit plasma and a fraction of human serum (*7*). Another difference has already been noted between squid nerve DFP-hydrolyzing enzyme and that from many other sources, namely, a lack of $Mn^{2+}$ stimulation in the former (*6*). The Michaelis constant ($K_m$) for DFP hydrolysis by squid head ganglion is estimated at $6.25 \times 10^{-3} M$, with a maximum velocity of approximately 1280 $\mu$mole of DFP hydrolyzed per gram of fresh tissue per hour. This should be regarded as an apparent $K_m$ inasmuch as a crude homogenate is, so far, the enzyme source. Figure 1 shows the relative rates of hydrolysis of DFP, of Tabun, and of mixtures of the two by identical amounts of head ganglion enzyme. The enzymatic hydrolysis of the mixture in terms of $CO_2$ released from bicarbonate buffer is equal, within the limits of the standard deviations, to the mean for the separate hydrolyses. This suggests that squid nerve contains a single enzyme that

Table 1. Enzymatic hydrolysis of DFP and Tabun. Values obtained from squid and rat were determined manometrically in 3.0 ml of a solution of the following molar concentrations: NaCl, 0.16; KCl, 0.005; $NaHCO_3$, 0.0165. Gas phase: 95 percent $N_2$, 5 percent $CO_2$; temperature, 30°C. Nerve was homogenized in this medium to provide 10 to 18 mg of tissue per Warburg vessel or 0.1 to 0.2 ml of rat serum per vessel. Substrate concentration, 0.01*M*. Other values were determined under similar conditions. Results are expressed as micromoles of substrate hydrolyzed per hour per gram of nerve or milliliter of serum ± standard deviation.

| Enzyme source | DFP | Tabun |
|---|---|---|
| Squid axon | 80–120 | < 10 |
| Squid stellate ganglion | 394 ± 4 | |
| Squid head ganglion | 750 ± 9 | 83 ± 18 |
| Rat serum | 12 ± 1 | 71 ± 6 |
| Rabbit plasma* | 80 | 281 |
| Human serum, fraction IV* | 80† | 205‡ |

* See (*7*). † Arbitrary. ‡ Relative to DFP = 80.

Table 2. Enzymatic hydrolysis of Sarin and Soman relative to DFP. Enzyme was from homogenized squid head ganglion. Other conditions and expression of results as in Table 1.

| Substrate | Hydrolysis rate |
|---|---|
| DFP | 750 ± 9 |
| Sarin | 256 ± 18 |
| Soman | 313 ± 44 |
| DFP plus Sarin | 830 ± 17 |
| DFP plus Soman | 909 ± 45 |

hydrolyzes DFP, and that an analogous compound, Tabun, while competing for the same site on this enzyme, is hydrolyzed much more slowly. Table 2 gives the rates of hydrolysis of Sarin and Soman relative to that of DFP by squid head ganglion. Unlike Tabun, here the immediate hydrolysis products are all moderately strong acids, thus permitting the expression of the mixed substrate hydrolysis rates in molar terms. However, since the hydrolysis rates of the Sarin-DFP and Soman-DFP mixtures are between the means and the sums of the separate rates, a choice between a single enzyme hydrolyzing several substrates and several enzymes with different or overlapping specificities is not possible from these data, but must await further enzyme purification. Squid head ganglion also hydrolyzes *p*-nitrophenyl acetate, but this reaction is 96 to 97 percent inhibited by a low concentration of DFP (*6*). It may be speculated that squid nerve contains enzymes corresponding to A- and B-esterase (*10*), but considering the unique phylogenetic source of this enzyme, relative to earlier reports, it is probable that neither previously attempted purifications nor previous speculation about the natural substrate for the DFP-hydrolyzing enzyme can be applied to the squid nerve enzyme (*11*).

The penetration of Tabun into squid giant axons was determined after a 1-hour exposure to a $10^{-3}M$ solution of the agent in artificial seawater made $0.001M$ in tris(hydroxymethyl)aminomethane, *p*H 7.4, at 20° to 22°C. In four experiments, the concentration inside the axons, that is, in the extruded axoplasm, was 55 percent of the external concentration with a standard deviation of 17 percent. The olive oil–water partition coefficient for Tabun was 2.6. The corresponding value found in this laboratory for DFP was 11.5; the reported (*12*) value for DFP is 9.5. In the course of these determinations, the bimolecular rate constants for the inhibition of *Electrophorus* acetylcholinesterase by Tabun and DFP are $1.3 \times 10^5$ and $2.5 \times 10^4$ liters mole$^{-1}$ min$^{-1}$, respectively; reported value (*13*) for DFP is $1.9 \times 10^4$. The Tabun synthesized in this laboratory was found to have a mouse intraperitoneal toxicity [$LD_{50}$ (lethal dose, 50 percent effective)] of 0.65 mg/kg, in close agreement with the reported value of 0.6 mg/kg (*8*). Tabun and DFP inhibit squid axon acetylcholinesterase at rates approximately comparable to those found with *Electrophorus* acetylcholinesterase, although the actual values are considerably more uncertain because of the small amount of tissue available for the determinations and the low level of acetylcholinesterase normally present (*14*). These results indicate that Tabun is both water and lipid soluble, that it penetrates into squid axons in its acetylcholinesterase-inhibiting form, and that, unlike DFP and in agreement with data presented in Table 1 and Fig. 1, it is only slowly detoxified by squid nerve enzyme. Table 2 indicates that Sarin and Soman are not hydrolyzed rapidly enough to make them more suitable substrates than DFP, but too rapidly to make them useful agents for the study of squid nerve function.

