Oxidative Metabolism of Phosphamidon in Rats and a Goat

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The vinyl phosphate insecticide phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*,*N*-diethyl-3-hydroxycrotonamide) was administered orally to white rats and a female goat. Analysis by chromatographic procedures revealed the presence of nine organoextractable metabolites in goat urine, eight in rat urine, and six in goat milk. Only trace amounts of phosphamidon were found. Desethyl phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*-ethyl-3-hydroxycrotonamide) was detected only in the urine, while phosphamidon amide (dimethyl phosphate, ester with 2-chloro-3-hydroxycrotonamide) and deschloro phosphamidon amide (dimethyl phosphate, ester with 3-hydroxycrotonamide) were found in the milk as well. These metabolites are at least as toxic as phosphamidon itself. In addition, the hydrolytic product, α-chloro-*N*,*N*-diethylacetoacetamide, was detected in the urine and milk. Additional information is presented on the structures of other metabolites, although the complete structures have not been elucidated. Over 90% of the radioactivity in urine was unextractable with chloroform and is, therefore, assumed to be nontoxic, polar metabolites.

Since the discovery that trialkylphosphites react with α-halogen ketones and esters to form vinyl phosphate esters, a number of these compounds have appeared as potentially good insecticides. When a trialkyl phosphite reacts with an α-chloro-β-keto acid amide, vinyl phosphates with particularly high water solubilities are produced (Anliker *et al.*, 1961). This high water solubility leads to interest in these compounds as plant systemic insecticides. Three such plant systemics are phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*,*N*-diethyl-3-hydroxycrotonamide), Bidrin (dimethyl phosphate, ester with *N*,*N*-dimethyl-3-hydroxycrotonamide), and Azodrin (dimethyl phosphate, ester with *N*-methyl-3-hydroxycrotonamide). The metabolism of Bidrin (Bull and Lindquist, 1964; Menzer and Casida, 1965) and Azodrin (Lindquist and Bull, 1967; Menzer and Casida, 1965) in plants, insects, and mammals has been fairly well established. While the metabolism of phosphamidon in plants has been dealt with (Anliker *et al.*, 1961; Bull *et al.*, 1967; Jaques and Bein, 1960), little is known about the behavior of the compound in mammals.

Phosphamidon has shown good potential activity against certain forest pests, insect pests of field crops, certain cotton insects, some fruit and vegetable pests, and ornamental pests (Clemons, 1968). In some instances, phosphamidon demonstrates a certain degree of specificity (Bonnemaison, 1962; Kehat and Swirski, 1964; Rai *et al.*, 1964; Shorey, 1963), although this is not always the case (Bartlett, 1964; Finley, 1965; Prakash *et al.*, 1965).

The metabolism of phosphamidon in plants has been investigated by a number of workers. Jaques and Bein (1960) found that phosphamidon was partially converted to desethyl phosphamidon, a compound that is as toxic as phosphamidon to mice and rats. Anliker *et al.* (1961) also found the desethyl analog of phosphamidon in plants. The hydrolytic metabolites Anliker extracted from bean plants were the α-chloro-*N*,*N*-diethylacetoacetamide, α-chloro-*N*-ethylacetoacetamide, and dimethyl phosphate. All three of these metabolites are considered to be innocuous. The only toxic metabolite isolated (desethyl analog) is more unstable than phosphamidon itself and breaks down rather rapidly (Anliker *et al.*, 1961). Bull *et al.* (1967) have reported the presence of methyl phosphate and desmethyl phosphamidon as metabolic products in cotton plants. They also report limited evidence for the appearance of a hydroxyethyl phosphamidon metabolite.

With the exception of the recent paper of Bull *et al.* (1967), little is known of the metabolism of phosphamidon in animals. The most important metabolites of organophosphorus insecticides from a toxicological point of view are the neutral phosphate esters formed. However, the hydrolysis products commonly account for about 90% of the metabolites, and are nontoxic, being rapidly eliminated from the animal in the urine and feces. Bidrin, which closely resembles phosphamidon in structure, was shown to undergo a series of *N*-demethylation conversions, resulting in the production of toxic metabolites (Menzer and Casida, 1965). Phosphamidon differs structurally from Bidrin only in that it contains a vinyl chlorine and has ethyl groupings on the amide nitrogen. The amide moiety of the molecule is of prime interest in the study of its metabolism. The compound has already been shown to be de-ethylated in plants, and might well follow a similar dealkylation scheme as Bidrin in animals. A further suggestion that these oxidative *N*-dealkylations occur in the case of phosphamidon is derived from the fact that the compound is synergized by methylene dioxyphenyl compounds (Bull *et al.*, 1967; Sun and Johnson, 1965). The behavior of the vinyl chlorine is also of concern. The present investigation deals with the fate of that portion of the administered dose that has not been hydrolyzed at any time after administration.

