Metabolism and Selectivity of *O,O*-Dimethyl 2,2,2-Trichloro-1-hydroxyethyl Phosphonate and Its Acetyl and Vinyl Derivatives

B. W. ARTHUR and J. E. CASIDA

Department of Entomology, University of Wisconsin, Madison, Wis.

O,O-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (L13/59) and its acetyl derivative (O,O-dimethyl 2,2,2-trichloro-1-acetoxyethyl phosphonate), and vinyl derivative formed on dehydrochlorination (O,O-dimethyl 2,2-dichlorovinyl phosphate) were investigated with phosphorus-32–tagged materials in relation to their metabolism and selective toxicity. A marked variation in species susceptibility was demonstrated; the vinyl phosphate was generally more toxic but less selectively toxic than the two phosphonates. Antiesterase activity of the three compounds appeared to be due to dimethyl phosphorylation of the enzymatically active sites. Acetyl L13/59 may be enzymatically deacetylated in vivo to form the more active antiesterase, L13/59. The greater activity with the free α-hydroxyl may be due to hydrogen bonding between the L13/59 and the esterase. Although several different types of experimental approaches were utilized, no evidence was obtained for in vivo L13/59 dehydrochlorination and rearrangement to the more toxic vinyl phosphate. The low mammalian toxicity of L13/59 appears to be due to phosphonate hydrolysis by serum esterases and elimination of the trichloro- portion of the molecule in the urine as trichloroethyl glucuronide. Studies are reported on the rate and products of acid and alkaline hydrolysis of the three derivatives, as are observations on their phytotoxicity, volatility, and in vivo distribution in certain insects and plants.

As an insecticide, *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (L13/59) has attracted considerable interest for housefly control and for potential use as a systemic in the treatment of animals infested with endo- and ectoparasitic arthropods (*1*, *7*, *21*, *37*). In the presence of dilute alkali, this trichlorohydroxyethyl phosphonate undergoes a dehydrochlorination and rearrangement reaction, yielding the highly insecticidal vinyl derivative, *O,O*-dimethyl 2,2-dichlorovinyl phosphate (DDVP) (*5*, *29*, *31*). The formation of an acetyl derivative, *O,O*-dimethyl 2,2,2-trichloro -1- acetoxyethyl phosphonate, involves blocking the active hydrogen taking part in dehydrochlorination and possibly in hydrogen bonding reactions.

Mammals poisoned with L13/59 display a rapid recovery of cholinesterase activity of serum and brain compared with the phosphate and phosphorothioate insecticides now being used (*24*). DuBois and Cotter (*16*) postulated that this rapid in vivo reactivation of the cholinesterase and possibly the low toxicity to mammals may be due to the ease of metabolic degradation and a rapid dissociation of the enzyme-inhibitor combination.

This investigation concerns the metabolism and selective toxicity of L13/59 and its acetyl phosphonate and vinyl phosphate derivatives. Radio-phosphorus-labeled insecticides were used to investigate the in vivo and in vitro metabolism of these materials.

Materials and Methods

Synthesis of Radiophosphorus Insecticides

***O,O*-Dimethyl 2,2,2-Trichloro-1-hydroxyethyl Phosphonate** (designated as the hydroxyethyl phosphonate or L13/59 based on the Bayer code number). Red phosphorus-32 was chlorinated to yield phosphorus-32 trichloride (*19*), which was slowly added to 3 equivalents of anhydrous methanol to yield dimethyl hydrogen phosphite-32 (*6*, *30*). The addition of equimolar chloral to the dimethyl hydrogen phosphite-32 yielded *O,O*-dimethyl 2,2,2-trichloro-1-hydroxyethyl phosphonate (*6*, *28*) in 34% yield with a specific activity of approximately 1 mc. per gram. This hydroxyethyl phosphonate was purified on a silica gel column with chloroform as the elutriant (*40*). Investigation of the chromatographic characteristics and infrared analysis of the product indicated radiochemical purity, but a chloral hydrate contaminant. Further purification was not effected, as it was considered that this small percentage of a nonradioactive contaminant would not affect the interpretation of the radiotracer experiments.

