Metabolism of O-Phenyl-O′-(4-nitrophenyl) Methylphosphonothionate (Colep) in Plants and Animals

G. J. MARCO and E. G. JAWORSKI
Agricultural Division, Monsanto Chemical Co., St. Louis, Mo.

The time course metabolism of Colep was followed for 28 days in apple and cotton seedlings. Thin layer chromatography in several solvent systems of the benzene, chloroform, and water extracts of the apple and cotton leaves showed a variety of radioactive compounds other than Colep. Because acid hydrolysis converts most of the radioactivity in all extracts to phenol, the variety of radioactive compounds separated by chromatography represent phenolic-containing materials. Within 24 hours, most of the radioactivity from an oral dose of Colep is excreted in the urine of rats. No activity is found in the respired CO_2 or fat tissue. Little activity is found in the intestines, liver, kidneys, or feces. Thin layer chromatographic and ion exchange analyses of the urine indicate the presence of a mixture of polar phenolic conjugates.

While reports pertaining to the development, use, and toxicity of phosphonate insecticides have appeared in the literature (*5*, *8*, *14*, *15*), very little information has appeared regarding their metabolism in plants and animals. Arthur and Casida (*2*) reported that the metabolic breakdown of *O,O*-dimethyl-2,2,2-trichloro-1-hydroxyethyl phosphonate(Dipterex) involves direct cleavage of the phosphonate bond rather than a dehydrochlorination and rearrangement to form the vinyl derivative as had been speculated earlier. They suggested that its low relative toxicity to mammals might be due to the ease of hydrolysis of the phosphonate by mammalian serum esterases. Furthermore, enzymatic cleavage of this phosphonate yielded trichloroethanol which was conjugated with glucuronic acid and excreted as urochloralic acid. Ahmed *et al.* (*1*) indicated that bovine rumen fluid rapidly metabolized EPN, *O*-ethyl-*O*-*p*-nitrophenyl phenylphosphonothioate.

The results presented herein are based on radiotracer studies with a new organophosphonate insecticide, *O*-phenyl-*O′*-(4-nitrophenyl) methylphosphonothionate (Colep, registered Monsanto trademark). The metabolic fate of this highly active insecticide was studied in cotton, apples, and rats.

Experimental

Plant Methods. Application of Insecticide. *O*-Phenyl-C^{14}-*O′*-(4-nitrophenyl) methylphosphonothionate (hereafter referred to as Colep-C^{14}) was synthesized with uniformly labelled phenol to a specific radioactivity of 0.97 mc. per mmole. A solution of the compound in ethanol (5.2 mg. per ml.) was applied by microsyringe to the individual leaves. Four leaves per plant of a Red Delicious graft seedling about 1 foot tall and having 10 to 15 leaves were treated. Ten microliters of the above solution were applied uniformly over each leaf having a 1- to 2-sq. inch area. The second pair of true leaves (about 2 sq. inches in area) of 3- to 4-week-old cotton plants (D and PL Fox variety) were treated in the same manner as the apple leaves. The application rates represented about 0.75 pound per acre. The recommended field rate is 0.25 to 0.5 pound per acre. All plants were watered by subirrigation.

Harvesting of Plant Tissues. Harvests were made by cutting the plants at the soil line at varying time intervals as follows—apple: 4, 7, 14, 28 days following treatment; cotton: 4, 14, 28 days following treatment. The treated leaves were collected by cutting at the base, excluding the petiole.

Extraction of Plant Tissue. The treated leaves were first washed by passage through three 20-ml. portions of benzene. The benzene portions were pooled and brought to a 100-ml. volume. The washed leaves were combined and homogenized by the method of Bligh and Dyer (*3*) in a conical, ground glass Kontes homogenizer with 6 ml. of CH_3OH–$CHCl_3$ (2:1, v./v.), using a solvent-to-tissue ratio of 6 to 1. Two milliliters of chloroform were added and the tissue rehomogenized. Finally 2 ml. of water were added, followed by homogenization. The resulting mixture was vacuum-filtered on a Büchner funnel. The residue was rehomogenized by the above procedure. The combined filtrates were separated into the chloroform and water layers. The chloroform fraction was washed with distilled water and the water fraction with chloroform. The chloroform fractions were combined and brought to a 25-ml. volume, as were combined water fractions.

The residue was further homogenized in 80% aqueous ethanol at a solvent-to-tissue ratio of 20 to 1. The homogenate was filtered on a Büchner funnel and the residues washed with additional aqueous ethanol until they were gray to pale yellow in appearance. The filtrate and washings were pooled and brought to a 25-ml. volume. All fractions (benzene, chloroform, ethanol, and water) and remaining fibrous residues were assayed for radioactivity.