EXPERIMENTAL

Materials. ^{32}P-Phosphamidon of specific activities 0.75, 1.50, and 7.00 mc. per mmole was obtained from the Radio-chemical Centre, Amersham, England. Phosphamidon-*N*,*N*-α-diethylamide-^{14}C was prepared by reacting *N*,*N*-diethylacetoacetamide-α-diethylamide-^{14}C with sulfuryl chloride and phosphorous acid, trimethyl ester, a modification of the method of Anliker *et al.* (1961). *N*,*N*-

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Diethylacetoacetamide-α-diethylamide-^{14}C (104.3 mg., 0.66 mmole) reacted with 147.15 mg. (109 mmoles) of sulfuryl chloride. The reaction mixture was held at room temperature for 30 minutes. Dilution was carried out with 15 ml. of benzene, and the reaction mixture was washed with ice-cold saturated sodium bicarbonate solution until the washes were neutral. The benzene was stripped with a rotary evaporator, and the residue was diluted in 5 ml. of chlorobenzene. The α,α-dichloro-*N*,*N*-diethylacetoacetamide solution was heated to boiling, 90.5 mg. (0.73 mmole) of phosphorous acid, trimethyl ester, were dripped in, and the mixture was refluxed for 1 hour. The resulting products were purified on a partitioning chromatographic column system described later. Two radioactive peaks resulted, one of which cochromatographed with known phosphamidon. Infrared spectra showed one peak to be the unreacted α,α-dichloro-*N*,*N*-diethylacetoacetamide and the other to be phosphamidon. The unreacted α,α-dichloro-*N*,*N*-diethyl acetoacetamide was reacted with excess phosphorous acid, trimethyl ester, three times. A total yield of 155 mg. (59.2%) of ^{14}C-phosphamidon was collected from all three reactions; specific activity was 0.49 mc. per mmole. Nuclear magnetic resonance studies showed the product to be 70% *trans*- and 30% *cis*-phosphamidon. All radiolabeled phosphamidon used in the treatment of animals consisted of a mixture of the geometric isomers. The chromatographic procedure employed in this study did not resolve geometric isomers of phosphamidon nor any metabolites found which retain the isomerism. The commercially available phosphamidon is also a mixture of the trans and cis isomers (Anliker, 1961).

The unsubstituted amide analog of phosphamidon, dimethyl phosphate, ester with 2-chloro-3-hydroxycrotonamide, was prepared by the following procedure. Diketene (14.4 grams, 0.171 mole) was added to 10 ml. of distilled water in an ice bath with the subsequent formation of two layers. Ammonia gas was bubbled through the mixture until the two phases became one. The reaction mixture was neutralized with 1*N* HCl and was extracted with an equal volume of chloroform three times. The chloroform solution was dried with sodium sulfate and filtered. The solvent was stripped off, leaving a residue of 5.1 grams (29%) of acetoacetamide. The amide (50 mmoles) was diluted in 20 ml. of chloroform and reacted with 14.2 grams (105 mmoles) of sulfuryl chloride (dropwise over a 10-minute period while the reaction vessel was cooled to room temperature in a water bath). The reaction temperature was increased to 40° to 50°C. and held for 3 hours. Volatile components were evacuated under vacuum and the residue was diluted in 50 ml. of chloroform and rinsed twice with 10-ml. portions of ice-cold saturated sodium bicarbonate (chloroform layer was neutral to litmus). The chloroform layer was dried with sodium sulfate and filtered. After the solvent was stripped, the product weighed 5.1 grams (59%). The chlorinated amide was dissolved in 20 ml. of chloroform and heated to boiling. Phosphorous acid, trimethyl ester (4.1 grams, 33 mmoles) was added dropwise over a 5-minute period. A water-cooled condenser was attached to the reaction vessel to collect any methyl chloride given off. The reaction continued for a total of 10 minutes. Upon evaporation of the solvent, 5.05 grams of crude products remained which were purified on a Celite column. The column yielded two large phosphorus-containing peaks and one smaller peak. Infrared spectra of the peaks showed that only the smaller one contained the unsubstituted amide moiety of the proposed metabolite. Nuclear magnetic resonance studies, showing an isomer ratio of 60% cis and 40% trans, were also consistent with this structure. Elemental analysis of this product gave the following results: Calculated for $C_6H_{11}NO_5ClP$: C 29.72%, H 4.12%, N 5.77%; found, C 29.95%, H 4.71%, N 5.68%. The total over-all yield was 2.55%.

