allows quantitative assessment of the input of specific solutes from the natural pool of dissolved organic material and should greatly facilitate assessment of the importance of this transport pathway in energy flow in marine communities.

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Hydrolysis of Nerve Gas by Squid-Type Diisopropyl Phosphorofluoridate Hydrolyzing Enzyme on Agarose Resin

Abstract. *An enzyme purified from squid nerve that hydrolyzes the cholinesterase inhibitor diisopropyl phosphorofluoridate (DFP) has now been coupled to agarose beads. A column of this agarose-DFPase hydrolyzes the nerve gas 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman). Although the more inhibitory of the four diastereoisomers of Soman are hydrolyzed least rapidly, a column of sufficient length will accomplish 95 percent hydrolysis whether measured by fluoride release or loss of cholinesterase-inhibiting power. The results suggest a means for detoxifying unwanted chemical warfare agents.*

From a perusal of scientific commentary and informed popular writing it appears that the powerful cholinesterase inhibitor 1,2,2-trimethylpropyl methylphosphonofluoridate (Soman) is a potential chemical warfare agent (*1*). A similar compound, diisopropylphosphorofluoridate (DFP), is widely used for research and has even been used for clinical purposes (*2*). The discovery of an enzyme or enzymes capable of hydrolyzing DFP (*3*), the seeming absence of a natural substrate or physiological role for the enzyme, and the discovery of a particular variety of the enzyme in the squid giant axon (*4*) has led us to use the term "squid-type DFPase" (*5*). This enzyme will also hydrolyze Soman (*6*). In contrast to DFP, Soman has a property that makes it especially insidious as a nerve gas. Its powerful inhibition of acetylcholinesterase (AChE) is irreversible by any known means because of the very rapid loss of the alkoxy side chain after the initial phosphonylation of AChE (*7*). Data showing that Soman may act on components of the cholinergic system other than AChE (*8*) have renewed our interest in Soman hydrolysis by DFPase. Since one of the two phosphorus-centered stereoisomers (there are a total of four diastereoisomers) is enzymatically hydrolyzed more rapidly than the other (*9*), the use of squid-type DFPase for ascribing these other actions to one or another isomer seemed an attractive possibility. Although this has not yet been realized, another accomplishment of some possible practical significance is presented here. This is the covalent linking of squid-type DFPase to a resin, and the use of this immobilized enzyme for the detoxication of Soman.

Prior to the immobilization of the enzyme, we determined the molecular weight of 2000-fold purified squid-type DFPase (*5*) by gel filtration on Sephadex G-100 resin (*10*). The elution volume of the DFPase, determined with DFP and a fluoride electrode (*11*), was compared to the elution volumes of cytochrome *c*, myoglobin, α-chymotrypsinogen, ovalbumin, and bovine serum albumin, determined by their absorbancies at 280 nm. From these determinations the molecular weight of squid-type DFPase was estimated to be 26,600 ± 1,000 (± standard deviation).

The coupling of this relatively low molecular weight enzyme to an agarose resin has been carried out three times by the method of Cuatrecasas and associates (*12*) with consistent results. In a typical preparation, 2 g of Sepharose-6B resin was activated with cyanogen bromide and was then added to squid-type DFPase (approximately 2 mg of protein capable of hydrolyzing 12 μmole of DFP per minute; the DFPase of highest purity has not been used for coupling) contained in 2 ml of 0.2*M* sodium bicarbonate, *p*H 7.5, and the suspension was shaken overnight in the cold. The resulting agarose-DFPase was collected on a coarse sintered glass filter and washed with 40 ml of a solution made 0.5*M* in sodium chloride, 0.1*M* in tris(hydroxymethyl)aminomethane, *p*H 7.5, all by gravity filtration over about 30 minutes. Finally, the agarose-DFPase was water-washed repeatedly by low-speed centrifugation. Approximately 80 percent of the enzyme was bound to the resin, and about 20 percent was accounted for in the filtrate. With either DFP or Soman as substrates, the Michaelis constants (K_M) and relative maximum velocities (V_{max}) of a dilute slurry of agarose-DFPase are essentially the same as those reported by us for the soluble enzyme (*6*).

