Nature of Neutral Phosphorus Ester Metabolites of Phosphamidon Formed in Rats and Liver Microsomes

George W. Lucier[1] and Robert E. Menzer*

Phosphamidon underwent complete oxidative *N*-deethylation in rats *in vivo* coupled with the formation of *N*-hydroxyethyl intermediates. Phosphamidon, its mono-*N*-ethylamide analog, and the unsubstituted amide analog were also apparently dechlorinated to form vinyl hydroxy compounds. The major neutral phosphate ester metabolite of phosphamidon in rats was its unsubstituted amide derivative. Rats deactivated phosphamidon very rapidly with most of the radioactivity excreted as innocuous hydrolysis products. Rabbit and rat liver microsomes fortified with NADPH *N*-deethylated phosphamidon, although the *N*-hydroxyethyl intermediates were not detected. Microsomal oxidation of phosphamidon *in vitro* was not enhanced by *in vivo* pretreatment of animals with sodium phenobarbital.

Phosphamidon (dimethyl phosphate, ester with 2-chloro-*N,N*-diethyl-3-hydroxycrotonamide) is a plant systemic insecticide which is prepared by the Perkow reaction (Perkow *et al.*, 1952). The material prepared by this reaction is a mixture of 30% *trans*-phosphamidon and 70% *cis*-phosphamidon, with the cis isomer the more biologically active (Ciba, 1968). Phosphamidon and its metabolite, des-*N*-ethyl phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*-ethyl-3-hydroxycrotonamide), are very potent inhibitors of fly head, rat brain, horse plasma, and human plasma cholinesterase, with the cis isomers of phosphamidon and des-*N*-ethyl phosphamidon being more potent than the corresponding trans isomers (Bull *et al.*, 1967; Ciba, 1968). The acute toxicity of phosphamidon and its previously reported metabolites have been determined following oral and intraperitoneal treatments to mice and rats (Jaques and Bein, 1960; Ciba, 1968; Clemons and Menzer, 1968). The cis isomers of phosphamidon and its des-*N*-ethyl metabolite were approximately 40 times more toxic than the trans isomers. The unsubstituted amide analog of phosphamidon was the most toxic compound tested, indicating that *N*-deethylation of phosphamidon increases its toxicity.

The metabolism of phosphamidon in plants and animals has been extensively studied, although neither the oxidative *N*-deethylation sequence nor the fate of the vinyl chlorine has been clearly established. Since metabolites resulting from *N*-deethylation and dechlorination would be likely potent anticholinesterase agents, a careful study to detect the presence of these types of metabolic conversions is of primary importance in evaluating mammalian health hazards.

Plant enzyme systems have been shown to be active in both bioactivation and deactivation conversions of phosphamidon. Jaques and Bein (1960) reported that plants metabolized phosphamidon to its des-*N*-ethyl derivative. Anliker *et al.* (1961) and Bull *et al.* (1967) confirmed that *N*-deethylation of phosphamidon occurs in several plant systems. Phosphamidon and its des-*N*-ethyl metabolite were rapidly hydrolyzed by plants to innocuous polar compounds such as α-chloro-*N,N*-diethylacetoacetamide, α-chloro-*N*-ethylacetoacetamide, and dimethyl phosphate. Bull *et al.* (1967) reported speculative evidence for the presence of the *N*-hydroxyethyl intermediate in the *N*-deethylation reaction of phosphamidon. *O*-Demethylase activity, resulting in a polar metabolite, des-*O*-methyl phosphamidon (methyl phosphate, ester with 2-chloro-*N,N*-diethyl-3-hydroxycrotonamide), has been found in cotton plants (Bull *et al.*, 1967). Subsequent enzymatic hydrolysis of this metabolite formed methyl phosphate and α-chloro-*N,N*-diethylacetoacetamide. Just as with other organophosphate insecticides, hydrolytic metabolites of phosphamidon have relatively low toxicity in both *in vivo* and *in vitro* systems.

Phosphamidon metabolism in animals parallels some aspects of plant metabolism but there are some conversions which appear to be peculiar to animal systems. Des-*N*-ethyl phosphamidon was the principal metabolite isolated following incubations of phosphamidon-^{14}C with rat and rabbit liver homogenates (Anliker, 1963; Ciba, 1964). Under certain conditions the hydrolysis products α-chloro-*N,N*-diethylacetoacetamide and α-chloro-*N*-ethylacetoacetamide were recovered from the liver incubations. Hydrolysis products identified by Bull *et al.* (1967) following treatment of boll weevils with phosphamidon-^{32}P and -^{14}C were phosphamidon acid, des-*O*-methyl phosphamidon, dimethyl phosphate, and methyl phosphate. *N*-Deethylation was the major route of metabolism in the boll weevil.

Clemons and Menzer (1968) conducted a study of the neutral phosphorus ester metabolites following phosphamidon-^{32}P and phosphamidon-α-diethylamide-^{14}C administrations to rats and a goat. Des-*N*-ethyl phosphamidon, phosphamidon amide (dimethyl phosphate, ester with 2-chloro-3-hydroxycrotonamide) and deschloro phosphamidon amide (dimethyl phosphate, ester with 3-hydroxycrotonamide) were identified. The diethylamide portion of the molecule was shown to be deethylated stepwise by mammals to form the unsubstituted amide analog of phosphamidon which was apparently dechlorinated. Several unknown neutral phosphate ester metabolites were also isolated from rats and a goat. Over 90% of the radioactivity in the urine was unextractable with chloroform and was therefore assumed to be nontoxic polar metabolites.