The effects of DFP and of Tabun on the externally recorded action potentials of squid giant axons are given in Table 3. Because of the external recording, the observed action potentials are diphasic. Before treatment the average voltage change of all axons from peak to trough was 52 ± 15 mv at a stimulus strength of 30 mv and a duration of 0.1 msec. On addition of compounds, no changes were observed other than those noted in Table 3. The results obtained with DFP are essentially those summarized previously (*5*). By comparison Tabun appears to be characterized by its lack of effects on conduction.

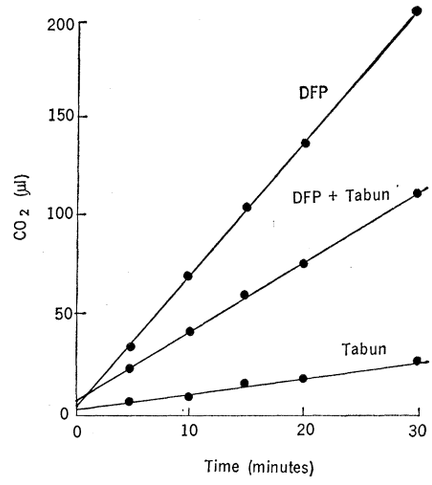


Fig. 1. Hydrolysis of DFP and Tabun by homogenized squid head ganglion at 30°C and *p*H 7.4. The concentration of each compound is $0.01M$.

These results indicate that the blocking of conduction obtained with some acetylcholinesterase inhibitors, notably DFP at concentrations greater than $10^{-3}$ mole/liter, may be due to the formation of acidity in the axon through the action of the DFP-hydrolyzing enzyme, as has been suggested (*15*). For other nerves which appear to have less DFP-hydrolyzing enzyme (*16*) per gram of tissue, there may nevertheless be sufficient acidity produced in localized regions to affect and eventually block conduction. It has also recently been reported that DFP has detergent properties (*17*). When Tabun is applied to squid axons, the substrate specificity of squid DFP-hydrolyzing enzyme and the nature of the products of Tabun hydrolysis would result in an accumulation of acidity at a rate only about 5 percent of that encountered with DFP. Thus, blocking of conduction in squid axon due to Tabun might be expected at concentrations greater than $10^{-2}$ mole/liter. It may

Table 3. Effects of DFP and Tabun on externally recorded action potentials of squid giant axon. Bathing medium, artificial seawater buffered to *p*H 7.4 with $10^{-3}M$ tris.

| Compound | Concentration ($M$) | Time (min) | Axons (No.) | Response |
|---|---|---|---|---|
| DFP | $5 \times 10^{-4}$ | 120 | 1 | No effect |
| | $10^{-3}$ | 113 | 1 | Blocked |
| | $5 \times 10^{-3}$ | 5–25 | 4 | Blocked |
| | $10^{-2}$ | 11 | 1 | Blocked |
| Tabun | $10^{-3}$ | 60–120 | 3 | No effect |
| | $5 \times 10^{-3}$ | 60 | 2 | No effect |
| | $7 \times 10^{-3}$ | 100 | 1 | 30 percent spike height decrease; tenfold threshold increase |
| | $10^{-2}$ | 60 | 2 | 15 percent spike height decrease; tenfold threshold increase |

be supposed that these concentrations of DFP or Tabun are in excess, by orders of magnitude, of what would have caused complete inhibition of axonal acetylcholinesterase. However, the difficulties in attempting to measure unambiguously the degree of inhibition of acetylcholinesterase in intact tissue are considerable and have frequently been reviewed (*1*, *4*, *16*).

Neither a role for DFP-hydrolyzing enzyme in nerve function, if indeed any exists, nor a natural substrate for this enzyme can be inferred from these results. In a more immediately practical context, it seems reassuring that toxic organophosphorus compounds such as DFP and related nerve gases and insecticides may be detoxified by enzymes in the squid and possibly in other inhabitants of the ocean. Such reassurance must be tempered by the marked substrate specificity described here and indicated previously for other classes of organophosphates (*2*).