Fractionation of Extracts. A 12-ml. bed of an anion exchange column was converted to its formate form (*4*, *19*). A 12-ml. bed of Rexyn AG 1 (Cl^{-1}, SO_4^{-2}) (Fisher Scientific Co., St. Louis, Mo.), 40- to 100-mesh, analytical grade, was treated with 250 ml. of 1*N* sodium formate to convert the resin to the formate form and rinsed with 5 bed volumes of distilled water. An aliquot of the aqueous plant extract was passed through, followed by a distilled water wash of 5 bed volumes. The pooled effluents represent the water-soluble organic base–neutral fraction. The column was eluted with 5 bed volumes of 6*N* HCl, the eluate representing the organic acid fraction.

An aliquot of organic base–neutral effluent was passed through a 12-ml. bed of a cation exchange column, Rexyn AG 50 (Na) (Fisher Scientific Co., St. Louis, Mo.), 40 to 100 mesh, analytical grade. The column was rinsed with 4 bed volumes of distilled water. The pooled effluents represent the organic neutral fraction. The column was eluted with 2 bed volumes of 1*N* NaOH, the eluate representing the organic base fraction.

The exchange, wash, and elution steps were all carried out at flow rates of 0.5 to 1 ml. per minute.

The distribution of radioactivity within fractions, and its association with definite

compounds, was achieved by thin layer chromatography followed by radioactive assay.

Animal Methods. METABOLIC CAGE STUDIES. Female albino rats, Wistar strain, weighing 150 to 160 grams, were maintained *ad libitum* on Purina Rat Chow and water in standard cages before use. Each rat was preconditioned for two 24-hour periods in an all-glass metabolism cage. While in the metabolism cage, water but no food was available. Air, made CO_2-free by passage through a soda-lime column, was drawn by suction through the cage at a rate of 500 to 600 ml. per minute. The respiratory gases were bubbled through 250 ml. of 1*N* NaOH to trap CO_2. The feces were separated from urine by a grooved, solid-glass tear covering the flask contained in the collection well. A plastic corset was placed around the rat's midsection to prevent turning. A solution of Colep-C^{14} was prepared in undenatured ethanol (1.0 mg. per ml.), and the dose was administered by a stomach tube which consisted of a 6-cm., size 17, thin-wall Teflon tube attached to a 1-ml. glass syringe.

EXTRACTION OF ANIMAL COMPONENTS. After a 24-hour period in the metabolism cage, the rat was sacrificed by cervical dislocation. The liver, kidneys, intestines, and abdominal fat layer were immediately excised. The tissues and feces were homogenized by the method of Bligh and Dyer (*3*) in a conical, ground glass Kontes homogenizer. While the following values are expressed on the basis of 100 grams wet weight, the actual volumes used were proportional to the wet weight of tissue. Each 100 grams of tissue was homogenized for 2 minutes with 300 ml. of CH_3OH–$CHCl_3$ (2:1, v./v.). To the mixture was added 100 ml. of $CHCl_3$, and after rehomogenization for 30 seconds, 100 ml. of distilled water was added and rehomogenization continued for another 30 seconds. The homogenate was vacuum-filtered through Whatman No. 1 filter paper on a Büchner funnel. The combined filtrates were separated into the chloroform and water layers. The chloroform fraction was washed twice with one-third its volume of distilled water and the water fraction with one-third its volume of chloroform. The chloroform fractions were combined and brought to an appropriate minimum volume. The combined water fractions were treated in a similar manner.

The urine was rinsed from the collection flask with distilled water and brought to an appropriate minimum volume.

FRACTIONATION OF URINE. The weak cation exchange resin, Rexyn CG 51 (H^+) (Fisher Scientific Co., St. Louis, Mo.), 100- to 200-mesh, chromatographic grade, was converted to the sodium form with 1*N* NaOH. A 10-cm. × 3-mm. i.d. glass column with Teflon stopcock was filled to 6 cm. with the resin representing 0.3-gram dry weight and a 0.4-ml. bed volume. A 0.5-ml. aliquot of urine was passed through, followed by a 1.5-ml. wash of 80% aqueous methanol at a rate of 0.05 ml. per minute for a 2.0-ml. total effluent. The column was then eluted with 2.0 ml. of 0.1*N* NaOH.

The weak anion exchange resin Rexyn CG 8 (Cl^{-1}, SO_4^{-2}) (Fisher Scientific Co., St. Louis, Mo.), 100- to 200-mesh, chromatographic grade, was converted to the formate form with 1*N* sodium formate (*2*, *3*). A column similar in size to the one for the cation resin was prepared.