Earlier studies with similar compounds (Menzer and Casida, 1965) indicated that phosphamidon should be *N*-deethylated through the formation of the *N*-hydroxyethyl intermediates. Two of the four unknown metabolites reported by Clemons and Menzer (1968) could represent the *N*-hydroxyethyl derivatives. This study on the metabolism of phosphamidon

Department of Entomology, University of Maryland, College Park, Maryland 20742
[1] Cell Biology Branch, National Institute of Environmental Health Sciences, Research Triangle, North Carolina 27709

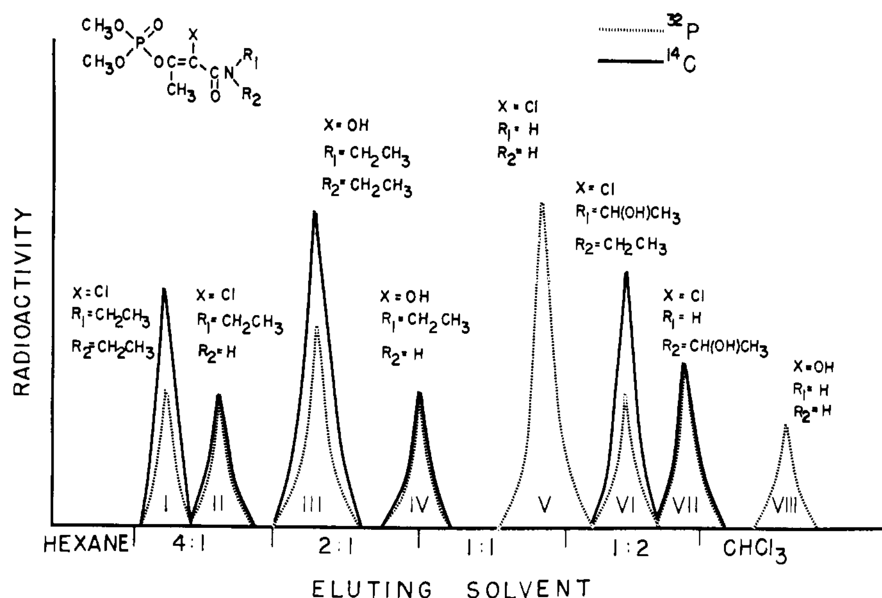

Figure 1. Representation of the separation of chloroform-extractable phosphamidon metabolites achieved on a Celite column based on partitioning between water and hexane–chloroform mixtures. Metabolites A and B are not shown for simplicity since they were not identified

in rats and mammalian liver microsomes was conducted with the aim of characterizing unknown metabolites in the oxidative *N*-deethylation sequence and investigating the occurrence and mechanism of dechlorination of phosphamidon.

MATERIALS AND METHODS

Radioactive Chemicals. To prepare phosphamidon-^{32}P, α,α-dichloro-*N*,*N*-diethylacetoacetamide was reacted with trimethyl phosphite-^{32}P. The chlorinated acetoacetamide was prepared according to the procedure of Anliker *et al.* (1961). Sulfuryl chloride (516 mg, 3.84 m*M*), dissolved in 5 ml of methylene chloride, was added dropwise to 10 ml of a chilled methylene chloride solution containing *N*,*N*-diethylacetoacetamide (300 mg, 1.92 m*M*), prepared by Clemons and Menzer (1968). The mixture was refluxed for 3 hr at 42° C, neutralized with two washings of 10 ml of saturated sodium bicarbonate solution, and dried with anhydrous sodium sulfate. The solvent was removed under vacuum, leaving 349.8 mg of a yellowish-brown oil, α,α-dichloro-*N*,*N*-diethylacetoacetamide (80.7% yield). Purity was determined by thin-layer chromatography and structure confirmed by infrared spectra. Trimethyl phosphite-^{32}P (58 mg, 47 m*M*, 4.35 mCi/m*M*) was added dropwise over a period of 10 min to the chlorinated acetoacetamide (113 mg, 0.5 m*M*), while it was refluxing in 10 ml of chlorobenzene at 120° C. The mixture was refluxed for an additional hour, the chlorobenzene removed under vacuum, and the labeled product purified on a Celite partitioning column. The radioactive peak which corresponded to unlabeled phosphamidon was further purified on thin-layer plates. The radioactive spot cochromatographing with unlabeled phosphamidon was extracted, washed with 200 ml of chloroform, and stored at 4° C. The yield of pure phosphamidon was 7.2%, as determined by recovery of radioactivity. Nuclear magnetic resonance spectra demonstrated the product to be 30% *trans*-phosphamidon and 70% *cis*-phosphamidon (Clemons and Menzer, 1968).

Phosphamidon-α-diethylamide-^{14}C prepared by Clemons and Menzer (1968) was purified on thin-layer plates prior to use. Phosphamidon-1,3-^{14}C obtained from Ciba, Ltd., Agrochemical Division, Basle, Switzerland, also required purification by thin-layer chromatography.