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## Salicylate: Effect on Membrane Permeability of Molluscan Neurons

Abstract. *Identified cells in the buccal ganglion of the marine mollusk* Navanax inermis *were exposed to salicylate (1 to 30 millimoles per liter) for short periods. Salicylate increased the permeability to potassium and decreased the permeability to chloride in a reversible, dose-dependent manner, producing a concomitant increase in membrane potential and a decrease in membrane resistance. These events would reduce the output from, as well as the effectiveness of synaptic input to, a particular neuron.*

Although salicylates are used daily as analgesics and antipyretics, little research has been reported on their effect on single neurons (*1*). We report here the results of experiments on the effect of salicylates at the membrane of molluscan neurons.

The buccal ganglion from the marine mollusk *Navanax inermis* (*2*) was isolated, mounted in a 1-ml Lucite chamber and perfused with normal physiological saline (*3*) at room temperature (22° to 24°C). After opening the capsule enveloping the ganglion, we impaled identified cells (*4*) with double-barreled micropipettes filled with 3*M* KCl (2 to 10 megohm resistance). One barrel was used to record membrane potential while the other allowed passage of current across the membrane.

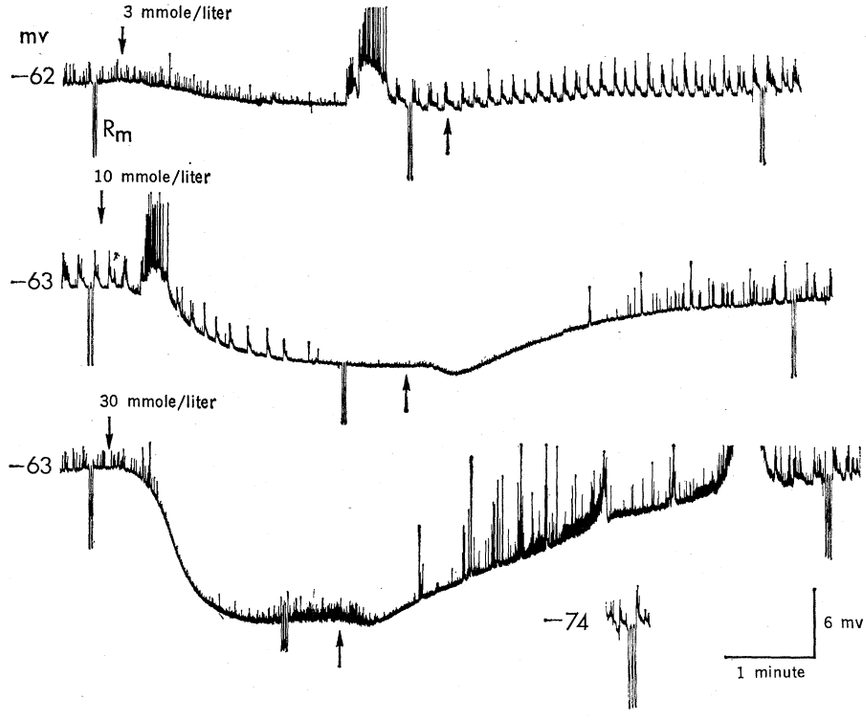


Fig. 1. Salicylate increases transmembrane potential and decreases membrane resistance. The results illustrated were obtained from cell M-R and are representative of all cells studied. The input resistance of the cell was obtained in normal physiological saline at a resting membrane potenital of −62 mv by passage of 4-na hyperpolarizing pulses 800 msec long ($R_m$) through a second intracellular electrode. Perfusion of the 1-ml chamber with 50 ml of physiological saline containing 3 m*M* sodium salicylate (downward arrow) caused a 2-mv hyperpolarization of the membrane potential with little change in resistance. Upon washing (upward arrow) with saline free of salicylate both the membrane potential and resistance returned to their control values. Subsequent tests with 10 m*M* and 30 m*M* salicylate produced greater hyperpolarizations (6 and 12 mv, respectively) and decreases in resistance (from 1.6 to 1.4 and 0.7 megohm, respectively). The upward deflections from the baseline are due to superimposed synaptic activity. The amplitude of these postsynaptic potentials is reduced in salicylate and readily recovers upon washing. (The record showing the recovery from 30 m*M* salicylate is interrupted briefly by a burst of synaptic activity which saturated the pen-recorder.) The membrane does not show rectifying properties over this range of potentials, and so in the absence of salicylate the resistance at −74 mv is the same as that at −63 mv (inset, lower right).