A 1.5-ml. aliquot of the above effluent was passed through, followed by a 1.0-ml. wash of 80% aqueous methanol at 0.05 ml. per minute to a 2.5-ml. total effluent. The column was then eluted with 2.0 ml. of 1*N* HCl.

Analytical Methods. THIN LAYER CHROMATOGRAPHY. Ascending one-dimensional thin layer chromatography was performed on glass slips (8 × 1/2 × 3/16 inches) coated with a 250- to 275-micron layer of Silica Gel G (Brinkmann Instruments, Inc., and Merck and Co., Darmstadt, Germany) of about 250-mesh. The sample was applied 2 cm. from one end and developed to a 15-cm. solvent front. Development was performed in 24/22 glass-stoppered test tubes (1 × 9 1/2 inches). The following solvent systems were used:

A: ether–hexane–acetic acid	100:50:1 (v./v.), running time 30 minutes at 25° C.
B: [chloroform–1*M* NH_4OH (10:1, v./v.)]–methanol	97:3 (v./v.), running time 45 minutes at 25° C.
C: epiphase: butanol–water–acetic acid	50:48:2 (v./v.), running time 5 hours at 25° C.
D: ethyl acetate–[isopropanol–water (2:1, v./v.)]	65:35 (v./v.), running time 45 minutes at 25° C.
E: chloroform–methanol–water	65:38:8 (v./v.), running time 2 hours at 25° C.

For collection of fractions for further identification, large plates (8 × 8 × 3/16 inches) coated with 250 to 275 microns of Silica Gel G were used. Development was performed in glass containers (9 × 9 1/2 × 4 inches). Running times of the solvents were the same as the small slips. Desired areas, determined from analyses of a small slip, were removed with a razor blade and eluted with the appropriate solvent.

Table I. Thin Layer Chromatography of Possible Metabolites of Colep

Compound	R_f Value[a] in Solvent System A	B	C	D	E
Colep	0.53	0.70	0.73	0.77	0.90
Oxon	0.20	0.60	0.77	0.70	0.91
Phenol	0.53	0.27	0.77	0.73	0.80
Phenyl glucoside	0	0	0.49	0.35	0.30
Phenylmethyl phosphonate	0	0	0.25	0.10	0.13

[a] The R_f value was taken at the center of the developed spot.

R_f values for the insecticide, some potential breakdown products, and various naturally occurring metabolites are presented in Table I.

COLOR REAGENTS. Colep and its oxon [*O*-phenyl-*O'*-(4-nitrophenyl) methylphosphonate] were located by spraying with 10% methanolic KOH. A yellow color developed from released *p*-nitrophenol.

Phenol was located by spraying with 10% methanolic KOH followed by a 1:1 mixture of 1% aqueous sulfanilic acid and 1% sodium nitrite. A bright yellow spot developed.

Phenyl glucoside and monophenyl methyl phosphonate were stained brown in iodine vapors at 50° C.

RADIOACTIVITY ASSAYS. Solid residues dried in a vacuum desiccator over P_2O_5 were gound in a Wiley mill to 60-mesh. A weighed sample of the resulting powder was counted as a suspension in counting solution containing 4% Cab-O-Sil (*12*). Liquid samples were counted as solutions using the Packard Tricarb liquid scintillation counter.

Aqueous solutions were counted by using a solution of xylene (5 parts), dioxane (5 parts), and ethanol (3 parts) in which naphthalene (80 grams per liter), 2,5-diphenyloxazole (5 grams per liter), and dimethyl POPOP [1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene] (200 mg. per liter) were dissolved (*10*). Nonaqueous solutions were counted in toluene containing 2,5-diphenyloxazole (4 grams per liter) and dimethyl POPOP (200 mg. per liter) (*7*).

The 1/2-inch thin layer chromatogram slips were divided into 16 1-cm. fractions bracketing both the origin and solvent front. The thin silica gel layer of each fraction was scraped with a flat-tipped spatula into a 15-ml. scintillation vial and suspended in counting solution containing 4% Cab-O-Sil.

All radioactivity determinations were carried out to give a probable error of

less than 1%, and counting data were corrected for background and counter efficiencies.