Other Chemicals. Phosphamidon, des-*N*-ethyl phosphamidon, deschloro phosphamidon amide, α-chloro-*N*,*N*-diethylacetoacetamide, and α-chloro-*N*-ethylacetoacetamide were supplied by Ciba Agrochemical Co., Vero Beach, Fla. Phosphamidon amide was prepared according to the procedure of Clemons and Menzer (1968). Deschloro phosphamidon (diethyl phosphate, ester with *N*,*N*-diethyl-3-hydroxycrotonamide) and deschloro desethyl phosphamidon (dimethyl phosphate, ester with *N*-ethyl-3-hydroxycrotonamide) were synthesized by a modified procedure of Anliker *et al.* (1961). Trimethyl phosphite was reacted with α-chloro-*N*,*N*-diethylacetoacetamide or α-chloro-*N*-ethylacetoacetamide to prepare the above two compounds.

Treatment of Rats and Liver Microsomes. Female and male rats, 150 mg, Sprague–Dawley derived (Flow Labs, Inc., Bethesda, Md.), were treated with phosphamidon for analysis of radioactive metabolites in the urine. Rats were anesthetized with ether and treated with phosphamidon-^{32}P and/or phosphamidon-^{14}C by stomach tube. Rats received 2.0 mg/kg of phosphamidon-1,3-^{14}C in one treatment and 5.1 mg/kg of a mixture of phosphamidon-α-diethylamide-^{14}C and phosphamidon-^{32}P in a second treatment. Twice as much phosphamidon-^{14}C as phosphamidon-^{32}P was administered in the double-label experiments so that the fate of the diethylamide portion of the molecule could be determined by the ^{14}C to ^{32}P ratio of each metabolite. After treatment, rats were placed in metabolism cages designed for the separate collection of urine and feces. The feces contained only trace quantities of organoextractable metabolites and therefore were not chromatographed. Samples were taken at 6, 12, 24, 36, 48, and 72 hr in the phosphamidon-1,3-^{14}C treatments and 10, 30, and 100 hr in the double-label studies.

In another experiment, rats were administered a mixture of phosphamidon-^{32}P and phosphamidon-α-diethylamide-^{14}C (0.5 mg/kg) each day for 5 days to evaluate the excretion pattern of phosphamidon and its metabolites from animals exposed to multiple doses of the insecticide. Samples were collected 24 hr after each treatment and 48 hr after the last administration.

Rabbit and rat liver microsomes prepared according to the procedure of Remmer *et al.* (1967) were incubated with phos-

Table I. Common Name, Chemical Name, and R_f Values for Phosphamidon and its Proposed and Theoretical Metabolites on Three Thin-Layer Chromatographic Systems

Common name	Metabolite no.	R_f in system[a] A	B	C	Chemical name
Phosphamidon	I	0.90	0.70	0.68	Dimethyl phosphate, ester with 2-chloro-*N*,*N*-diethyl-3-hydroxycrotonamide
Des-*N*-ethyl phosphamidon	II	0.85	0.70	0.65	Dimethyl phosphate, ester with 2-chloro-*N*-ethyl-3-hydroxycrotonamide
Phosphamidon amide	V		0.50	0.58	Dimethyl phosphate, ester with 2-chloro-3-hydroxycrotonamide
N-Hydroxyethyl phosphamidon	VI		0.45		Dimethyl phosphate, ester with 2-chloro-*N*-ethyl-*N*-hydroxyethyl-3-hydroxycrotonamide
N-Hydroxyethyl des-*N*-ethyl phosphamidon	VII		0.45		Dimethyl phosphate, ester with 2-chloro-*N*-hydroxyethyl-3-hydroxycrotonamide
Vinyl hydroxy phosphamidon	III		0.60	0.62	Dimethyl phosphate, 3-ester with *N*,*N*-diethyl-2,3-dihydroxycrotonamide
Vinyl hydroxy des-*N*-ethyl phosphamidon	IV		0.50	0.55	Dimethyl phosphate, 3-ester with *N*-ethyl-2,3-dihydroxycrotonamide
Vinyl hydroxy phosphamidon amide	VIII		0.35	0.35	Dimethyl phosphate, 3-ester with 2,3-dihydroxycrotonamide
Deschloro phosphamidon		0.72	0.55	0.60	Dimethyl phosphate, ester with *N*,*N*-diethyl-3-hydroxycrotonamide
Deschloro desethyl phosphamidon		0.55	0.50	0.55	Dimethyl phosphate, ester with *N*-ethyl-3-hydroxycrotonamide
Deschloro phosphamidon amide		0.35	0.40	0.45	Dimethyl phosphate, ester with 3-hydroxycrotonamide
α-Chloro-*N*,*N*-diethylacetoacetamide		0.80	0.95	0.80	
α-Chloro-*N*-ethylacetoacetamide		0.85	0.95	0.80	

[a] Thin-layer chromatography on silica gel G using 8 to 1 chloroform–methanol in system A, 2 to 1 acetone–hexane in system B, and 4 to 1 acetone–benzene in system C.

phamidon-1,3-^{14}C and phosphamidon-α-diethylamide-^{14}C and appropriate cofactors (Lucier and Menzer, 1970) in separate experiments. In one experiment the rabbit was pretreated for 3 days with intraperitoneal injections of sodium phenobarbital of 75 mg per kg per day.

Extraction and Chromatography. Each urine sample and microsomal incubate was extracted five times with equal volumes of chloroform, using centrifugation to separate the two phases when necessary. The chloroform extracts were evaporated to 0.5 ml and placed directly on a Celite partitioning column (Lucier and Menzer, 1970). Elution was accomplished with hexane–chloroform mixtures, beginning with pure hexane and ending with pure chloroform. Twenty-milliliter fractions were collected at a flow rate of about 2 ml per min and each fraction was assayed for radioactivity in a Packard Tri-Carb Liquid Scintillation Spectrometer. Resolution of phosphamidon and its neutral phosphate ester metabolites on Celite columns is depicted in Figure 1. The water fractions consisting of hydrolysis products were assayed for radioactivity but were not chromatographed.