We refer to the immobilized enzyme as agarose-DFPase; our interest is predominantly in its Soman-hydrolyzing properties. Two measures of this have been used: the release of fluoride ion measured by a fluoride-sensitive electrode (*11*) and the loss of AChE-inhibiting power in which AChE activity is measured by the method of Ellman *et al.* (*13*).

Figure 1 shows that when $10^{-4}M$ Soman is passed through a 5 by 50 mm agarose-DFPase column at a flow rate of 0.1 ml/min, fluoride ion appears in the column effluent at almost the rate of fluoride appearance if $10^{-4}M$ fluoride is passed through the column. By contrast, $10^{-4}M$ Soman passed through a simple agarose column of the same dimensions releases fluoride ion at a rate compatible

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with nonenzymatic aqueous hydrolysis. The fluoride release curves of Fig. 1 are presented only for comparison since they are a complex function of such factors as the volume of the collecting vessel and the flow rate, as well as the falling substrate concentration and increasing product inhibition (*9*) throughout the column. However, the consistent lag of column effluent fluoride originating from the enzymatic degradation of Soman compared to that originating from applied fluoride led to an examination of fluoride concentration and AChE-inhibiting power in undiluted column effluent. About 20 minutes after the application of Soman to the column shown in Fig. 1, that is, at about t = 10 minutes, column effluent was collected directly into a second vessel. That effluent, diluted as required, was tested for its ability to inhibit a standard solution of AChE (Worthington Electrophorus AChE, 150 U/mg, suitably diluted), and was compared to the AChE-inhibiting power of the original Soman before passage through the column. In practice, this involves the determination of the second-order rate constant for inhibition, and the use of that value and the same equation for the determination of Soman concentration in the column effluent (*14*). Finally, the fluoride concentration in the undiluted effluent in the second collecting vessel was determined. The results [see Table 1 (A)] suggest that about 80 percent of the applied Soman is hydrolyzed when judged by effluent fluoride (in fairly good agreement with the lag noted for Fig. 1), but that only about half of the AChE-inhibiting power is lost. This is in marked contrast to the results shown with DFP, in which the loss of AChE-inhibiting power is virtually complete, and is approximately equal to the loss of DFP as judged by the appearance of fluoride ion.

This diffference between DFP and Soman hydrolysis can be explained only in part by the lower K_M and higher V_{max} for DFP. The discrepancy between fluoride appearance and AChE-inhibiting power disappearance is probably due to the stereospecificity of DFPase with respect to Soman, previously noted by us (*9*), and the stereospecificity of AChE with respect to Soman noted by Keijer and Wolring (*15*). From their findings and nomenclature combined with our previously reported optical rotational changes and our present second-order rate constant for inhibition, Table 2 has been constructed to account for the discrepancy. These data show that the enzymatic hydrolysis rates for the two $(R)_P(-)$ diastereoisomers are slower than for the two $(S)_P(+)$ members (*16*). This relative stereospecificity also agrees with the relatively rapid rotational change in the (−) direction, and then the slower return toward the original value observed during enzymatic hydrolysis in solution (*9*). Some stereospecificity could also be imputed to the asymmetric carbon with similar results; if this were to be done, then the limitation imposed by the experimental data would be that the very powerful AChE-inhibiting diastereoiso-

Table 1. Enzymatic hydrolysis of Soman and DFP on an agarose-DFPase column determined by fluoride release and by loss of AChE-inhibiting power. At t = 10 minutes (see Fig. 1) the suction line was removed, a new collecting vessel was substituted, and sufficient column effluent was collected to permit measurements of AChE inhibition and of fluoride.

Substrate	Concentration (M)	Hydrolysis measured by: Fluoride release (%)	Hydrolysis measured by: AChE-inhibitory* loss (%)
(A) 5 by 50 mm agarose-DFPase column; 0.1 ml/min; 10 minutes void time			
Soman	10^{-3}	81	62.0
	10^{-4}	85	38.0
DFP	10^{-3}	96	99.7
	10^{-4}	95	99.2
(B) 5 by 150 mm agarose-DFPase column; 0.067 ml/min; 45 minutes void time			
Soman	10^{-3}	95	95.7
	10^{-3}	95	95.0

*Second-order rate constant for unhydrolyzed Soman, 1.125×10^8 liter/mole-min (± 20 percent standard deviation, six determinations); for unhydrolyzed DFP, 2.98×10^4 liter/mole-min (± 29 percent standard deviation, five determinations).