An attempt to increase the yield of the unsubstituted amide by reaction of the unreacted chlorinated acetoacetamide from the above reaction with phosphorous acid, trimethyl ester, in chlorobenzene yielded an unknown phosphorus-containing peak. Infrared and NMR spectra showed that this unknown closely resembled the unsubstituted amide of phosphamidon, but lacked the chlorine. The infrared spectrum of a known sample of dimethyl phosphate, ester with 3-hydroxycrotonamide (deschloro phosphamidon amide) suggested that this structure was the same as the unknown. Apparently any unreacted chlorinated acetoacetamide remaining from the synthesis of the unsubstituted amide was α-chloroacetoacetamide and required the more severe reaction conditions of the chlorobenzene solvent to react with phosphorous acid, trimethyl ester. The infrared spectra of the phosphamidon amide and deschloro phosphamidon amide are presented in Figure 1. The primary amide moiety shows typical absorption in the range of 2.9 to 3.6 microns. The other notable feature in these spectra is the absence of the chlorine absorption in the deschloro phosphamidon amide spectrum. This absorption is present in the spectrum of the phosphamidon amide at approximately 10.6 microns. Nuclear magnetic resonance spectra were consistent with these structures also.

Other potential metabolites were obtained from the following sources: Deschloro phosphamidon (SD 5911; dimethyl phosphate, ester with *N*,*N*-diethyl-3-hydroxycrotonamide) was supplied by the Shell Development Co., Modesto, Calif. Samples of desethyl phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*-ethyl-3-hydroxy-

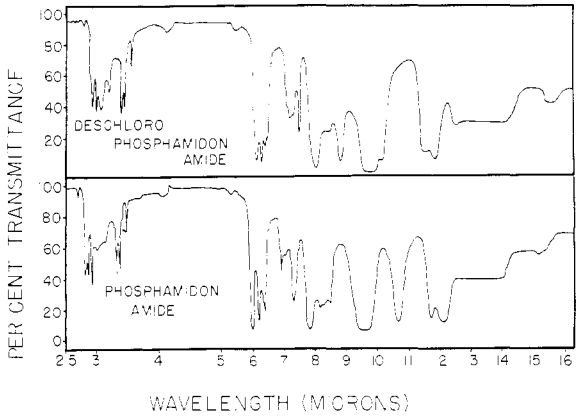

Figure 1. Infrared spectra of phosphamidon amide and deschloro phosphamidon amide

Spectra determined from 10% solutions in chloroform using Beckman IR8 spectrophotometer. Compounds were synthetic materials of proposed structures

crotonamide) and the chloroform-extractable hydrolysis products, α-chlorodiethylacetoacetamide and α-chloroethylacetoacetamide, were obtained from CIBA Agrochemical Co., Vero Beach, Fla. The dechlorinated forms of these acetoacetamides were prepared by classical procedures (Noller, 1957).

Treatment of Animals. White rats of both sexes (140 to 150 grams, Sprague-Dawley derived, Drug Detection and Development Organization, Inc., Bethesda, Md.) were used as test animals. The rats were anesthetized with ether, and the phosphamidon was administered via a stomach tube. The dosage was either 2.0 or 4.0 mg. per kg. The rats were placed in metabolism cages which were designed to provide for separate collection of urine and feces samples. Samples were collected at 6, 12, 18, 24, 36, 48, and 72 hours. The rats were killed at 72 hours, the livers removed and frozen at −20° C., and later fractionated.

A lactating Togenburg goat weighing 44.5 kg. was treated with ^{32}P-phosphamidon at an oral dosage of 3 mg. per kg. Five months later, the experiment was repeated on the same animal using a mixture of ^{32}P-phosphamidon and ^{14}C-phosphamidon. In the second treatment, the animal weighed 51.7 kg. The goat was placed in a metabolism stall and catheterized to allow continuous collection of urine. The radioactive material was divided equally among five gelatin capsules containing a small amount of a commercial cow feed. A balling gun was used to administer the capsules. Milk, urine, feces, and blood samples were collected at frequent intervals up to 72 hours after treatment, when only trace amounts of radioactivity could be detected. The blood sampling was omitted in the second treatment after the first had shown insignificant amounts of radioactivity present. The goat was killed immediately after the second treatment period by a vascular injection of pentabarbitol; the liver and kidneys were immediately removed and stored at −20° C.

Extraction and Analytical Procedures. Rat and goat urine samples were extracted three times with an equal volume of chloroform, using centrifugation to separate the two phases. The chloroform was removed on a rotary evaporator, and the residue containing chloroform-extractable metabolites was placed on Celite columns.

Milk samples were fractionated using a modified procedure of Timmerman *et al.* (1961); 100 ml. of fresh milk, except in early samples when 100 ml. were not obtained, were mixed with 150 ml. of warm (40° C.) acetonitrile. This resulted in the precipitation of the milk solids. The precipitate was washed with 150 ml. of chloroform, followed by 150 ml. of acetone. All solvents were combined, resulting in two layers—the acetonitrile-chloroform-acetone and the water from the milk. The two phases were separated, and the organic layer was dried with about 5 grams of anhydrous sodium sulfate. This procedure resulted in the formation of three fractions: the organic extractables, water-solubles, and the protein or solids fraction. An aliquot of each fraction was mixed in the appropriate scintillation counting mixture and assayed for radioactivity. The organic fractions, which contained enough radioactivity to make chromatographic procedures feasible, were stripped of the solvent and placed on Celite columns.