Three thin-layer chromatographic systems were developed for metabolite isolation and cochromatography with known standards with the following solvents being used for the mobile phase: 2:1 acetone–hexane; 4:1 acetone–benzene; and 8:1 chloroform–methanol. R_f values for phosphamidon and its potential and actual metabolites are listed in Table I.

Detection and Identification of Metabolites. Phosphamidon-^{32}P, phosphamidon-^{14}C, and their metabolites were detected on thin-layer plates by spotting known unlabeled standards with the radioactive extracts. After resolution the location of the standard metabolites was determined by exposing the plate to bromine vapor for 1 min, followed by spraying with a 0.5% solution of brilliant green in acetone. The presence of the compounds was indicated by yellow spots on a green background, and when plates were again exposed to bromine vapor the spots became bluish-green on a white background. Phosphamidon (50 μg) or its metabolites gave an easily detectable spot. To ascertain the location of radioactive spots, the chromatograms were exposed to Kodak No-Screen Medical X-ray film for a 2-hr to 7-day period, depending on the isotope used and the amount of radioactivity applied to the plates. The coincidental chromatographic position of the radioactive spots and those resulting from the brilliant green spray was indicative of the identity of the radioactive metabolites. Radioactivity measurement of metabolites on silica gel was accomplished by forming a gel with Cab-O-Sil (Packard Instrument Co.) in scintillation counting mixture. Cochromatography of radioactive metabolites with known standards on Celite columns and counting procedures were the same as previously described (Lucier and Menzer, 1970).

N-Hydroxymethyl derivatives of dicrotophos and monocrotophos have been shown to liberate formaldehyde upon acid hydrolysis (Menzer and Casida, 1965). Therefore, *N*-hydroxyethyl intermediates in the deethylation of phosphamidon-α-diethylamide-^{14}C would be expected to yield acetaldehyde-^{14}C when subjected to similar conditions. To determine the presence of acetaldehyde-^{14}C, the following derivative isotope dilution procedure was used. A portion of the metabolite to be evaluated and 2 ml of a 2% hydrochloric acid solution were placed in a glass tube. The tube was sealed and placed in boiling water 1 hr, and the contents were neutralized by dropwise addition of a saturated sodium bicarbonate solution. A labeled dimedone derivative of the resulting acetaldehyde-^{14}C was prepared as follows (Shriner *et al.*, 1965): 300 mg (0.9 m*M*) of dimedone (5,5-dimethyl-1,3-cyclohexanedione) in 4 ml of 50% aqueous ethanol, 20 mg (0.45 m*M*) of acetaldehyde and 2 to 3 drops of pyridine were added to the reaction tube. Crystal formation was accomplished by boiling the solution slowly for 2 min followed by cooling to 4° C. The products were separated by suction filtration and recrystallized in 50% aqueous methanol. The resulting white powder had a melting point of 140° C and weighed 82.6 mg (51.9% yield). A portion of the above condensation product was cyclized to its octahydroxanthene derivative by boiling for 5 min a solution of 40 mg of the dimedone derivative in 3 ml of 80%

ethanol to which 1 drop of concentrated hydrochloric acid had been added. The product was chilled and the substituted octahydroxanthene was removed by suction filtration. The white crystal melted at 175° C and weighed 21 mg. Dimedone derivatives were assayed for radioactivity.

Hydrolysis Rates of Metabolites and Known Standards in Base. To aid in the identification of unknown metabolites, the hydrolysis rates of radioactive metabolites in base were compared with those from known standards. The half-life of each compound was measured in 0.05 *N* NaOH. Since phosphorus-containing hydrolysis products remain in neutral aqueous solutions upon chloroform extraction, hydrolysis of the organophosphates can be evaluated by assaying total phosphorus in each fraction. Table II compares half-lives of ^{32}P-derived metabolites with some known standards.

Glucuronidase Activity. In rats glucuronide formation occurs with free hydroxy groups of several pesticides. Based on this knowledge, it was felt a similar reaction could take place with *N*-hydroxyethyl and vinyl hydroxy analogs of phosphamidon. To determine the presence of glucuronides in the water fraction of rat urine, the method of Bull and Ridgway (1969) was followed. An aliquot of the water fraction of rat urine containing 100,000 cpm was incubated with 25 mg of β-glucuronidase (70,000–100,000 units per g) in 5 ml of phosphate buffer (pH 4.4) at 37° C for 10 hr. However, no radioactivity was found in a chloroform extract of the incubation mixture, indicating that the chloroform extractable analogs of phosphamidon are not excreted as β-glucuronides in rat urine. Results were also negative when 5 ml of 1% HCl was incubated at 37° C with the radioactive water fraction.

RESULTS

Chemical Nature of Metabolites. Ten radioactive compounds derived from the double-label studies were isolated on Celite columns. Six contained both isotopes, two contained only ^{32}P, and two others had only the carbon label. Resolution of the phosphorus-containing metabolites is depicted in Figure 1 and R_f values for each metabolite on thin-layer plates are listed in Table I.