CHEMICAL AND BIOCHEMICAL TREATMENT OF EXTRACTS AND FRACTIONS. For emulsin hydrolysis, a 1- to 2-ml. aliquot of a particular extract or fraction was evaporated to dryness by a stream of nitrogen gas in small test tubes (75 × 10 mm.). After drying under nitrogen, a 0.2-ml. solution of 1% emulsin (β-glucosidase from almonds obtained from Sigma Chemical Co., St. Louis, Mo.) in 0.15*M* phosphate–citrate buffer (*11*) (pH 4.7) containing 3*M* methanol (*6*) was added. The mixture was incubated for 20 hours at 38° ± 0.5° C. in a constant temperature shaker bath. After incubation, 0.4 ml. of 0.5*M* methanolic KOH was added and the mixture immediately evaporated to dryness by a nitrogen gas stream. A 0.2-ml. aliquot of 1*M* methanolic HCl containing carrier phenol (0.1 mg. per ml.) was added, contents mixed with a microstirrer, and centrifuged at 1500 r.p.m. for 5 minutes. A 100-μl. aliquot of the clear supernatant liquid was spotted on a 1/2-inch thin layer chromatography slip. After development, the slips were immediately sprayed with 10% methanolic KOH and diazotized sulfanilic acid. A 100-μg. sample of phenyl β-glucoside was also run, and the released phenol was chromatographically identified as a check on emulsin activity.

For base hydrolysis, a 1- to 2-ml. aliquot of an extract or fraction was evaporated to dryness by a stream of nitrogen gas in small test tubes (75 × 10 mm.). A 0.1-ml. portion of 0.5*M* methanolic KOH was added, the tube stoppered with a cork and the mixture incubated for 4 hours on a constant temperature shaker bath held at 38° ± 0.5° C. After incubation, the mixture was evaporated to dryness and a 0.2-ml. portion of 1*M* methanolic HCl containing carrier phenol (0.1 mg. per ml.) was added. After stirring with a thin glass rod, the mixture was centrifuged at 1500 r.p.m. for 5 minutes and 100 μl. of clear supernatant spotted on 1/2-inch thin layer chromatography slips. After development, the slips were immediately sprayed with 10% methanolic KOH and diazotized sulfanilic acid.

For acid hydrolysis, a 1- to 2-ml. aliquot of extract or fraction was evaporated to dryness by nitrogen gas in a 15-ml. conical centrifuge tube. The residue was transferred with a minimum amount of methanol–water (1:3, v./v.) solution to an 8-cm. × 7-mm. o.d. tube and again evaporated to dryness by nitrogen. A 0.1-ml. portion of 1*N* aqueous HCl was added, the mixture frozen in dry ice, and the tube sealed. The sealed tube was placed in a 15-cm. metal centrifuge tube and immersed in a 95° to 100° C. oil bath for 22 hours. The mixture was refrozen in dry ice, opened, and 0.1 ml. of 2*M* methanolic KOH added and stirred with a microstirrer. The mixture was evaporated to dryness by nitrogen gas. A 0.2-ml. aliquot of 1*N* methanolic HCl containing carrier phenol (0.1 mg. per ml.) was added. After stirring with a microstirrer, the mixture was centrifuged at 1500 r.p.m. for 5 minutes and 100 μl. of clear supernatant spotted on 1/2-inch thin layer chromatography slips. After development, the slips were immediately sprayed with 10% methanolic KOH and diazotized sulfanilic acid.

A 1- to 2-ml. aliquot of urine was treated as for plant extracts except that 0.1 ml. of 2*N* aqueous HCl was used.

Table II. Time Course Study of Colep-C^{14} in Apple and Cotton Leaves

Extract	Per Cent of Total Applied Radioactivity Recovered: Days after treatment 4	7	14	28
	APPLE			
Benzene	56.5	33.3	19.8	14.2
Chloroform	7.1	11.6	12.6	8.4
Water	5.0	15.9	21.2	21.7
80% Ethanol	0.2	0.7	1.1	3.1
Residue	1.1	0.6	1.7	1.2
Total recovered	69.9	62.1	56.4	48.6
	COTTON			
Benzene	25.4	16.0	9.2	7.4
Chloroform	14.4	11.0	9.6	8.7
Water	21.3	24.0	42.2	51.0
80% Ethanol	0.8	1.5	3.6	4.5
Residue	1.0	2.0	1.9	1.3
Total recovered	62.9	54.5	66.5	72.9

Table III. Thin Layer Chromatography of Benzene Extracts

R_f	Per Cent of Total Radioactivity Chromatographed[a] with Solvent A: Days after treatment 4	14	28	B: Days after treatment 4	14	28
	APPLE					
0 –0.05	28	59	69	12	9	22
0.10–0.20	17	..	..	9	6	25
0.35–0.40	..	..	6	19	24	..
0.55–0.60	47	37	18	8	..	..
0.75–0.80	..	..	..	55	57	21
	COTTON					
0 –0.05	40	27	65	35	18	10
0.15–0.20	..	41	..	..	40	10
0.30–0.40	..	..	..	5	..	35
0.50–0.60	44	26	24	..	..	..
0.58–0.63	..	..	..	55	34	35

[a] The percentages represent the major radioactive components.