Liver and kidney fractionation was accomplished with a procedure outlined by Long (1961). Four fractions were obtained, designated as follows: nuclei and cell debris, mitochondria, microsomes, and supernatant. Each of the four fractions was assayed for radioactivity. Blood fractionation was accomplished by centrifuging 20 ml. of heparinized blood at 2900 r.p.m. (1490 G) in a Beckman Model L-2 ultracentrifuge using a 50T rotor to separate red blood cells from plasma. The red blood cells were counted after being washed by resuspension in isotonic saline (0.85%) and recentrifugation. The plasma, in 10 volumes of acetone, was centrifuged at 2900 r.p.m. (1490 G) to precipitate and separate the protein. The protein was then washed with acetone and the radioactivity determined. The acetone rinse was added to the supernatant from the protein precipitation and evaporated to 20 ml. total volume. A 2-ml. sample was assayed for radioactivity.

Partitioning coefficients (chloroform-water) were determined by phosphate analysis, radioactivity assay, or total weight. They are as follows: phosphamidon, 21.6; desethyl phosphamidon, 7.27; phosphamidon amide, 2.18; deschloro phosphamidon amide, 0.40; α-chloro-*N*,*N*-diethylacetoacetamide, 22.25; and α-chloro-*N*-ethylacetoacetamide, 6.56. Urine samples were extracted three times with chloroform as described later. Theoretical recoveries of these metabolities, if found, would be as follows (in per cent): phosphamidon, 99.99; desethyl phosphamidon, 99.82; phosphamidon amide, 96.89; deschloro phosphamidon amide, 63.56; α-chloro-*N*,*N*-diethylacetoacetamide, 99.99 and α-chloro-*N*-ethylacetoacetamide, 99.78. All reported recoveries are corrected for the partitioning characteristics of the metabolites.

The partitioning characteristics of phosphamidon plus the knowledge that phosphamidon partitioned from *n*-hexane into water suggested the use of a partitioning column to separate metabolites. The use of a partitioning column was further indicated by the strong resemblance of phosphamidon to the insecticide Bidrin. Menzer and Casida (1965) used a Celite partitioning column system to resolve the oxidative metabolites of the above insecticide. After trial of various modifications of their method the following system was found to be most satisfactory; 20 ml. of distilled water was added to 32 grams of Celite and mixed until it was homogeneous. Further homogeneity was achieved by addition of *n*-hexane and blending in a Waring Blendor at medium speed for about 3 minutes. This slurry was packed to a height of 50 cm. in a 19-mm. I.D. column. Material to be resolved was stripped of solvent to a volume of approximately 4 ml.; sufficient Celite was added to adsorb the solvent, and the material was dried with air. This Celite plus metabolites was added to the packed column, and the container was rinsed once with about 1 ml. of chloroform. Hexane, followed by varying ratios of hexane and chloroform to yield increasing polarity in the solvent, was used for elution. The final solvent was pure chloroform. The material in the column was removed and washed with methanol to extract any material remaining after elution. This washing process was aided by suction filtration. Ten-milliliter fractions from the columns were collected and assayed for radioactivity and/or phosphorus content. The method of Allen (1940) was utilized for phosphorus determination on

individual fractions obtained from column chromatography.

Two descending paper systems were used for cochromatographing certain metabolites with known samples of theoretical metabolites. In system A (Anliker and Menzer, 1963), the material was spotted on Whatman No. 1 filter paper and developed in the upper phase of a mixture of petroleum ether (b.p. 90–100° C.)–toluene–methanol–water (5:5:7:3). The developing chamber was saturated beforehand with both the upper and lower phases of the above mixture. In system B (Bull *et al.*, 1967) the organo-extractable products were spotted on Whatman No. 3 filter paper which had previously been saturated with dimethylformamide (20% in acetone, by volume), and developed with cyclohexane also saturated with dimethylformamide.

Radioactivity was assayed in a Packard Model 3375 liquid scintillation spectrometer. Three different scintillation counting solutions were employed. Organosoluble products were assayed in a standard scintillation solution consisting of 0.3 gram of dimethyl POPOP [1,4-bis-2-(methyl-5-phenyloxazolyl)-benzene] and 5.0 grams of PPO (2,5-diphenyloxazole) per liter of toluene. For the counting of water-soluble materials, two counting mixtures were employed. The first was a solution of 60 grams of naphthalene, 4 grams of PPO, 0.2 gram of dimethyl POPOP, 100 ml. of absolute methanol, 20 ml. of ethylene glycol, and 1,4-dioxane to make a total volume of 1 liter (Bray, 1960). A more efficient aqueous counting solution which would accommodate more water in the sample was used in the later stages of this problem. It consisted of 5.5 grams of PPO and 200 mg. of dimethyl POPOP brought up to 1 liter with a mixture of toluene–Triton-X-100 (2 to 1). Counting of solid materials such as milk solids, feces, and crystalline derivatives was accomplished by using thixotropic gel powder (CAB-O-Sil, Packard Instrument Co., Downers Grove, Ill.). The sample in the proper scintillation mixture was gelled by the addition of the above powder, and assayed for radioactivity. The efficiency of this method was superior to a thin window gas flow counting procedure tried earlier.