The first peak eluted from the column (I) contained both ethyl groups, since the isotope ratio was 2:1 ^{14}C:^{32}P. This compound cochromatographed with unlabeled phosphamidon on Celite columns and thin-layer plates. The hydrolysis rate of standard phosphamidon in 0.05 *N* NaOH was comparable to compound I and this fraction did not liberate acetaldehyde-^{14}C upon acid hydrolysis. From the above evidence compound I was designated unmetabolized phosphamidon. Metabolite II was detected in only small quantities, had a 1:1 ^{14}C:^{32}P ratio, and did not produce acetaldehyde-^{14}C. It cochromatographed with and had a similar half-life in base to des-*N*-ethyl phosphamidon.

Table II. Base Hydrolysis Rates of Phosphamidon and its Metabolites Determined as Half-Life in Minutes in 0.05 *N* NaOH

Compound $(CH_3O)_2P(O)OC(CH_3) = C(X)C(O)R$		^{32}P Partition[a]	Phosphate partition[b]
X = Cl	R = $N(CH_2CH_3)_2$	44	38
X = Cl	R = $N(CHOHCH_3)(CH_2CH_3)$	13	
X = Cl	R = $NHCH_2CH_3$	15	12
X = Cl	R = $NHCHOHCH_3$	6	
X = Cl	R = NH_2	16	16
X = OH	R = $N(CH_2CH_3)_2$	14	
X = OH	R = $NHCH_2CH_3$	8	
X = OH	R = NH_2	4	

[a] Percentage hydrolysis at any one time determined by partition coefficient of radioactivity between chloroform and water. [b] Percentage hydrolysis at any one time determined by partition of total phosphate between chloroform and water.

Compound III was comparable to the unknown metabolite III isolated by Clemons and Menzer (1968) from goat and rat urine, in that both had a 2:1 ^{14}C:^{32}P ratio, they eluted in approximately the same position on Celite columns, and neither liberated acetaldehyde-^{14}C. Metabolite III did not cochromatograph with any of the known standards. To determine if metabolite III contained a free hydroxyl group, the compound was reacted with acetic anhydride. The resulting product (51.8%) chromatographed at R_f 0.70 on the acetone–hexane thin-layer system, compared to R_f 0.60 for unreacted metabolite III. This alteration to a less polar material indicated the acetylation of a free hydroxyl group. After metabolite III had been incubated with 1 *N* HCl for 30 min at 23° C, 90% of the radioactivity cochromatographed with standard phosphamidon on Celite columns and thin-layer plates, with 10% remaining as metabolite III. Also the hydrochloric acid derivative was comparable in its base hydrolysis rate to standard phosphamidon. Apparently metabolite III had been formed by a dechlorination reaction resulting in a vinyl hydroxyl on the 2-crotonamide position, since chlorination by a substitution reaction with 1 *N* HCl reformed phosphamidon. Metabolite III had a 14-min half-life in base compared to 44 min for standard phosphamidon. This relationship would be expected of the proposed structure of dimethyl phosphate, 3-ester with *N*,*N*-diethyl-2,3-dihydroxycrotonamide (vinyl hydroxy phosphamidon).

To investigate the mechanism of dechlorination, the supernatant fraction from microsome preparations was incubated with phosphamidon-1,3-^{14}C in the presence of 4.4×10^{-3} *M* glutathione. After incubation, metabolite III was not detected in the chloroform extract, indicating that the proposed dechlorination is not a glutathione-dependent reaction.

Metabolite IV had a 1:1 ^{14}C:^{32}P ratio, could be acetylated to a less polar compound, forms des-*N*-ethyl phosphamidon when treated with 1 *N* HCl, and did not yield acetaldehyde-^{14}C. Therefore, the proposed structure of metabolite IV is dimethyl phosphate, 3-ester with *N*-ethyl-2,3-dihydroxycrotonamide (vinyl hydroxy des-*N*-ethyl phosphamidon). The chromatographic position and base hydrolysis rate were consistent with the expected polarity and stability of such a molecule.

Metabolite V cochromatographed with phosphamidon amide, contained only the phosphorus isotope, did not produce acetaldehyde-^{14}C, and its base hydrolysis rate was similar to that of the phosphamidon amide. From this evidence, metabolite V is identified as the unsubstituted amide analog of phosphamidon.

Metabolite VI had both ethyl groups and the phosphorus intact. After hydrolysis it formed a dimedone-^{14}C derivative in stoichiometric amounts, which could be cyclized to its labeled octahydroxanthene. The hydrolysis rate in basic solution and chromatographic position of this metabolite was consistent with that expected of dimethyl phosphate, ester with 2-chloro-*N*-ethyl-*N*-hydroxyethyl-3-hydroxycrotonamide (*N*-hydroxyethyl phosphamidon).

Metabolite VII had a 1:1 ^{14}C:^{32}P ratio, liberated acetaldehyde-^{14}C in stoichiometric quantities, and the dimedone derivative could be cyclized to its labeled octahydroxanthene. This evidence, plus the base hydrolysis rate and chromatographic position, indicates the identity of metabolite VII to be dimethyl phosphate, ester with 2-chloro-*N*-hydroxyethyl-

3-hydroxycrotonamide (*N*-hydroxyethyl des-*N*-ethyl phosphamidon).