Results and Discussion

Plant Metabolism. From the time course studies shown in Table II, the radioactivity in the extracts is found to shift from the benzene fraction at 4 days toward the water fraction by 28 days. This indicates a conversion of the relatively nonpolar Colep to polar, water-soluble materials with time. The total recovery of radioactivity applied to the plants varied between 49 and 73% and could be due to surface hydrolyses of Colep followed by subsequent volatilization of released phenol. A 100-μg. (3252 d.p.m.) (decompositions per minute of absolute counts corrected for background and counter efficiency) portion of Colep-C^{14} when spread over a 0.5- to 1.0-sq. inch area on a watch glass lost one third of its activity (2094 d.p.m. recovered) in a humid atmosphere at room temperature in 20 hours. The transpiration occurring in the leaf would provide such an atmosphere. Since Colep is a solid melting at 80° to 81° C., loss of activity at room temperature by evaporation or sublimation would be unexpected other than by hydrolyses and volatility of phenol.

In Table II, a consistent drop of recovered radioactivity is noted for apple but not cotton. There is also a rapid movement of radioactivity into the water phase in cotton even by the fourth day. Such a transformation for apple is much slower. This suggests a longer residence time of Colep on the apple leaf surface while movement through the cotton cuticle is more rapid, allowing a greater possibility for surface hydrolyses and loss of radioactivity in the case of apple.

Table IV. Thin Layer Chromatography of Chloroform Extracts

	Per Cent of Total Radioactivity Chromatographed[a] with Solvent								
	B			C			E		
	Days after treatment			Days after treatment			Days after treatment		
R_f	4	14	28	4	14	28	4	14	28
				Apples					
0 –0.05	55	62	70	..	..	12	14	5	8
0.20–0.35	12	18	14	27	6	15	..	15	19
0.35–0.50	..	14	..	32	..	18	16	..	14
0.55–0.65	31	..	10	33	79	53	18	..	10
0.80–0.90	..	..	..	..	..	..	47	77	46
				Cotton					
0 –0.05	30	46	56	6	..	..			
0.20–0.30	14	15	12	5	10	18			
0.30–0.40	7	..	..	..	16	15			
0.50–0.60	50	..	..	..	..	..			
0.70–0.80	..	33	25	81	69	60			

[a] The percentages represent the major radioactive components.

Table V. Thin Layer Chromatography of Water Extracts

	Per Cent of Total Radioactivity Chromatographed[a] with Solvent					
	C			D		
	Days after treatment			Days after treatment		
R_f	4	14	28	4	14	28
			Apple			
0 –0.10	86	74	90	82	66	58
0.20–0.30	5	13	..	17	6	..
0.30–0.40	7	11	..	..	13	40
0.40–0.50	2	..	..	..	13	..
			Cotton			
0.10–0.20	51	25	19	45	51	27
0.20–0.40	35	66	76	42	38	65
0.50–0.60	12	7	4	11	9	3

[a] The percentages represent the major radioactive components.

No evidence of tissue disruption with benzene wash of cotton leaves was observed. Little or no pigments were removed, indicating that only some of the cuticular constituents were removed by benzene. This extract should contain Colep remaining on the leaf surface plus nonmetabolic breakdown products such as could occur by hydrolysis.

Thin layer chromatography of the benzene fractions in two solvent systems is shown in Table III. In solvents *A* and *B*, the region associated with Colep (R_f 0.5 to 0.6, and R_f 0.7 to 0.8 for *A* and *B*, respectively) decreased in radioactivity by the 28th day, while activity at or near the origin increased. For cotton, Colep moved slower in solvent *B* than expected, and its identity was therefore verified by cochromatography. After 28 days, the remaining amount of originally applied radioactivity associated with Colep represents 2.8 and 2.5% for apple and cotton, respectively.

Similar analyses of the chloroform extracts are shown in Table IV. The retention of a large portion of the radioactivity at the origin in solvent *B* (a basic medium) with movement away from the origin in solvent *C* (a highly polar system) suggests that a polar acidic product contributes to a large portion of the radioactivity in both apple and cotton leaves. However, by the 28th day in apple, the 53% activity at R_f 0.55 to 0.65 (representing Colep) equals only 4% of the total applied activity.