***O,O*-Dimethyl 2,2,2-Trichloro-1-acetoxyethyl Phosphonate** (acetyl derivative). A portion of the radiophosphorus-labeled L13/59 was acetylated with equimolar acetic anhydride to yield the acetyl derivative in 70% yield with a specific activity of about 1 mc. per gram. The radioactive product, following purification on a silica gel column with *n*-hexane, yielded an infrared spectrogram identical with that of a known acetyl sample.

***O,O*-Dimethyl 2,2-Dichlorovinyl Phosphate** (vinyl derivative). A portion of the radiophosphorus-labeled L13/59 was dehydrochlorinated with equimolar alcoholic potassium hydroxide (*5*, *31*) to form a radiochemically homogeneous material in 62% yield, which readily reduced permanganate and had

a specific activity of about 1 mc. per gram. The partitioning properties of this vinyl phosphate between chloroform and water or *n*-hexane and water were identical to those of a known column-purified sample of *O,O*-dimethyl 2,2-dichlorovinyl phosphate.

Biological Assays LD_{50} determinations were made with the adult female housefly, *Musca domestica* (L.), adult German cockroach, *Blattella germanica* (L.), 3rd instar imported cabbageworm, *Pieris rapae* (L.), and 4th instar nymphs of the pea aphid, *Macrosiphum pisi* (Harr.). All insects were from laboratory cultures except the imported cabbageworms, which were field-collected from an insecticide-free area. Contact insecticidal toxicity was based on 24-hour mortality counts following application of desired amounts in 1.3 μl. of acetone to the individual insects (housefly mortality was measured 8 hours after treatment). The aphid systemic bioassay method has been described (*14*). Serial dilutions (dilution factor of 2) were used with three or four replications of 10 insects each per replicate. Dosage-mortality plots (*27*) yielded the LD_{50} values shown in Table I. Mosquito larvae bioassays [3rd instar *Aedes aegypti* (L.)] were utilized in a study of L13/59 metabolism in the dog. Approximately 20 larvae per 3.5 ml. of the diluted biological fluid with three replicates per treatment were introduced by a method similar to that previously described (*10*).

In vivo Distribution The distribution among the organ systems of the American cockroach, *Periplaneta americana* (L.), was studied following topical application of radiolabeled L13/59 and its acetyl and vinyl derivatives. The radioactive materials were applied to the pronotum in 20 μl. of acetone with approximately 50 γ or 5000 counts per minute (c.p.m.) of insecticide per gram of insect. Dissections were made with the tissues submerged under mineral oil at 10, 45, and 240 minutes after insecticidal application. The various insect parts were freed of the mineral oil by blotting on filter paper, weighed on a Roller-Smith torsion balance, and the counts per minute of phosphorus-32 were determined with a Geiger-Müller counter. The results were calculated as insecticide (or its equivalent degradation products) per milligram of wet weight of tissue [corrected for decay and molecular weight differences (see Table II)].

A distribution study was made with uniform seedling Perfection pea plants following 24 hours absorption through the roots from aqueous solutions containing approximately 35,000 c.p.m. of the insecticide per ml. At the end of the absorption period, the plants were subdivided into roots, stems, and leaves, and four plants were analyzed for distribution of the radioactivity. The remaining peas were transplanted into silica sand and held at 25° to 30° for 4 days in the absence of further insecticide

Table I. Biological and Chemical Properties of O,O-Dimethyl 2,2,2-Trichloro-1-hydroxyethyl Phosphonate and Its Acetyl and Vinyl Derivatives

