idative cleavage of the C-5–C-6 single bond in I followed by a cyclization reaction. It is notable that some minor metabolites could be isolated in the bacterial culture medium, formed by secondary reactions with metabolites of metamitron: Compound VIII is the formal condensation product of metamitron and benzaldehyde derived from metabolite IV by decarboxylation. Compound VIII may be cleaved hydrolytically, decarboxylated, and oxidized to the triazole IX prior to ring contraction (Figure 1). Dibenzalhydrazine (X) results from the reaction of hydrazine and benzaldehyde which are formed enzymatically during the herbicide degradation as discussed above.

In the presence of light, the hypothetic intermediate II is converted to the tetrazine derivative (VI) after decarboxylation and oxidation. Traces of compound VI are oxidized further to the 4'-hydroxyphenyl compound VII. The comparatively high amounts of compound VI (Table I), even in experiments run in the dark, are most probably due to aboitic secondary reactions during the extraction and cleanup procedures.

The reason why tetrazine could usually not be found in metamitron-treated soils may be that the herbicide is too rapidly transformed to its desamino analogue (V) either by the action of UV light at the soil surface or by various soil fungi and bacteria (Engelhardt and Wallnöfer, 1978). Desaminometamitron with its three nitrogen atoms, however, cannot be transformed to a tetrazine derivative.

Within soil, microbial degradation of metamitron certainly proceeds to desaminometamitron as well as to the hypothetic ring fission product II which is decomposed further to products similar to those identified in this study. The formation of compound VI seems most unlikely because of the absence of light; also, the chemical structure of II makes its binding to soil organic matter most probable.

Whether metamitron is degraded in the environment by the same reaction sequence as shown by the in vitro studies must be confirmed by further experiments.

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Mechanism of Cholinesterase Inhibition by Methamidophos

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[*O-methyl*-^{14}C]Methamidophos and [*S-methyl*-^{14}C]methamidophos were synthesized and used to determine the nature of the phosphorylating moiety in the inhibition of electric eel acetylcholinesterase by methamidophos. Gel filtration chromatography served to separate the phosphorylated enzyme from excess labeled inhibitors. The results revealed that the methylthio–phosphorus linkage is cleaved during the phosphorylation process.

Although methamidophos (*O,S*-dimethyl phosphoramidothioate) is one of our most important insecticides and has been in agricultural use for a number of years, relatively little is known about its mechanism of action. For example, methamidophos is a relatively poor anticholinesterase yet is highly toxic to animals which die of typical cholinergic symptoms of poisoning (Quistad et al., 1970). Further, in its inhibition of acetylcholinesterase, it is not known whether the P–O or P–S linkage in methamidophos is cleaved during the inhibition process. While cleavage of the P–S linkage in the inhibition reaction had been previously suggested (Quistad et al., 1970), subsequent work revealed that the P–O linkage was also highly labile and either P–S or P–O cleavage occurred during alkaline hydrolysis, depending on the solvent system employed (Fahmy et al., 1972).

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This report is concerned with a study designed to determine the nature of the phosphorylating moiety when electric eel acetylcholinesterase (EEAChE) is inhibited by methamidophos.

MATERIALS AND METHODS

^{14}C-Labeled Methamidophos. [*O-methyl*-^{14}C]Methamidophos was prepared according to Lubkowitz et al. (1974). [^{14}C]Methanol (specific activity 55 mCi/mmol; 0.58 mg; ICN) diluted with nonlabeled methanol (1.74 mg) was added to 22 mg (0.141 mmol) of *S*-methyl phosphorothioic dichloride (Hilgetag et al., 1960) in 15 mL of dichloromethane at 0 °C. After being stirred for 30 min, the solution was saturated with dry ammonia and then filtered to remove ammonium chloride. The resulting [*O-methyl*-^{14}C]methamidophos was purified by preparative thin-layer chromatography (TLC) using silica gel G-1000 plates (Analtech, Newark, DE) with acetone–dichloromethane (1:1 v/v) as the developing solvent. The area between R_f 0.3 and R_f 0.45 was scraped and eluted with

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ethyl acetate, yielding a product of greater than 99% purity, as determined by TLC, with a specific activity of 13.75 mCi/mmol.

[*S-methyl*-^{14}C]Methamidophos was prepared according to Mel'nikov and Zen'kovich (1955) by reacting 3 mg of sodium *O*-methyl phosphoramidothioate (2.04×10^{-2} mmol) with 1.93 mg of [^{14}C]methyl methanesulfonate (specific activity 58 mCi/mmol; Amersham) in refluxing acetonitrile for 1.5 h. The resulting product was purified in the same manner as the *O*-methyl-labeled analogue. The final purified product (specific activity 58 mCi/mmol) was greater than 99% radiochemically pure.