While phenyl methyl phosphonate is a possible breakdown product of Colep, and the radioactivity at R_f 0.20 to 0.35 (Table IV) in solvent *C* suggests its presence in both plants, plant pigments interferred with color development of the phenyl methyl phosphonate in cochromatographic studies. The mild base hydrolysis (0.1*N* methanolic KOH at 4° C.) of Colep-C^{14} showed a steady decrease of Colep and steady increase of phenol at 5, 10, 15, and 30 minutes. There was evidence of less than 1% of intermediate phenyl methyl phosphonate formation at 15 minutes, and none at 30 minutes. Thus, if Colep hydrolyzes in a similar manner on the plant, the formation of large amounts of phenyl methyl phosphonate would not be anticipated.

A large portion of the 28-day apple leaf chloroform fraction was chromatographed on large plates in solvent *E*, and the R_f 0.8 to 1.0 region was isolated for chemical treatment and rechromatography, since a considerable portion of activity resided in this area. While a nontreated sample moved only to R_f 0.1 in solvent *A*, acid and base hydrolyses released 70 to 75% of the activity as phenol. The rapid movement of this fraction in solvent *E* with slow movement in solvent *A*, combined with hydrolysis by both basic and acidic conditions, suggests the presence of the oxon [*O*-phenyl-*O'*-(4-nitrophenyl) methylphosphonate], an intermediate oxidation product of Colep. No activity was noted for the Colep region in solvent *A*. Since the R_f 0.8 to 1.0 fraction represents 46% of the total activity of the 28-day chloroform fraction, the oxon would represent only 2.9% of the original total applied radioactivity. The oxon would be a logical preliminary breakdown product of Colep. Since the water-insoluble Colep would be expected in the chloroform phase if it had penetrated the cuticle, these results indicate the rapid metabolism of Colep upon movement into the leaf tissue.

Thin layer chromatography of the water fractions is shown in Table V. For both apple and cotton, the major amount of activity remained at or near the origin for the polar solvents *C* and *D*. Since the activity remained at the origin in solvent *C* for the 28-day apple water fraction, a larger portion was chromatographed on large plates and the R_f 0 to 0.2 region isolated for emulsin, base, and acid hydrolyses. Both emulsin and acid treatment released 70 to 80% of activity as phenol (Table VI), while base treatment released none. These results indicate the presence of a component containing a β-glycosidic linkage. Since the radioactivity in the water extract did not move to R_f 0.4 to 0.5 in solvent *C* (Table V), phenyl β-glucoside is eliminated. This low mobility and resistance to base, but hydrolysis by emulsin, suggests a phenyl β-glycuronoside (*13*). Stewart (*18*) has suggested that uronic acids may serve as "detoxication" agents in plants as well as in animals. The presence of phenyl β-glycuronoside would appear reasonable.

Similarly, a large portion of the 28-day cotton water extract was chromatographed on large plates in solvent *D* and the R_f 0.2 to 0.4 fraction isolated, since a major portion of activity was located in this region (Table V). The results of emulsin, acid, and base hydrolyses (Table VI) would eliminate phenyl methylphosphonate, since there is resistance to base hydrolysis. From the nonhydrolyzed control, there appear to be two major materials in this fraction. Acid hydrolysis causes both to disappear with production of phenol. Emulsin causes the R_f 0.40 to 0.50 portion (Table VI) to disappear, suggesting phenyl β-glucoside, whose R_f is 0.49 in solvent *C*. The material at R_f 0.20 to 0.30 could be an alpha glycoside which would be resistant to emulsin and base hydrolysis.

The solvent D, R_f 0 to 0.2 fraction was also isolated from the above large plate separation and subjected to the same hydrolysis treatments (Table VI). There is appreciable acid and base hydrolysis but little from emulsin. The small amount of emulsin hydrolysis could be due to the pH 4.7 reaction conditions. A compound that would fit such reaction responses is phenyl sulfate, a known detoxication product in animals and insects (*10*). The high polarity of the compound would cause slow movement, even in polar solvents.

An aliquot of the apple leaf 28-day water extract was fractionated on anion and cation resin columns. The recovered radioactivities in the various effluents are shown in Table VII. Since the originally applied Colep-C^{14} had a specific activity of 0.97 mc. per mmole, the 62.5% retained on the column represents only 0.03 μmole of material. Such a small amount of material could be tightly adsorbed on the resin and be difficult to elute. Of the radioactive effluent from the anion column, 74.9% passed through a cationic column, showing the components to be largely neutral. Table VIII shows the thin layer chromatographic analysis of the original water extract, organic base–neutral effluent from the anion column, and organic neutral effluent from the cation column. About two thirds of the radioactivity at R_f 0.1 to 0.2 was removed by the anionic column and would represent an acidic compound in agreement with the postulated phenyl β-glycuronoside discussed above. Since phenol is evidenced in the neutral effluent at R_f 0.8 to 0.9 (see Table I, solvent E) but not in the organic base–neutral effluent, the cationic column may have caused some hydrolysis of either a neutral or basic phenolic derivative. However, essentially the same components, R_f 0.2 to 0.3 and 0.4 to 0.5, passed both columns, showing them to be neutral compounds. Phenyl glycosides or phenolic esters would show such characteristics.