Isotope Dilution Procedure. The hydroxymethyl intermediates in biological *N*-demethylation schemes are acid and heat labile and yield formaldehyde upon degradation (Casida *et al.*, 1954a, 1954b). Hydroxyethyl intermediates in the oxidative metabolism of phosphamidon would be expected to liberate acetaldehyde in a similar fashion. The following isotope dilution procedure was devised to obtain evidence of radiolabeled acetaldehyde. Approximately 200 c.p.m. of the resolved metabolite from the Celite column was added to a glass tube, and the solvent was evaporated with air. Unlabeled acetaldehyde (0.1 ml.) was added along with 1 ml. of a 1% HCl solution. The tube was sealed and placed in a boiling water bath for 1 hour. The tube was broken, and enough sodium bicarbonate was added to neutralize the solution. Semicarbazone formation was accomplished by adding 0.1 gram of semicarbazide hydrochloride and 0.15 gram of sodium acetate, resealing the tube, placing it in a boiling water bath for 5 minutes, and then allowing the tube to reach room temperature in the water bath. The tube was placed at 4° C. and allowed to stand until crystals of the semicarbazone formed. The crystals were removed by suction filtration and assayed for radioactivity. The per cent yield of the semicarbazone of acetaldehyde (m.p. 160° C.) was 40.6% when the procedure was carried out using known ^{14}C-acetaldehyde.

RESULTS

Chemical Nature of Metabolites. Five chloroform-extractable metabolites were isolated from goat and rat urine and goat milk. Two additional metabolites, metabolites I and II, were present in rat and goat urine that were not present in milk. In addition, metabolites IV and VII were present in goat milk and urine but not in rat urine. Unchanged phosphamidon occurred only in trace amounts in a few instances. The chromatographic positions of the isolated metabolites are shown in Figure 2.

Four of the nine metabolites have been characterized. Metabolite I cochromatographed with known unlabeled desethyl phosphamidon in the column chromatographic system and in paper system B. In addition, the ^{32}P to ^{14}C isotope ratio indicated that an ethyl group had been lost. Metabolites V and VIII contained the radioactive phosphorus, but both ^{14}C-ethyl groups had been removed. Metabolite V cochromatographed with the synthesized unsubstituted amide analog of phosphamidon. Metabolite VIII cochromatographed with deschloro phosphamidon amide. Metabolite IX, in chromatographic systems A and B and the column system, cochromatographed with α-chloro-*N*,*N*-diethylacetoacetamide. Two of the above metabolites, the phosphamidon amide and the deschloro phosphamidon amide, have not been previously reported.

Of the remaining five products, metabolite III was most interesting. It contained both labeled carbon atoms and the phosphorus; furthermore, it was one of the major metabolites in all systems studied. It did not cochromatograph with deschloro phosphamidon, and the isotope dilution procedure indicated that this material did not liberate ^{14}C-acetaldehyde upon hydrolysis. The other unknown metabolites occurred in lesser amounts, and the isotope ratios were as indicated in Figure 2. Metabolites

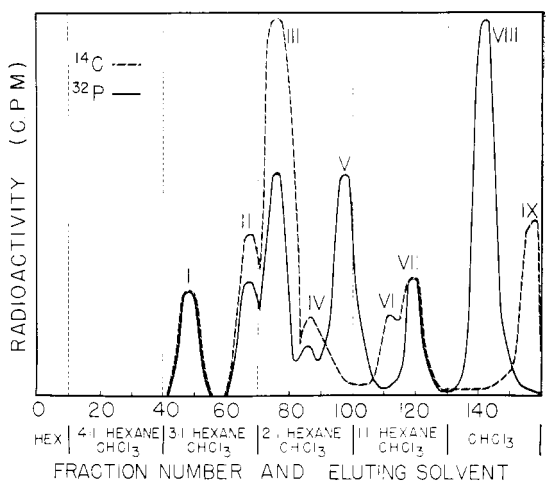

Figure 2. Chromatographic characteristics of phosphamidon metabolites on Celite column based on partitioning between water and hexane-chloroform mixtures

Reproduction of results obtained from chromatography of chloroform-extractable materials from 1-hour sample of goat urine

II and IV occurred in minor amounts and contained the same isotope ratio as III. Metabolite VI contained only the labeled carbon and did not cochromatograph with α-chloro-*N*-ethylacetoacetamide, *N*,*N*-diethylacetoacetamide, or *N*-ethylacetoacetamide. Metabolite VII lost one of the ^{14}C-ethyl groups and did not appear to be acetaldehyde-liberating.