Metabolite VIII, eluted in pure chloroform, contained only the phosphorus label, could be acetylated to a less polar compound, and formed the phosphamidon amide when reacted with 1 *N* HCl. It did not yield acetaldehyde-^{14}C and was rapidly degraded in dilute base. Therefore, the proposed structure of metabolite VIII is dimethyl phosphate, 3-ester with 2,3-dihydroxycrotoamide (vinyl hydroxy phosphamidon amide).

Metabolites A and B contained only the carbon label, but neither cochromatographed with the following hydrolytic products on the Celite columns: α-chloro-*N*,*N*-diethylacetoacetamide, α-chloro-*N*-ethylacetoacetamide, and *N*,*N*-diethylacetoacetamide. The identity of metabolites A and B was not further investigated, since they do not contain the phosphorus moiety and are therefore not potential anticholinesterases.

Recovery of Radioactivity. Phosphamidon-^{32}P and -^{14}C degradation was extremely rapid in rats, with none of the parent compound detectable 10 hr after treatment. Good recoveries were achieved from rat urine, with most of the radioactivity excreted as hydrolytic products in the water fraction. By 72 hr 64.1% phosphamidon-1,3-^{14}C equivalents from females and 66.3% from males were excreted in the urine. The recovered percentage of phosphamidon-^{32}P equivalents was higher, with 81.3% of the administered radioactivity excreted in the urine during the 100-hr sampling period. The levels of radioactivity at each sampling time were consistent with those found by Clemons and Menzer (1968).

The percentage of phosphamidon-^{32}P equivalents recovered as chloroform-extractable compounds after 10 hr was 5.49% and only trace quantities were detected at the later sampling times. Similar recoveries of chloroform-extractable compounds were observed for the phosphamidon-1,3-^{14}C treatments.

Phosphamidon-^{32}P and -^{14}C equivalents of individual metabolites at each sampling time are shown in Tables III and IV. The phosphorus-containing metabolite consistently occurring in the highest concentration was the unsubstituted amide analog of phosphamidon. In females it comprised

Table III. Percentages of Administered Dose of Labeled Compounds Recovered after Treatment of Three Female Rats with 5.1 mg/kg Phosphamidon-^{32}P and Phosphamidon-α-diethylamide-^{14}C

Compound $(CH_3O)_2P(O)OC(CH_3) = C(X)C(O)R$	Hours after treatment[a]		
	0–10	10–30	30–100
X = Cl R = $N(CH_2CH_3)_2$	0.094		
X = Cl R = $N(CHOHCH_3)(CH_2CH_3)$	0.147	0.014	
X = Cl R = $NH(CH_2CH_3)$	0.067		
X = Cl R = $NH(CHOHCH_3)$	0.435	0.042	0.005
X = Cl R = NH_2	1.854	0.103	0.008
X = OH R = $N(CH_2CH_3)_2$	0.537	0.040	
X = OH R = $NH(CH_2CH_3)$	0.185	0.009	
X = OH R = NH_2	0.173	0.015	
Unknown A	1.933	0.553	0.027
Unknown B	0.072	0.117	0.008
Water fraction	58.633	10.167	6.424

[a] Percentage recovery based on phosphamidon-^{32}P equivalents, except for unknowns A and B, which are based on phosphamidon-α-diethylamide-^{14}C equivalents.

Table IV. Percentages of Administered Dose of Labeled Compounds Recovered after Treatment of Three Female and Three Male Rats with 2.0 mg/kg Phosphamidon-1,3-^{14}C

Compound $(CH_3O)_2P(O)OC(CH_3) = C(X)C(O)R$	Males, hours after treatment			Females, hours after treatment		
	0–6	6–12	12–24	0–6	6–12	12–24
X = Cl R = $N(CH_2CH_3)_2$	0.019			0.090		
X = Cl R = $N(CHOHCH_3)(CH_2CH_3)$	0.144	0.007		0.159	0.015	0.003
X = Cl R = $NH(CH_2CH_3)$	0.084			0.531	0.021	
X = Cl R = NH_2	0.548	0.013		0.935	0.224	0.048
X = OH R = $N(CH_2CH_3)_2$	0.133			0.487	0.052	
X = OH R = $NH(CH_2CH_3)$	0.072			0.142	0.009	
X = OH R = NH_2	0.090	0.016		0.234	0.034	
Unknown A[a]	1.743	0.695	0.227	2.346	0.701	0.388
Unknown B	0.044	0.025	0.004	0.103	0.086	0.040
Water fraction	31.499	18.310	5.946	39.311	14.433	5.119

[a] The *N*-hydroxyethyl des-*N*-ethyl metabolite eluted at the same position on Celite columns as Unknown A and is therefore included in the recoveries of Unknown A.

Table V. Percentages of Administered Dose of Labeled Compounds Recovered from Daily Treatment of Three Female Rats for 5 Days with Phosphamidon-^{32}P and Phosphamidon-α-diethylamide-^{14}C (0.5 mg/kg/Day)