LD_{50}	L13/59	Acetyl	Vinyl
White rats (i-p, mg./kg.)	400	800	6
Housefly (topical, mg./kg.)	11	12	0.6
German roach (topical, mg./kg.)	28	32	4
Imported cabbageworm (topical, mg./kg.)	10	56	8
Pea aphid			
Topical, mg./kg.	20	150	7
Systemic 1 day, p.p.m.	14	2600	3
Systemic 4 days, p.p.m.	120	500	100
Time till death of rats following i-p LD_{50} dosage, hr.	0.5	12	0.1
Antiesterase activity (pI_{50})			
Purified acetylcholinesterase	5.5	3.2	6.0
30% whole blood cholinesterase	4.1	3.5	3.7
Chymotrypsin (crystalline)	3.9	2.5	3.0
Volatility, % loss in 24 hr.	38	12	88
Hydrolytic stability (28° C.)			
Acid (5*M* HCl, % hyd. in 24 hr.)	1	11	43
Alkaline (1*M* Na_2CO_3, half life, in min.)	[a]	6	21
Partition coefficients (org. sol./H_2O)			
$CHCl_3$	1.2	>100	>100
CCl_4	0.040	20	5.1
n-Pentane	0.002	0.69	1.5
n-Hexane	0.002	0.89	1.7
Benzene	0.077	25	5.5

[a] Concurrent phosphonate bond cleavage and vinyl phosphate formation so result similar to vinyl derivative (see Figure 2).

Table II. Distribution among Organ Systems of American Cockroach of O,O-Dimethyl 2,2,2-Trichloro-1-hydroxyethyl Phosphonate and Its Acetyl and Vinyl Derivatives Following Topical Application to Pronotum of 50 γ per Gram (P^{32} Analysis)

		γ/Mg. Tissue after Minutes Indicated[b]								
		L13/59			Acetyl			Vinyl		
Tissue	Wt.[a]	10	45	240	10	45	240	10	45	240
Salivary gland	2.2	0.055	0.091	0.172	0.210	0.328	0.074	0.097	0.036	0.052
Foregut	10.6	0.081	0.688	0.037	0.066	0.266	0.066	0.092	0.076	0.066
Crop	2.9	0.032	0.048	0.068	0.098	0.076	0.102	0.022	0.014	0.061
Gastric caeca	10.0	0.009	0.008	0.028	0.008	0.034	0.044	0.008	0.004	0.015
Midgut	9.5	0.010	0.014	0.058	0.112	0.038	0.104	0.009	0.018	0.097
Malphighian tubules	1.2	0.003	0.121	0.126	0.000	0.001	0.000	0.041	0.090	0.154
Hindgut	6.7	0.021	0.041	0.201	0.062	0.153	0.614	0.025	0.078	0.396
Nerve cord	2.4	0.044	0.096	0.109	0.156	0.150	0.168	0.121	0.073	0.158
Muscles	40.4	0.005	0.005	0.057	0.024	0.026	0.020	0.018	0.042	0.120
Fat body	6.2	0.027	0.007	0.092	0.040	0.034	0.010	0.011	0.048	0.067
Reproductive organs	4.2	0.015	0.050	0.043	0.057	0.115	0.044	0.006	0.044	0.026
Tracheae	3.6	0.212	0.240	0.036	0.072	0.040	0.044	0.032	0.026	0.032
Head	26.7	0.023	0.025	0.024	0.028	0.054	0.070	0.021	0.038	0.055
Pronotum	8.2	0.218	0.176	0.107	0.460	0.374	0.164	0.908	0.165	0.110
Wings	32.7	0.070	0.105	0.544	0.110	0.118	0.103	0.061	0.139	0.158
Cuticle	32.0	0.008	0.009	0.021	0.020	0.022	0.008	0.009	0.008	0.012
Blood	0.05 ml.	0.249	0.013	0.173	0.154	0.410	1.040	0.255	0.520	0.580

[a] Average weight of tissue sample, mg. per roach. [b] Each figure is the average of four replications.

source. Samples of four plants each were removed at 1, 2, and 4 days after transplanting to determine the distribution and extent of metabolic degradation of the tagged insecticides.

In vivo and in vitro Metabolism Organophosphate insecticides can usually be extracted from aqueous solutions into chloroform, whereas their hydrolytic products remain in the aqueous phase. Partition coefficients between several organic solvents and water for the three insecticidal materials under investigation are shown in Table I. Metabolism of these phosphate insecticides was determined in vivo and in vitro by the change in solubility of the total radiophosphorus as hydrolysis occurred. The percentage hydrolysis was determined from standard curves based on the change in partition coefficient resulting from varying degrees of hydrolytic decomposition. For example L13/59 with a chloroform-water partition coefficient of 1.22 would change in its coefficient to 0.38 following 50% hydrolysis, as the hydrolysis products remaining in the aqueous layer would not affect the partitioning of the residual L13/59.