Table 2. A combining of the configurational assignments and findings of Keijer and Wolring (*15*), and previously reported optical rotation during enzymatic hydrolysis of Soman (*9*) to account for the seeming discrepancies in Table 1(A), Soman.

Configuration at α-C	Configuration at P	Optical rotation	K_{inhib} (liter/mole-min)	Enzymatic hydrolysis	Fluoride release (percent of total)	AChE-inhibitory power lost (percent of total)
R	*S*	+	10^4*	Fast	25	†
S	*S*	+	10^4*	Fast	25	†
S	*R*	−	1.8×10^8‡	Slow	15	48§
R	*R*	−	4.5×10^7‡	Slow	15	12§
				Apparent hydrolysis	80	60

*From (*15*). †Relatively little to begin with. ‡1.125×10^8 liter/mole-min found by us for racemic Soman; virtually all of that, or 2.25×10^8 liter/mole-min, creditable to $(R)_P$ half; of that, 80 percent (*15*) or 1.8×10^8 liter/mole-min, creditable to $(S)_C(R)_P$ diastereoisomer; same reasoning for $(R)_C(R)_P$. §Sixty percent (that is, 15 percent of the 25 percent possible) of the 80 percent lost; same reasoning for $(R)_C(R)_P$.

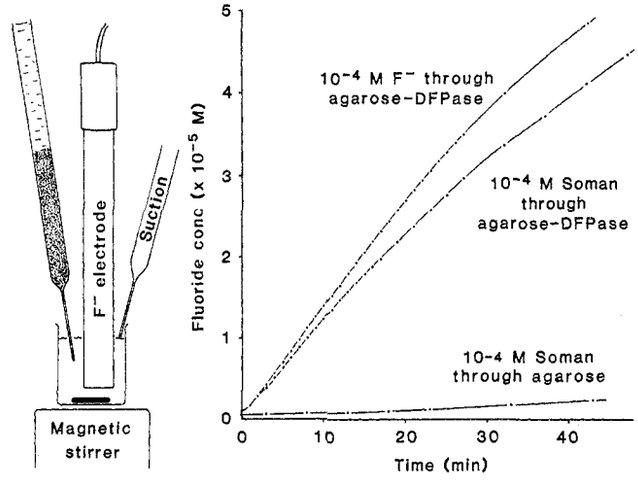

Fig. 1. Enzymatic hydrolysis of Soman by passage through an agarose-DFPase column, shown at the left. Comparison is made with the passage of sodium fluoride through the same agarose-DFPase column, and with the passage of Soman through an agarose column of the same dimensions and flow rate. Zero time is taken as 1 minute before the first detectable rise on the fluoride electrode meter (Orion 901). The medium used for making the Soman or fluoride solutions, for washing the column, or for standardizing the fluoride electrode was 400 mM in KCl, 50 mM in NaCl, and 5 mM in 1,3-bis[tris(hydroxymethyl)methylamino]propane (bis-tris-propane), pH 7.2.

mer, $(S)_C(R)_P$, should be enzymatically hydrolyzed least rapidly.

The evidence that there is some enzymatic hydrolysis of this isomer, albeit more slowly, suggested that a longer exposure to squid-type DFPase would result in more nearly complete hydrolysis. Table 1(B) shows that a $10^{-3}M$ solution of Soman is almost completely hydrolyzed by a 5 by 150 mm agarose-DFPase column with a flow rate of 0.067 ml/min and a time until fluoride appearance of 45 minutes. It can also be seen that now the extent of hydrolysis is virtually the same whether measured by the production of fluoride or by the loss of AChE-inhibiting power.