Compound $(CH_3O)_2P(O)OC(CH_3) = C(X)C(O)R$	Days after first treatment[a]						
	1	2	3	4	5	6	7
X = Cl R = $N(CH_2CH_3)_2$	0.026	0.041	0.082	0.033	0.027		
X = Cl R = $N(CHOHCH_3)(CH_2CH_3)$	0.093	0.090	0.131	0.259	0.181	0.021	
X = Cl R = $NH(CH_2CH_3)$	0.089	0.063	0.070	0.051	0.059		
X = Cl R = $NH(CHOHCH_3)$	0.153	0.078	0.077	0.075	0.081	0.009	
X = Cl R = NH_2	1.163	1.044	1.110	0.875	0.960	0.111	0.016
X = OH R = $N(CH_2CH_3)_2$	0.513	0.388	0.217	0.134	0.138	0.007	
X = OH R = $NH(CH_2CH_3)$	0.156	0.103	0.209	0.170	0.207	0.022	
X = OH R = NH_2	0.083	0.095	0.126	0.079	0.126	0.009	
Unknown A	2.404	1.843	1.898	2.035	1.699	0.422	0.091
Unknown B	0.237	0.235	0.331	0.314	0.392	0.108	0.014
Total chloroform extract	4.917	3.980	4.251	4.025	3.870	0.709	0.121

[a] Percentages based on recovery of phosphamidon-^{32}P equivalents, except for Unknowns A and B, which are based on phosphamidon-α-diethylamide-^{14}C equivalents.

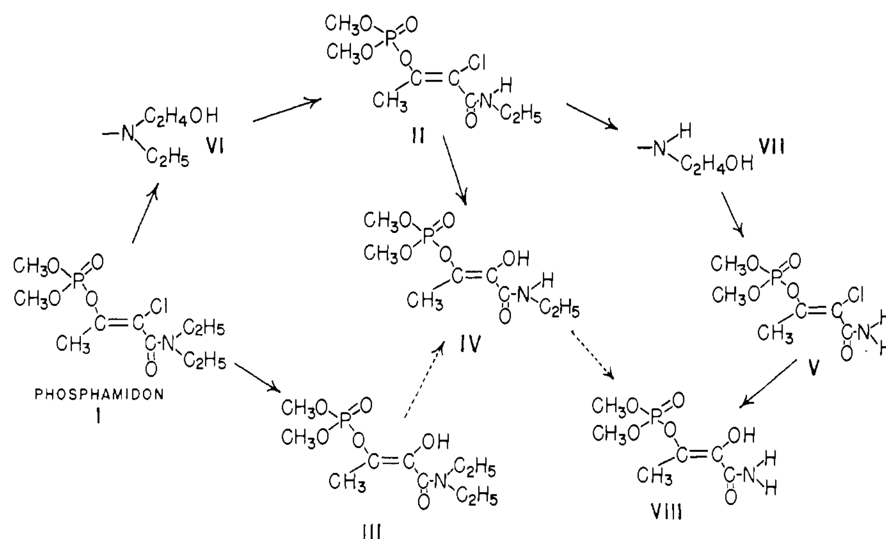

Figure 2. Proposed partial metabolic route of phosphamidon in rats

nearly 2% of the administered radioactivity in the ^{32}P treatments and 1.2% from phosphamidon-1,3-^{14}C treatments, and was detectable in trace quantities even 100 hr after treatment. Recoveries for all metabolites were less for males than females. Metabolite III, vinyl hydroxy phosphamidon, occurred in the next highest concentration, but was not detectable after 12 hr in the ^{14}C administrations and 30 hr in the double-label studies. The remaining phosphorus-containing metabolites were found in lesser quantities. Metabolite A, which did not contain phosphorus, was found in higher levels than any phosphorus-containing metabolite. The *N*-hydroxyethyl des-*N*-ethyl compound, the phosphamidon amide, and metabolites A and B were the only radioactive compounds isolated at the 100-hr sampling time.

Table V shows the recovery of metabolites derived from daily treatments of phosphamidon-^{32}P and -^{14}C. The daily recovery of chloroform-extractable metabolites decreased during the course of the treatments from the first through the fifth day. The percentage of the daily dose recovered which was chloroform-extractable dropped from 4.92% 24 hr after the first treatment to 3.87% 24 hr after the fifth daily treatment. The amount of metabolites detected rapidly decreased when treatments were stopped. The level of phosphamidon increased during the second and third day, but then decreased to its original level by the fifth day. Metabolites IV, VI, VIII, and B showed an increase in concentration during the course of the treatment, while metabolites II, III, V, VII, and A decreased in concentration. The most striking change was demonstrated by metabolite III, which steadily decreased during the 5-day sampling period. On the other hand, metabolite VI, *N*-hydroxyethyl phosphamidon, doubled in concentration during the same period.

Both rat and rabbit liver microsomes metabolized phosphamidon (Table VI). However, most of the degradation products were nonorganoextractable and were not further investigated. Des-*N*-ethyl phosphamidon and the phosphamidon amide were found in small quantities but neither the *N*-hydroxyethyl analogs nor the proposed vinyl hydroxy compounds were detected. Rabbit liver microsomes were more effective in degrading phosphamidon than rat liver microsomes, but the activity was not enhanced in liver microsomes prepared from rabbits pretreated with sodium phenobarbital.