Adult houseflies were topically treated on the abdomen with 50 γ per gram of phosphorus-labeled L13/59 and its vinyl derivative. At various intervals following treatment, groups of 100 flies were homogenized (*35*) in 5 ml. of water and the homogenates centrifuged to separate 3 ml. of clear supernatant from the particulate materials. This clear supernatant was extracted with an equal volume of chloroform in a 15-ml. centrifuge tube and the emulsion centrifuged so that 1.0 ml. of each phase could be removed for determination of phosphorus-32 content. Percentage-hydrolysis figures were then derived from the partition coefficient of the phosphorus-32 compared with standard curves as described above. The in vivo hydrolysis curves for houseflies are shown in Figure 1.

Decomposition rates for L13/59 and its acetyl and vinyl derivatives were studied in pea plants by a similar method (Figure 1). Pea plants treated and sampled as described in the section on in vivo distribution were homogenized, extracted with chloroform, and radioassayed to derive the per cent hydrolysis.

Human blood plasma was also investigated as to its ability to hydrolyze the three organophosphorus insecticides (Figure 1). In a 15-ml. centrifuge tube, 0.2 ml. of plasma at pH 6.25 was added to approximately 80 γ of each radiolabeled material. Following incubation for varying periods at 37° C., 2.8 ml. of water and 3.0 ml. of chloroform were added to each tube and the partitioning properties were utilized to derive the per cent hydrolysis. Each determination consisted of six replications.

A further in vivo decomposition study was made by intravenous injection of 150 mg. per kg. of L13/59 in isotonic saline into a 9.2-kg. dog. Blood samples of 15 ml. were withdrawn after various time intervals, oxalate was added as an anticoagulant, and the blood was chilled to 5° C. The plasma from each sample was removed by centrifugation at 5° C., and the amount of residual L13/59 and hydrolytic products present in the plasma were determined by partition coefficients based on phosphorus-32, anticholinesterase activity (purified serum acetylcholinesterase by manometric determination), and bioassay with mosquito larvae. Samples of urine from the dog were collected at regular intervals and the concentration of L13/59 and its degradation products determined in a similar manner as with the plasma.

Miscellaneous Methods All insecticidal materials used were purified by column chromatography. The column-purified L13/59 was recrystallized three times from a chloroform–*n*-hexane mixture. Total phosphorus was determined as reduced phosphomolybdate following perchloric acid oxidation (*13*). Cholinesterase activity was determined manometrically with purified serum acetylcholinesterase (*12*) or potentiometrically with 30% whole human blood (*14*). The inhibitors in the manometric assays were preincubated at 30° C. with the enzyme for 30 minutes prior to addition of the substrate; the whole blood assays utilized a 2-hour preincubation of enzyme and inhibitor at 37° C. Activity-pS curves (*3, 4, 11, 32*) shown in Figure 3 were made with housefly head cholinesterase by the general method of Metcalf and March (*33*). The methods for chymotrypsin assay and Lineweaver-Burk inhibition plots have been described (*12*).

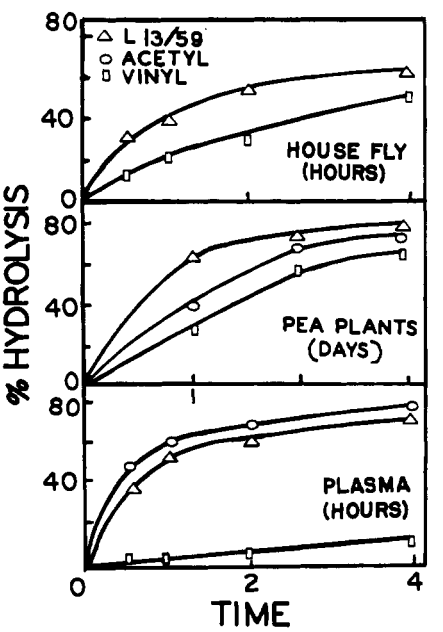

Figure 1. Rate of detoxification for Bayer L13/59 and its acetyl and vinyl derivatives by houseflies, pea plants, and human plasma