Biological Activity of Phosphamidon and Its Metabolites. LD_{50} values determined in male white mice by intraperitoneal injections of aqueous solutions of phosphamidon and its metabolites are as follows: phosphamidon, 6.0 mg. per kg.; desethyl phosphamidon, 7.0 mg. per kg.; phosphamidon amide, 2.5 mg. per kg.; and deschloro phosphamidon amide, 2.5 mg. per kg. An LD_{50} value of the α-chloro-*N*,*N*-diethylacetoacetamide was not determined; it has been reported to exhibit a low level of mammalian toxicity (Jaques and Bein, 1960).

Urine Results. The majority of the recoverable dose was excreted in the urine within 24 hours after treatment (Figure 3). Higher per cent recoveries were obtained in rat urine than in goat urine. In both male and female rats and in the goat there was always less radioactive carbon recovered than labeled phosphorus. Per cent recoveries calculated from data of all rats used in the study (48 animals of each sex) showed that there was a definite sex difference in the amount of recoverable ^{32}P in the urine. The females gave an average recovery of 64.75% while the males gave 52.31%. Table I shows that the majority of the radioactivity in the urine was in the form of water-soluble hydrolysis products. This table also lists the per cent recovery of each metabolite in the urine. The methanol extraction of the Celite removed from the column (hereafter designated methanol fraction), although not investigated further, is believed to consist of hydrolysis products which were trapped as emulsions in the chloroform fraction during extraction of the urine. In rats, the major product was metabolite III; in the goat, the deschloro unsubstituted amide, metabolite VIII, predominated, followed by metabolite III. The only breakdown product not occurring in both species was metabolite IV.

Results of Milk Studies. Treatment of the goat with ^{32}P- and ^{14}C-phosphamidon resulted in very small amounts of labeled compounds in the milk. The percentages of the administered radiolabels appearing in the milk within the 72-hour sampling period were 1.95 for ^{32}P-phosphamidon and 2.08 for ^{14}C-phosphamidon. Secretion of ^{14}C into the milk reached a maximum between 8 and 12 hours after treatment when 0.37% of the administered dose was recovered. However, the ^{32}P activity was secreted more slowly, the maximum being reached between 36 and 48 hours after treatment with 0.36% recovered. Figure 4 illustrates the secretion rate of each radioisotope in the

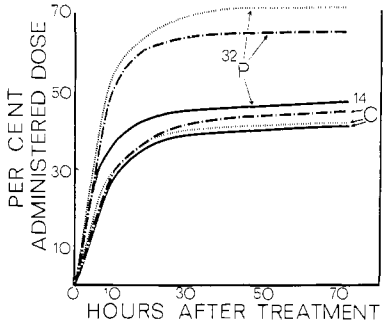

Figure 3. Cumulative per cent recovery of administered ^{32}P- and ^{14}C-phosphamidon in whole urine of white rats and a goat

———Goat urine
· · · · · · · ·Female rat urine
—·—·—Male rat urine

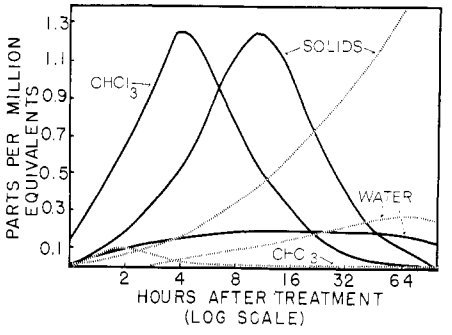

Figure 4. Parts per million equivalents of ^{32}P- and ^{14}C-phosphamidon in fractionated goat milk following oral administration of 3 mg. per kg.

Addition of warm acetonitrile precipitated the solids fraction. Extraction of the supernatant with chloroform yielded chloroform and water fractions
———^{14}C; · · · · · · · · ^{32}P

Table I. Occurrence of Metabolites of Phosphamidon in Goat and Rat Urine after Oral Administration of ^{32}P- and ^{14}C-Phosphamidon

	6 Female Rats		6 Male Rats	1 Goat		
Metabolites[a]	6 hr.	12 hr.	6 hr.	1 hr.	2 hr.	6 hr.
I	0.025	...	0.004	0.003	0.001	0.004
II	0.007	...	...	0.001	0.002	...
III	0.063	0.006	0.026	0.011	0.010	0.014
IV	...	...	...	0.001	0.001	0.003
V	0.003	0.002	0.011	0.012	0.014	0.016
VI	0.008	...	0.017	0.001	0.004	0.002
VII	...	...	...	0.003	0.005	0.014
VIII	0.004	0.001	0.021	0.011	0.048	0.148
IX	0.008	0.005	0.014	0.005	0.004	0.033
Methanol fraction	0.021	0.013	0.018	0.002	0.364	0.711
Hydrolysis products, %	41.24	14.48	31.85	2.34	4.14	16.57