Enzyme Analysis. The inhibition of electric eel acetylcholinesterase (EEAChE; Type VI-S; Sigma Chemical Co.; EC 3.1.1.7) by methamidophos was monitored by the method of Ellman et al. (1961) with acetylthiocholine as the substrate. The enzyme was inhibited to greater than 95% of its initial activity in pH 7.6, 0.1 M phosphate buffer at 37 °C. The regeneration of inhibited enzyme also was monitored by Ellman's procedure. Regeneration experiments were carried out by adding 20 μL of 0.01 M aqueous 2-pyridinecarboxaldoxime methiodide (2-PAM; Aldrich) to the inhibited enzyme fraction (0.5 mL), incubating for 30 min, and measuring the amount of active enzyme.

Sephadex Chromatography and Enzyme Inhibition. Sephadex G-25 (medium; Pharmacia Fine Chemicals) gel filtration was used to separate the inhibited enzyme from excess methamidophos. Column preparation and determination of void volume were by standard procedures (Pharmacia Fine Chemicals). The void volume was 6.5–7.0 mL for a flow rate of 0.5 mL/min.

EEAChE (500 or 1000 units) was diluted with 0.25 mL of pH 7.6, 0.1 M phosphate buffer and maintained at 37 °C for 15–20 min. Excess [^{14}C]methamidophos was added, and the mixture was incubated until the enzyme was inhibited to greater than 95% (about 90 min). The mixture was transferred to a Sephadex column (27 × 1.5 cm; Bio-Rad) with the aid of another 0.25–0.50 mL of phosphate buffer. The column was eluted with 0.1 M phosphate buffer, and 0.5-mL fractions were collected to a total volume of 60–80 mL. At this point most of the added radioactivity was eluted from the column. Extraction of the Sephadex resin with methanol–water did not remove any additional amount of radioactivity. Aliquots (25 μL) were taken from each 0.5-mL fraction, and radioactivity was estimated in 10 mL of scintillation cocktail with a Beckman LS-230 scintillation counter. The cocktail was a mixture of toluene–Triton X-100 (2:1) containing 6 g/L 2,5-diphenyl-1,3-oxazole (PPO) and 0.2 g/L 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (POPOP). Radioactive counts were corrected by using quench correction curves obtained with a [^{14}C]toluene standard.

RESULTS AND DISCUSSION

Chromatography. It was necessary first to establish the chromatographic behavior of EEAChE and methamidophos in the Sephadex G-25 column. EEAChE (5 units) and 2–3 mg of Blue Dextran, an indicator which chromatographs with high molecular weight proteins, were applied to the column and eluted with phosphate buffer. Enzymatic analysis of each fraction showed the enzyme to be associated with fractions 9–12 (see Figure 1). Blue Dextran also eluted with the enzyme fractions. This experiment was repeated 6 times, and the void volume preceding elution of the enzyme was determined to be 4.0–4.5 mL for a flow rate of 0.75 mL/min.

In analogous experiments [^{14}C]methamidophos was observed to elute in fractions 26–31 with a total recovery of radioactivity of >98%. TLC analysis of the eluted radioactivity showed it to be in the form of unchanged methamidophos. These experiments established the feasibility of Sephadex G-25 in separating EEAChE from methamidophos.

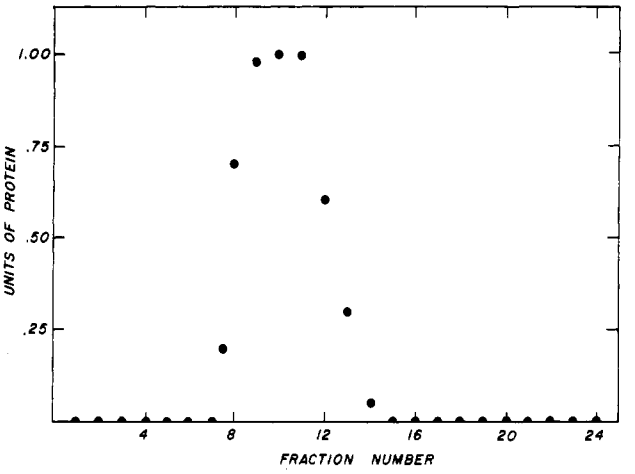


Figure 1. Plot of relative fraction number vs. units of protein for 5 units of EEAChE eluted from a Sephadex G-25 column.