Table VI. Percentages of Administered Dose of Labeled Compounds Recovered from Liver Microsomes Treated with 19.4 μg of Phosphamidon-1,3-^{14}C

Compound $(CH_3O)_2P(O)OC(CH_3) = C(X)C(O)R$	Rat	Rabbit Control	Rabbit Phenobarbital-treated
X = Cl R = $N(CH_2CH_3)_2$	66.41	49.63	44.80
X = Cl R = $NH(CH_2CH_3)$	0.09	0.67	0.74
X = Cl R = NH_2	0.02	0.11	0.14
Water fraction	34.49	42.55	46.43
Total recovery	101.01	92.96	92.11

DISCUSSION

The metabolites comprising the complete oxidative *N*-deethylation sequence of phosphamidon were detected in rat urine and liver microsomes, except that the *N*-hydroxyethyl intermediates were not isolated after microsomal incubations. The metabolite scheme is depicted in Figure 2. Hydroxylation in oxidative *N*-deethylation probably occurs on the α-carbon of the substituted amide moiety, since a β-hydroxylated intermediate would not liberate acetaldehyde-^{14}C in stoichiometric quantities when subjected to acid hydrolysis. The *N*-hydroxyethyl intermediates accumulated in rat urine to comparable levels as the corresponding deethylated compounds (Tables III, IV, and V), indicating that these intermediates are relatively stable in the rat system.

Attempts to prepare the *N*-hydroxyethyl metabolites for use in cochromatographic studies were unsuccessful. The procedure was analogous to that used to synthesize the *N*-hydroxymethyl intermediates of other similar compounds (Menzer and Casida, 1965; Lucier and Menzer, 1970), but none of the corresponding *N*-hydroxyethyl compounds could be isolated.

In addition to the metabolites of the oxidative *N*-deethylation series, three other neutral phosphorus ester metabolites of phosphamidon were isolated. These are proposed to be the vinyl hydroxy derivatives of phosphamidon, des-*N*-ethyl phosphamidon, and the phosphamidon amide. Their place in the metabolic scheme is illustrated in Figure 2. *N*-Hydroxyethyl intermediates of the vinyl hydroxy compounds were not detected, but it is probable that the vinyl hydroxy metabolites are *N*-deethylated. The chemical evidence for

dechlorination of phosphamidon indicates that the vinyl hydroxy metabolites are indeed formed. The best evidence in support of these metabolites is regeneration of the chlorinated compounds by the substitution reaction with HCl [analogous to Lucas test for alcohols (Noller, 1965)]. Thus phosphamidon is regenerated from the metabolite proposed to be the vinyl hydroxy phosphamidon following HCl treatment, des-*N*-ethyl phosphamidon is reformed from vinyl hydroxy des-*N*-ethyl, and the vinyl hydroxy phosphamidon amide yields phosphamidon amide. To definitely characterize the nature of these metabolites, it would be necessary to prepare known standards of the vinyl hydroxy compounds for cochromatographic purposes and to collect from animals a quantity of these metabolites sufficient to obtain spectral data. Repeated efforts to prepare these materials synthetically have been unsuccessful.

The mechanism of substitution of the vinyl chlorine with an hydroxyl group remains unclear. Since glutathione incubations with labeled phosphamidon and the $105,000 \times g$ supernatant fraction from homogenized mammalian liver do not produce any of the proposed vinyl hydroxy metabolites, it appears doubtful that the dechlorination reaction is mediated by glutathione, although Parke (1968) reports its involvement in hydrolytic dehalogenation of some compounds. None of the vinyl hydroxy metabolites were detected from mammalian microsomal incubations with phosphamidon, indicating that neither is dechlorination mediated by the mixed function oxidase system.

Simazine [2-chloro-4,6-*bis*(ethylamine)-*s*-triazine] is another compound which is reported to be dechlorinated in a biological system to form an hydroxyl group attached to a double-bonded carbon. Simazine is converted *in vivo* by corn seedlings and *in vitro* by corn extracts to hydroxysimazine [2-hydroxy-4,6-*bis*(ethylamino)-*s*-triazine] (Hamilton and Moreland, 1962; Montgomery *et al.*, 1969). Dechlorination in this case is mediated by a naturally occurring cyclic hydroxamate (2,4-dihydroxy-3-keto-7-methoxy-1,4-benzoxazine) or its glucoside. The reaction is thought to be primarily nonenzymatic, although Hamilton and Moreland (1962) suggested that dechlorination of simazine could be partially enzymatic. Perhaps dechlorination of the vinyl chlorine of phosphamidon is effected by an analogous mechanism in mammals.

Neither the proposed vinyl hydroxy metabolites nor the *N*-hydroxyethyl intermediates were excreted as conjugates in rat urine. This finding is somewhat surprising since the dicrotophos and monocrotophos *N*-hydroxymethyl metabolites were excreted as glucuronides in rat urine (Bull and Lindquist, 1964).

Metabolite III, proposed to be the vinyl hydroxy derivative of phosphamidon, corresponds to the metabolite III isolated by Clemons and Menzer (1968). Two other neutral phosphate ester metabolites (Clemons and Menzer, 1968), having isotope ratios of 2:1 and 1:1 $^{14}C:^{32}P$, compare with metabolites VI and VII, the *N*-hydroxyethyl intermediates of phosphamidon and des-*N*-ethyl phosphamidon. The deschloro phosphamidon amide reported by Clemons and Menzer (1968) corresponds to the proposed vinyl hydroxy phosphamidon amide. No deschloro phosphamidon amide was detected in the present study, as determined by cochromatography with a known standard on three thin-layer systems. Possibly the paper chromatographic system used by Clemons and Menzer (1968) was not adequate to resolve the deschloro phosphamidon amide from the vinyl hydroxy phosphamidon amide metabolite. Both the vinyl hydroxy and deschloro amide standards are eluted at the same position on the Celite column and the 2 compounds could only be resolved on thin-layer plates. Thus the earlier proposal by Clemons and Menzer (1968) for a reductive dechlorination reaction of phosphamidon was incorrect.

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