Acid and alkaline hydrolysis studies were made with L13/59 and its acetyl and vinyl derivatives. The partitioning properties were determined by total phosphorus colorimetric analysis after various periods of incubation of the organophosphorus compounds in 0.1*M* sodium carbonate or 5*M* hydrochloric acid. Results are indicated in Figure 2 and Table I. Relative volatility was determined by loss in total phosphorus content at room temperature from a 100-γ amount placed in 1 × 8 inch borosilicate glass combustion tubes (Table I). The vinyl phosphate derivative was more volatile than L13/59, and the acetyl derivative was the least volatile of the three.

Chemical analysis of the trichloroethyl portion of the L13/59 molecule was achieved by a modification of the Fujiwara method (*8, 9, 20, 22*). A tube containing the desired aliquot of the trichloro-containing material (chloroform, chloral, trichloroethanol, or L13/59) was chilled in an ice bath and 4 ml. of 5.5*N* sodium hydroxide was slowly added to the tubes; after the addition of 5 ml. of reagent grade pyridine, the color was developed by placing the reaction tube in boiling water for 2 minutes. The crimson color of the Fujiwara test shows absorption maxima at 360 and 530 mμ (*22*). Both L13/59 and trichloroethanol must be oxidized with dichromate to yield a positive test; these two may be distinguished because, prior to oxidation, trichloroethanol (but not L13/59) is extractable into pentane from an aqueous solution. The Fujiwara test was utilized in studying the acid, alkaline, and mammalian degradation products of L13/59.

Results and Discussion

Selective Toxicity L13/59 and its acetyl derivative were much less toxic to mammals than to any of the insects assayed (Table I). The vinyl derivative was found to yield a fly LD_{50} of 0.6 mg. per kg., whereas that for the other insects and the white rat were in the range of 4 to 8 mg. per kg. Of these materials, the phosphonates displayed the greater selectivity, although the vinyl phosphate was selective for houseflies. The toxic action of the vinyl derivative to rats was very similar, in rate and symptomology, to that reported for two other very toxic substituted-vinyl phosphate insecticides (*25, 34*). The acetyl L13/59 was much slower in poisoning mammals (Table I), but yielded the same symptomology as L13/59 [described by DuBois and Cotter (*16*)]. This might be expected if the acetyl group were enzymatically removed

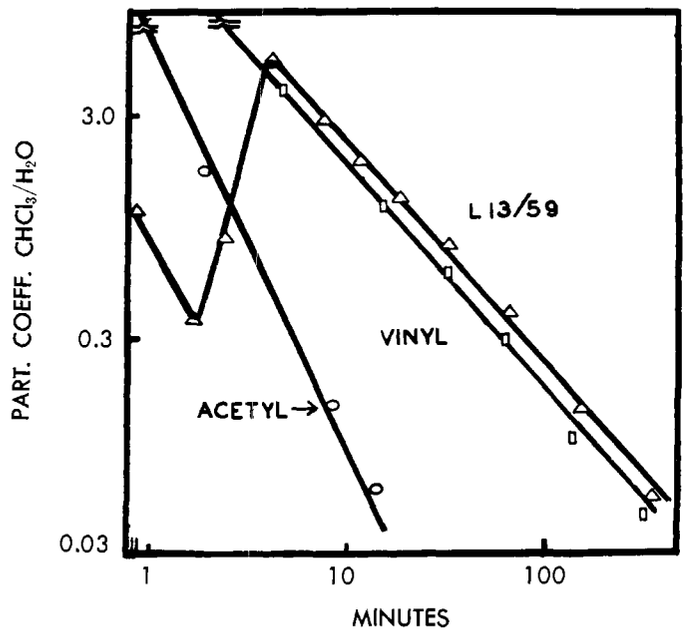

Figure 2. Rate of alkaline hydrolysis of L13/59 and its acetyl and vinyl derivatives in 0.1M sodium carbonate