[a] Expressed as per cent of administered dose. Calculations for all metabolites except VI and IX are based on ^{32}P content; calculations for metabolites VI and IX are based on ^{14}C activity.

three milk fractions. Chloroform-extractable ^{32}P activity was secreted in the largest amounts between 1 and 2 hours after treatment. ^{32}P activity in the water fraction and in the solids fraction reached maximum amounts much later. The phosphorus incorporation into the solids fraction was still increasing at 72 hours, while in the water fraction it was at a maximum between 60 and 72 hours after treatment. The ^{14}C activity reached approximately equal levels in the chloroform-extractable and solids fractions with the former reaching its maximum at 4 hours and the solids fraction peaking at 12 hours. The level of ^{14}C activity in the water fraction increased gradually through 16 hours and then declined.

Table II lists the recoveries of each metabolite resolved from the chloroform-extractable milk fractions on Celite columns. The 4-hour milk metabolites eluting after metabolite III were unresolved because of an improper solvent mixture and are not reported separately in Table II. They are included in the figure for the methanol fraction. The major product was metabolite III which occurred at a maximum level between 2 and 4 hours following treatment. Metabolites I, II, and VI were never present in the milk. Two peaks consisting almost entirely of ^{14}C activity occurred in the hexane elution of milk columns. Evaporation of solvents from these fractions yielded a lipoidal material that gave a positive test for phosphorus. The radioactivity in these peaks was not included in Table II and represented the following percentages of the administered dose of ^{14}C activity: 0.001, 0.005, 0.021, and 0.014 at 1, 2, 4, and 6 hours, respectively. Recoveries of administered ^{32}P activity were less than 0.001% of the administered dose in the above peaks mentioned at each time interval.

Total Recovery of Administered Radioactivity. Table III lists individual recoveries of ^{32}P- and ^{14}C-phosphamidon equivalents in the tissues or excretions studied for the total sampling periods. Less ^{14}C was recovered than ^{32}P in both sexes of rats and in the goat. In the goat liver and kidneys, the highest levels of activity were found in the microsomes and supernatant. In all tissues and excretions studied except the goat milk, ^{32}P activity predominated over ^{14}C activity.

DISCUSSION

Nine metabolites of phosphamidon have been isolated. Structural identification of four of these has been based on cochromatography with known compounds and isotope ratios. Of these four products, three are oxidative metabolites, while one is a hydrolysis product. From the evidence of the structures of these products, the phosphamidon molecule is apparently altered in at least two places which can result in toxic metabolites: The vinyl chlorine is replaced by a hydrogen, and the substituted alkyl amide moiety is de-ethylated. LD_{50} values obtained on compounds of known structure indicated that the de-ethylation reactions result in more toxic phosphorus esters. The mechanism of the dechlorination is not clear at this point. Of the five remaining unknown metabolites, two are major in relative amounts produced while three are minor and are not completely separated from the major unknowns. Metabolite III did not lose either of the ^{14}C-ethyl groups and does not appear to liberate acetaldehyde upon hy-

Table II. Occurrence of Metabolites of Phosphamidon in Goat Milk after Oral Administration of ^{32}P- and ^{14}C-Phosphamidon Expressed as P.P.M.-Phosphamidon Equivalents[a]

Time, Hr.	Metabolites									Methanol Fraction
	I	II	III	IV	V	VI	VII	VIII	IX	
1	...	...	0.012	0.002	0.014	...	0.012	0.006	0.006	0.104
2	...	...	0.008	...	0.004	...	...	0.006	0.006	0.014
4[b]	...	...	0.016	[b]	[b]	[b]	[b]	[b]	[b]	0.034[b]
6	...	...	0.004	...	...	...	...	0.004	0.009	0.011

[a] Calculations of p.p.m. of all metabolites except IX are based on ^{32}P content, while metabolite IX is based on ^{14}C content.
[b] Amounts of metabolites eluting after metabolite III are included in the figure for the methanol fraction since they were not resolved.

Table III. Recovery of ^{32}P- and ^{14}C-Phosphamidon Equivalents from Tissues and Excretory Products of Experimental Animals Expressed as Per Cent of Administered Dose (72-Hour Sampling Period)