Acid hydrolysis of the benzene, chloroform, and water extracts converted 75 to 80% of the radioactivity to phenol for both apple and cotton. Therefore, the radioactivity in all extracts is associated with phenol-containing materials. The separation by chromatography of several radioactive components in each extract indicates the active metabolism of *O*-phenyl-*O'*-(4-nitrophenyl) methylphosphonothionate into a variety of breakdown products, showing the plant's ready ability to degrade it.

Animal Metabolism. The dose and radioactive recoveries from the various animal components are shown in Table IX. The oral dose of Colep for Rat I (weight 158 grams) represents 0.45 mg. per kg., and for Rat II (weight 150 grams), 1.19 mg. per kg. These doses are well below the LD_{50} oral dose of 3 mg. per kg. (2.5 to 3.5 range) for female rats (*9*). The male rat LD_{50} oral dose is 8 mg. per kg. (5.25 to 12.5 range) (*9*). The major portion of radioactivity appears in the urine within a 24-hour period. The liver would be the expected major site of detoxification, and the kidney, the major excretory route. Both of these tissues contained low amounts of radioactivity, indicating that the detoxification and excretory processes were fairly well completed within 24 hours. What little activity remained was associated with the water extract,

Table VI. Hydrolyses of Isolated Chromatographic Fractions from 28-Day Cotton and Apple Water Extracts

R_f	Per Cent of Total Radioactivity Chromatographed[a]: Untreated control	Emulsin	Acid hydrolysis	Base hydrolysis
Apple (Solvent *C*, R_f 0–0.2 Recovered Fraction)				
0 –0.10[b]	100	21	12	99
0.70–0.80[b]	...	72[d]	82[d]	..
Cotton (Solvent *D*, R_f 0.2–0.4 Recovered Fraction)				
0.20–0.30[c]	63	68	..	83
0.40–0.50[c]	33	..	..	10
0.70–0.80[c]	..	25[d]	91[d]	2[d]
Cotton (Solvent *D*, R_f 0–0.2 Recovered Fraction)				
0 –0.10[c]	..	76	25	..
0.10–0.20[c]	75	..	..	23
0.30–0.50[c]	12	..	..	..
0.60–0.65[c]	..	..	5	16
0.70–0.80[c]	..	16[d]	63[d]	46[d]

[a] The percentages represent the major radioactive components.
[b] Developed in solvent *D*.
[c] Developed in solvent *C*.
[d] Identified by cochromatography with phenol.

Table VII. Ion Exchange of Apple 28-Day Water Fraction

Rexyn AG 1 (Formate) Exchange Column	Per Cent Radioactivity Recovered
Organic base–neutral effluent	37.5
Organic acid eluate (6*N* HCl)	15.2
Total anion recovery	52.7
Rexyn AG 50 (Na) Exchange Column	
Organic neutral effluent	62.3
Organic base eluate (1*N* NaOH)	12.6
Total cation recovery	74.9

Table VIII. Thin Layer Chromatography of Ion Exchange Effluents of Apple 28-Day Water Fraction

R_f[a]	Per Cent of Total Radioactivity Chromatographed: Untreated control	Anionic, organic base–neutral effluent	Cationic, neutral effluent
0.1–0.2	67		
0.2–0.3	23	76	67
0.4–0.5	8	20	7
0.8–0.9			19

[a] Developed in solvent *E*.

Table IX. Dosages and Radioactive Recoveries in Rat Metabolism of Colep

Dose of Colep: rat I, 4.94 × 10^5 d.p.m. = 0.071 mg.; rat II, 12.36 × 10^5 d.p.m. = 0.179 mg.

Animal Component	Extraction Phase	Per Cent of Administered Radioactivity: Rat I	Rat II
Kidney	H_2O	0.2	0.6
	$CHCl_3$	0	0
Liver	H_2O	3.3	1.4
	$CHCl_3$	0.3	0.1
Intestine	H_2O	2.2	5.7
	$CHCl_3$	1.1	1.3
Abdominal fat layer	H_2O	...	0
	$CHCl_3$	...	0
Feces	H_2O	0.7	2.0
	$CHCl_3$	0.7	0.6
Urine		79.3	68.0
CO_2		0	0
Total		87.8	79.7

Table X. Ion Exchange Separation of Rat Urine

Total d.p.m. applied to cationic column: rat I, 17,813 d.p.m.; rat II, 16,812 d.p.m.