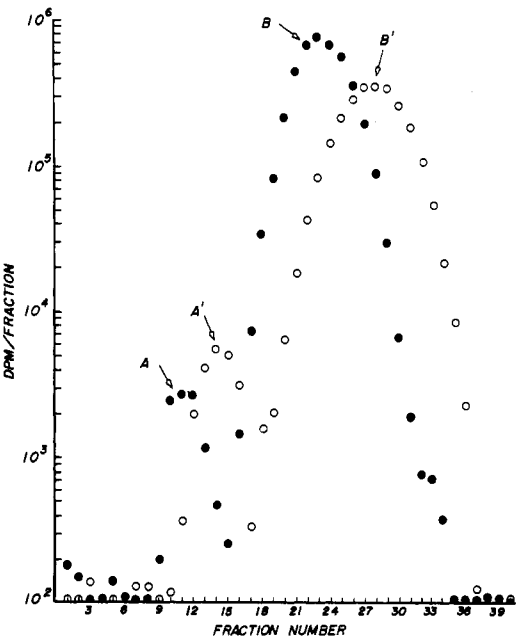


Figure 2. Plot of dpm/fraction vs. relative fraction number for $(^{14}CH_3O)(CH_3S)P(O)NH_2$: (●) 500 units of EEAChE; (○) 1000 units of EEAChE.

Enzyme Inhibition. EEAChE was inhibited by either [*O-methyl*-^{14}C] or [*S-methyl*-^{14}C]methamidophos, and the reaction mixture was subjected to Sephadex chromatography. When [*O-methyl*-^{14}C]methamidophos was used, radioactivity was recovered in two distinct peaks as shown by the chromatographic profiles in Figure 2. Two different runs of enzyme were employed. The small peak, consisting of fractions 9–14 (peak A; 500 units of enzyme) and fractions 12–17 (peak A′; 1000 units of enzyme), corresponds to EEAChE. The second peak, consisting of fractions 16–32 (peak B) and fractions 19–37 (peak B′), corresponds to methamidophos. Peak A′ was approximately twice the size of peak A, in general agreement with the relative amounts of EEAChE employed in the separate experiments.

The fractions in peak A were each examined for hydrolysis of acetylthiocholine and found to have little or no enzymatic activity. Addition of 2-PAM to each fraction, followed by a 30-min incubation, resulted in a substantial

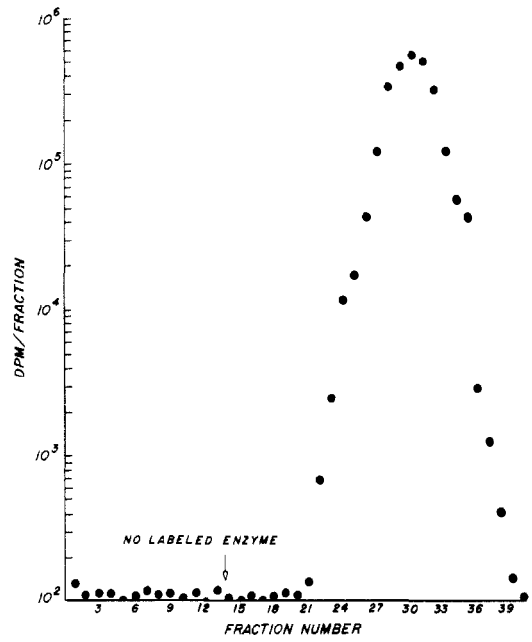
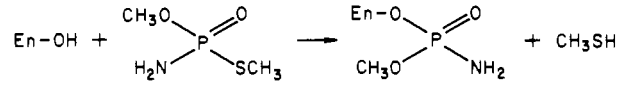


Figure 3. Plot of dpm/fraction vs. relative fraction number for $(CH_3O)(^{14}CH_3S)P(O)NH_2$: (●) 500 units of EEAChE.

increase in enzymatic activity in each fraction. This provided additional evidence for the presence of the enzyme in peak A. Because of the low level of radioactivity in the fractions and the large dilution which takes place in Sephadex gel filtration, it was not possible to determine whether radioactivity was removed from the enzyme in the regeneration process.

Incubation of EEAChE (500 units) with excess [*S-methyl*-^{14}C]methamidophos, followed by Sephadex chromatography, resulted in the chromatographic profile shown in Figure 3. In this case, only one radioactive peak was observed (fractions 22–36), i.e., the peak associated with methamidophos. As in the study with the *O*-methyl-labeled material, treatment of each enzyme fraction with 2-PAM resulted in increased enzymatic activity in fractions 9–12, indicating the presence of EEAChE in these fractions.

The results described above provide strong evidence for P–S bond cleavage in the reaction leading to the inhibition of acetylcholinesterase by methamidophos. The inhibition reaction may be depicted as



where En–OH is the enzyme. In the inhibition reaction, the serine hydroxyl group in the enzyme is phosphorylated by the amido(methoxy)phosphinyl moiety of methamidophos with simultaneous loss of the thiomethyl moeity.

Preferred loss of this thiomethyl moeity can thus support an oxidative activation mechanism previously suggested by Eto et al. (1977). We are currently investigating this possibility in further detail.

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