	Rats				Goat	
	Female		Male			
	^{32}P	^{14}C	^{32}P	^{14}C	^{32}P	^{14}C
Urine	71.823	42.741	67.432	44.296	47.113	41.684
Feces	...	12.642	...	10.093	...	0.072
Milk	...	...	...	...	1.946	2.083
Liver						
Nuclei and cell debris	...	0.210	...	0.474	0.056	0.021
Mitochondria	...	0.355	...	0.333	0.097	0.031
Microsomes	...	0.155	...	0.146	0.194	0.054
Supernatant	...	0.827	...	0.672	0.104	0.032
Kidney						
Nuclei and cell debris	...	...	...	...	0.016	0.002
Mitochondria	...	...	...	...	0.013	0.002
Microsomes	...	...	...	...	0.075	0.012
Supernatant	...	...	...	...	0.075	0.016
Ashes (residual activity after incineration)	...	...	...	...	0.005	0.001

drolysis. This latter evidence suggests that it is not an *N*-hydroxyethyl intermediate. Since it does not cochromatograph with deschloro phosphamidon, it is not simply the dechlorinated parent material. The two minor metabolites, II and IV, also contain both ^{14}C-ethyl groups, and if the *N*-hydroxyethyl analog of phosphamidon (dimethyl phosphate, ester with 2-chloro-*N*-ethyl-*N*-hydroxyethyl-3-hydroxycrotonamide) is produced, it would appear to be one of these metabolites. This suggestion is further supported by the fact that the *N*-hydroxyalkyl metabolites of other insecticides are unstable and generally do not occur in major amounts (Abdel-Wahab *et al.*, 1966; Bull and Lindquist, 1964; Hodgson and Casida, 1961; Menzer and Casida, 1965). Metabolite VI is not completely separated from metabolite VII. It contains only the carbon label and does not cochromatograph with any acetoacetamides. No speculation as to its identity is offered. Metabolite VII has lost one of the ^{14}C-ethyl groups and does not liberate acetaldehyde upon hydrolysis. From this information, it does not appear to be the *N*-hydroxyethyl analog of desethyl phosphamidon and would more likely be the dechlorinated form of desethyl phosphamidon. No known sample of deschloro desethyl phosphamidon was available for comparison.

To gain more information on the unknown metabolites, it would be valuable to synthesize known compounds for chromatographic and other comparisons. A synthesis procedure analogous to the one used by Menzer and Casida (1965) was employed to produce the *N*-hydroxyethyl intermediates in this study. The results were ambiguous, however; no compounds were produced that would cochromatograph with any of the metabolites.

Approximately 70% of the administered dose of phosphamidon was eliminated in the rat urine and 45% in the goat urine. The low recovery of the administered dose in the goat may be partially explained by difficulties encountered in the treatment of the animal. The animal regurgitated one of the capsules, which was then readministered. However, the capsule was partially dissolved and some of the material may have been lost in the process. All data are treated as if the entire dose was administered. A preliminary treatment of the goat with only ^{32}P-phosphamidon may provide more realistic figures. This treatment gave recoveries of 75.04% in the urine and 12.4% in the feces. Over 90% of the activity in the urine was present as hydrolysis products, but characterization of hydrolysis products was beyond the scope of this study. These materials could include such products as dimethyl phosphate, *O*-desmethyl phosphamidon, and phosphamidon acid (dimethyl phosphate, ester with 2-chloro-3-hydroxycrotonic acid). Bull *et al.* (1967) suggest acetic acid, acetoacetic acid, and chloroacetoacetic acid as additional hydrolysis products. Such materials are generally considered insignificant as toxic agents.

Milk was the only animal material where ^{14}C activity predominated over ^{32}P activity. This situation may be due to incorporation of the carbon radioactivity into lipoidal materials in the milk. The fact that all other tissues and excretions studied gave lower recoveries of ^{14}C indicates that de-ethylation is occurring.

The new metabolites, the phosphamidon amide and deschloro phosphamidon amide, must be considered in residue studies. They appear to create no new dangers in the field use of phosphamidon. They are more toxic than phosphamidon, but do not persist in large amounts for long periods of time in urine or goat milk.

This study has established that oxidative de-ethylation of phosphamidon does occur, and that it appears to be an important route in the metabolism of phosphamidon in animals. Only trace amounts of phosphamidon itself, larger amounts of desethyl phosphamidon, and significant quantities of the phosphamidon amide were recovered. Bull *et al.* (1967) report the presence of desethyl phosphamidon and a material claimed to be hydroxyethyl phosphamidon in cotton plants, alfalfa, and insects. They did not report the presence of phosphamidon amide or deschloro phosphamidon amide, although several unknown metabolites were present.

ACKNOWLEDGMENT

Invaluable materials, advice, and assistance were provided by W. F. Williams of the Dairy Science Department, W. Reeve and P. F. Alluotto of the Chemistry Department, and E. J. Schultz of the Livestock Sanitary Service, University of Maryland. Valuable technical assistance was provided by Norma Best and Thomas Shea of the Department of Entomology.

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Received for review October 20, 1967. Accepted January 8, 1968. Scientific article A1386, Contribution No. 3983, Maryland Agricultural Experiment Station, Department of Entomology. Part of a thesis presented to the Graduate School, University of Maryland, by the first author in partial fulfillment of the requirements for the master of science degree. Study supported by grant No. ES00121 from the National Institutes of Health, Division of Environmental Health Sciences.