Weak Cationic (Na form)	Per Cent of Applied Radioactivity: Rat I	Rat II
Effluent	73.7	75.1
0.1*N* NaOH eluate	19.1	9.7
Recovery	92.8	84.8
Weak Anionic (Formate form)		
Effluent	0.2	0.2
1*N* HCl eluate	0	0.2
Recovery	0.2	0.4

indicating polar breakdown products, since Colep is not water-soluble. The same was shown for the activity remaining in the intestine. This would indicate that some disruption occurred in the intestine also. Williams (*20*), in discussing the metabolic fate of phenol in mammals, gives pertinent references showing phenyl glucuronoside, phenyl sulfuric acid, and conjugated catechol and quinol to be the urinary metabolites. Phenol conjugation was shown in both liver and intestinal tissue, and more recently in the kidney as well (*17*). Since the radioactivity in Colep is incorporated in the phenol moiety, we would anticipate activity to occur in polar materials if it is degraded in the animal. The concentration of radioactivity in the urine of both animals indicates a disruption of Colep and release of phenol.

There was no measurable activity incorporated in the abdominal fat layer nor released as CO_2. Apparently Colep is not found in fat where the benzene- and $CHCl_3$-soluble and water-insoluble Colep might be expected to be transported. Mammals are not known to be able to oxidize phenol to CO_2 and water so the absence of respiratory $C^{14}O_2$ is not surprising.

Thin layer chromatography in solvent *E* of the urine resulted in a large broad band of radioactivity from R_f 0 to 0.6, peaking at R_f 0.25 to 0.35, suggesting a mixture of unresolved highly polar materials. No activity was found in the region associated with Colep.

To determine the ionic character of the excretory products, samples of urine were passed through ion exchange columns. The results are shown in Table X. While most of the activity passed through the cationic column, 25% was retained and could be partly recovered by NaOH elution. This suggests a basic radioactive component. The radioactivity in the effluent from the cation exchange column was completely retained by the anionic column resisting even 1*N* HCl for elution. Either such a small amount of material is involved as to be irreversibly adsorbed, or the component is strongly acidic and difficult to elute. A strongly acidic metabolite could be phenyl sulfuric acid. Williams (*20*) indicates that, in rabbits, the predominant phenolic excretion conjugate for low levels of phenol (as is present in this situation) is phenyl sulfuric acid.

Acid hydrolysis of the urine samples showed the release of phenol as in plant extracts. Further identification of the metabolic products was not attempted. The remainder of the animals was not analyzed, nor was the carcass washed for possible urine adhering to the legs and fur. This could account for some of the losses in recovery noted in Table IX.

These initial data indicate an effective degradation of Colep and rapid excretion of breakdown products within a 24-hour period, with little deposition of radioactivity in some of the key tissues of rats. The radioactivity excreted is in the form of polar molecules which liberate phenol when subjected to acid hydrolysis.

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INSECTICIDE METABOLISM

Metabolism of 3-Hydroxy-*N,N*-dimethylcrotonamide Dimethyl Phosphate by Cotton Plants, Insects, and Rats

D. L. BULL and D. A. LINDQUIST

Entomology Research Division, Agricultural Research Service, U. S. Department of Agriculture, College Station, Tex.

BIDRIN [(registered trademark, Shell Oil Co.), defined as the *cis* isomer only] (3-hydroxy-*N*, *N*-dimethylcrotonamide dimethyl phosphate), formerly known as compound SD-3562 (Shell Development Co., Modesto, Calif.), has shown promise as a short-residual, systemic insecticide that is toxic to a wide spectrum of phytophagous insects and mites. Foliar treatments with Bidrin are particularly effective for control of early-season insect pests of cotton (*2, 5*). This substituted-vinyl phosphate compound is soluble in water, of good stability (at 38° C. the half life is 1200 and 2400 hours at pH 9.1 and 1.1, respectively), and has a rat oral LD_{50} of 25 mg. per kg. (*14*). The technical product consists of a mixture of *cis* (85%) and *trans* (8%) isomers, of which the *cis* form is the more insecticidally active. Basic research on certain substituted-vinyl phosphate and other compounds structurally related to Bidrin has been reviewed in considerable detail in recent publications (*9, 11*). However, there have been no published reports of detailed investigations of the systemic action and metabolic behavior of Bidrin in plants and animals. This report presents the results of investigations of the rates of absorption, in vivo metabolism, and excretion of C^{14}- or