Plotted as logarithmic relationship

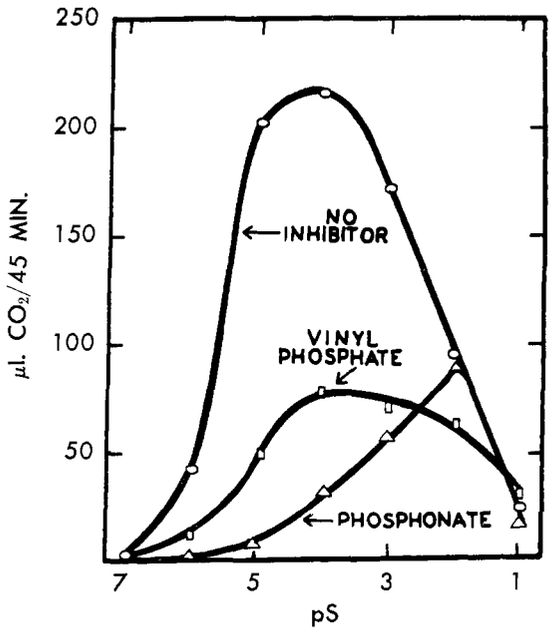

Figure 3. Effect of acetylcholine concentration on in vitro inhibition of fly head acetylcholinesterase by L13/59 (2×10^{-6} M) and its vinyl derivative (7×10^{-8} M)

in vivo to increase the anticholinesterase activity (approximately 100 × difference in activity, Table I) and finally accumulate sufficient L13/59 (about 50% of applied dose based on LD_{50}) to poison, as the hydroxyethyl phosphonate.

As chloral and trichloroethanol are possible degradation products of L13/59, the toxicity of these two compounds to houseflies was determined. The LD_{50} of trichloroethanol to houseflies was 4000 γ per gram, while the LD_{50} for chloral was greater than 4000 γ per gram.

The vinyl derivative was not phytotoxic to pea plants at 300 p.p.m. but the two phosphonates at this concentration or greater resulted in distinct marginal chlorosis after about 24 hours, which progressed towards the midrib producing necrotic areas by the fourth day.

In vivo Distribution in American Cockroaches and Pea Plants

A study on the absorption and distribution rates for the three radioactive insecticides in pea plants demonstrated that all three were absorbed to about the same degree in 24 hours (about 1000 p.p.m. within the plants absorbing from a 300-p.p.m. solution). When removed from the insecticide source, redistribution occurred into the leaves and tips for the first 24 hours with little further change in localization during the next 3 days, at which time there was with each material 500 p.p.m. residual insecticide or its degradation products.

The in vivo distribution of the radiolabeled insecticides in the American cockroach following topical application is summarized in Table II. The average concentration in the nerve cords after 45 minutes was about 0.07 to 0.16 γ per mg. with all three compounds. This represents about 0.5% of the applied dosage. Since a preliminary study by Casida (*11*) implicated only the acetylcholinesterase inhibition in the nerve tissue as being physiologically significant in L13/59 poisoning of roaches, this 0.5% of the applied toxicant in the nerve might be considered as the only portion of the insecticide arriving in proximity to the final site of action. As shown in Table II, L13/59 and derivatives were absorbed into the blood, then into the gut parts, and finally redistributed within the alimentary canal to appear in the posterior regions. This distribution route is different from that reported for other phosphate insecticides which showed a foregut accumulation (*18, 36*).

In vitro Antiesterase Activity

L13/59 and its vinyl derivative are much more active anticholinesterase agents in vitro than the acetyl derivative (Table I). Studies by Casida (*11*) with cockroaches indicated that L13/59 poisoning of these insects was due to in vivo anticholinesterase activity. In the present study, it was found that rat erythrocyte cholinesterase inactivated by L13/59 was not reactivated by repeated washings of the erythrocytes, according to the general procedure of Aldridge (*2*). However, when rat serum was added to the rat erythrocyte preparations, inhibited by either *O,O*-dimethyl 1,1,1-trichloro-2-hydroxyethyl phosphonate or *O,O*-dimethyl 2,2-dichlorovinyl phosphate, the rate of recovery of the erythrocyte cholinesterase activity due to the serum esterases was almost identical for the two materials [general experimental method of Davison (*15*)]. These results indicate a dimethyl phosphoryl cholinesterase as the inactive enzyme from inhibition by this phosphonate and vinyl phosphate. Further evidence of esterase phosphorylation resulted from reacting radiolabeled-L13/59 with chymotrypsin, which yielded a noncompetitive inhibition [Lineweaver-Burk determination (*12*)] and incorporation of phosphorus-32 into the enzyme molecule in a protein-bound, nondialyzable form.