In addition to confirming that the $(R)_P$ pair of phosphorus-centered isomers of Soman is a much more potent AChE inhibitor than the other, our results present the disturbing evidence that this pair is also the least rapidly enzymatically detoxified. Nevertheless, enzymatic detoxication can be accomplished by a longer exposure to the hydrolyzing enzyme. Although the use of DFPase from such a relatively limited source as the squid, bound to such a fragile support as agarose, is probably not practical for the large-scale detoxication of unwanted nerve gas, the potential exists for accomplishing this by a combination of the techniques of genetic manipulation (*17*) and biochemical engineering (*18*).

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Hepatic Glucose Production Oscillates in Synchrony with the Islet Secretory Cycle in Fasting Rhesus Monkeys

Abstract. *Oscillations in the concentration of plasma glucose were found to reflect large fluctuations in hepatic glucose production. The fluctuations in glucose production were synchronous with fluctuations in the concentration of plasma insulin and glucagon. This synchrony suggests that hepatic pathways are entrained to the islet cycle with a minimal time delay.*

Insulin and glucagon are secreted as regular synchronous pulses in nonhuman primates that have fasted overnight (*1*). These hormonal cycles are accompanied by smaller synchronous oscillations in the plasma glucose concentration. We have investigated the origin of the glucose fluctuations in unanesthetized rhesus monkeys (*Macaca mulata*) prepared for measurement of splanchnic glucose production by the method of Bowden and Bergman (*2*). We postulated that the glucose cycle originates in cyclic hepatic glucose production because, in the fasting state, the hepatic actions of the islet hormones predominate over peripheral actions. The phase relations between the glucose cycle and the hormonal signals

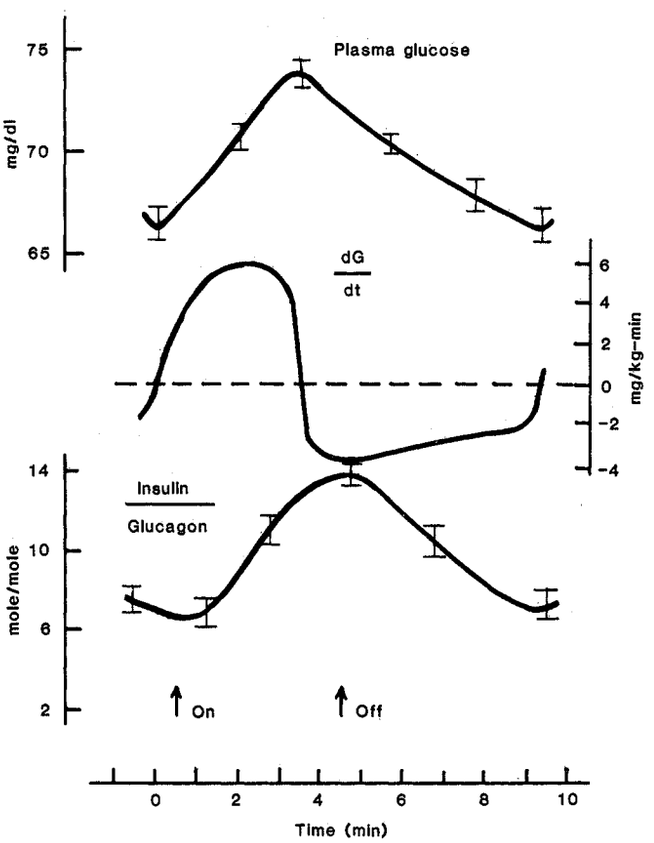

Fig. 1. The plasma glucose cycle compared with the cycles for insulin and glucagon, here combined into the insulin, glucagon molar ratio. The waveform for the plasma glucose cycle was derived by analysis of 94 separate glucose cycles from 48 experiments in 11 rhesus monkeys displaying significant glucose oscillations. The derivative of the glucose concentration cycle was used to calculate the rate of change of glucose in the exchangeable glucose pool (G) which occurs each cycle. This parameter, dG/dt, has been plotted in correct phase relation to the insulin, glucagon molar ratio. The arrows indicate points of maximum on or off signal to hepatic pathways for glucose production. The behavior of the bihormonal signal and the predicted change in entry of glucose into the glucose space correspond closely in the time domain.

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