A further study on the mechanism of in vitro inhibition of cholinesterase by L13/59 derivatives involved the effect of acetylcholine concentration on the efficiency of L13/59 and the vinyl phosphate as inhibitors using the experimental methods of Augustinsson (*3*). The results averaged from four activity-pS curves are shown in Figure 3. At high acetylcholine concentration, the cholinesterase appears not to be affected by the L13/59 in contrast to a definite inhibition by the vinyl phosphate. These results may be due to the vinyl phosphate's reacting more rapidly with cholinesterase than does L13/59, this being indicated by the rapid onset of symptoms in animals poisoned by the vinyl phosphate compared to those poisoned by L13/59. The free α-hydroxyl group in L13/59 and potential hydrogen bonding of this group with the enzyme may also contribute materially to differences observed in the mechanism of in vitro anticholinesterase activity of the three derivatives.

Table III. Metabolism of O,O-Dimethyl 2,2,2-Trichloro-1-hydroxyethyl Phosphonate in a Dog as Measured by Radiotracer, Anticholinesterase, and Insect Bioassay Analyses of Serum and Urine

Time after Treatment, Hours	μmoles/Ml. L13/59	μmoles/Ml. Hydrolysis product	Part. Coeff. $CHCl_3/H_2O$	% Mortality Mosquito Larvae[a]	% Inhibition ChE[a]
		Serum[b]			
0	6.90[c]	0.00	1.6	100	95
0.15	0.33	0.62	0.22	100	82
0.5	0.23	0.65	0.13	100	70
2	0.13	0.43	0.12	40	64
6	0.03	0.13	0.09	1	34
24	0.00	0.03	0.00	2	0
32	0.00	0.004	0.00	0	0
48	0.00	0.00	0.00	0	0
		Urine[d]			
0- 6	8.00	28.3	0.104	3	..
6-24	0.14	0.41	0.13	0	..
24-32	0.06	0.22	0.104	2	..
32-48	0.00	0.03	0.00	5	..

[a] % larval mortality after 24 hr. at 1 p.p.m. and cholinesterase inhibition after 0.5 hr. at 0.25 p.p.m. of L13/59 equivalent based on P^{32}.
[b] Posttreatment interval before blood removed for analysis.
[c] Initial concentration calculated from amount injected.
[d] Posttreatment increment within which urine sample collected.

Mammalian Detoxification of L13/59

Mammalian metabolism of L13/59 was studied by injecting 150 mg. per kg. of L13/59, intravenously, into a 9.2-kg. female dog, and analyzing subsequent blood and urine samples. The general anticholinergic symptoms first appeared 15 minutes after administration, with a marked diminution in symptoms by 30 minutes and an apparent total recovery 6 hours after administration of L13/59. The symptomology and course of poisoning for the dog were very similar to those reported for the rat by DuBois and Cotter (*16*).

The rapid recovery of the dog from L13/59 intoxication appeared to be correlated with the rapid destruction of the anticholinesterase agent in vivo (Table III). The 10-minute posttreatment blood sample contained 1 part of L13/59 per 2 parts of hydrolytic products. By 6 hours only 0.4% of the administered dose was detectable in the plasma as the unhydrolyzed phosphonate ester, with about four times this amount present as phosphorus-containing hydrolytic products. These radiotracer results on L13/59 decomposition were substantiated by mosquito larvae bioassay and anticholinesterase determination.

Less than 1% of the phosphonate ester administered was excreted in an unchanged form by the dog in the 2 days following treatment based on both radiotracer and bioassay determinations. The reported figures for unchanged L13/59 in the urine may be somewhat high, as no attempt was made to correct for the effect of the salts in the urine on the partitioning properties of the phosphonate insecticide. The first samples of urine, feces, and saliva recovered following injection of the radiolabeled-L13/59 were all highly radioactive.

Final metabolic degradation products of L13/59 were studied by fractionation of the urine in an attempt to recover potassium trichloroethyl glucuronides [isolation method of Kulz (*26*)]. A potassium salt was isolated and found to contain organically bound chlorine [analytical method of Stepanov (*39*)] in amounts equivalent to 67% of the administered dose of L13/59. The fractionation scheme utilized in isolating this salt excludes the possibility that the chlorine was due to L13/59, chloral, trichloroethanol, or inorganic chlorides. Hydrolysis of this salt for 4 hours with concentrated hydrochloric acid yielded a material which could be extracted into pentane and which responded to a Fujiwara color test after, but not before, dichromate oxidation. This Fujiwara-positive material partitioned between pentane and water or hexane and water in a manner identical to that found with known trichloroethanol following oxidation. The trichloroethanol recovered from the L13/59 metabolite was comparable to 63% of the original L13/59 injected into the dog.

Color tests were used to determine the nature of the material conjugated with trichloroethanol in the L13/59 metabolite. The salt recovered from the urine was dissolved in concentrated hydrochloric acid and distilled, and the products were tested for reaction with aniline acetate (*17*). A positive aniline acetate test indicated the presence of furfural derived from a pentose or glucuronic acid derivative (*38*); hexoses would respond negatively under these conditions as the hydroxymethyl furfural formed would be changed to levulinic acid on heating. The potassium salt from the urine also responded positively to a naphthoresorcinol test, showing that the carbohydrate derivative present is glucuronic acid rather than a pentose (*23*, *38*). Administration of chloral, trichloroethanol, or trichloroethylene to dogs, results in 40 to 60% excretion as the trichloroethyl glucuronide metabolite in the urine (*8*, *9*). All of the chemical properties noted for the L13/59 metabolite recovered in the urine agree with those for trichloroethyl glucuronide. Thus L13/59 appears to be metabolized in dogs to yield trichloroethanol which is conjugated with glucuronic acid and excreted in the urine in about 65% yield.

Metabolism of L13/59 and Derivatives by Insects, Pea Plants, and Blood Plasma

Radioactive materials were used to study the insect metabolism of L13/59 and its vinyl and acetyl derivatives. Houseflies poisoned with L13/59 were extracted with chloroform and the extract was chromatographed. A large proportion of the L13/59 was hydrolyzed but no other phosphorus-32-containing metabolites could be detected by solubility characteristics, change in antiesterase activity, or permanganate-reducing characteristics. Studies with cockroaches [*P. americana* (L.) and *Leucophaea maderae* (Fab.)] again failed to demonstrate the presence of any vinyl derivative (less than 0.1%) formed in vivo based on chromatography and permanganate reduction experiments. Further, the anticholinesterase activity of L13/59 was not affected by incubation with whole roach intestines [general method of Metcalf and March (*32*)]. With houseflies topically treated with L13/59 and its vinyl derivative, there was a more rapid in vivo metabolism of the phosphonate (Figure 1). If L13/59 dehydrochlorinated in vivo to yield the vinyl derivative, the over-all decomposition rate of L13/59 should have been less or equal to that for the vinyl compound. No evidence was obtained that the toxicity of L13/59 to flies was due to conversion to the more active vinyl derivative or that the in vivo detoxication rate can explain the relative susceptibility of houseflies to these two materials.

Attagenus piceus (Oliv.) larvae are highly resistant to L13/59. This resistance cannot be solely attributed to a rapid destruction of this chemical in vivo, as over 95% of L13/59 was recovered from larvae 4 hours after treatment with 4000 γ, a dosage level approaching the LD_{50} value. L13/59 and its acetyl and vinyl derivatives were incubated with homogenates of housefly heads, imported cabbageworm heads, rat serum, rat liver, and whole pea aphids in a Warburg respirometer with a

bicarbonate buffer at pH 7.6. No acid was liberated with either L13/59 or the vinyl derivative but a rapid enzymatic reaction formed acid from the acetyl L13/59. The acid liberated under these conditions is probably due to insect esterases capable of hydrolyzing the acetyl group from acetylated L13/59.

Plant metabolism of the three materials was studied in vivo (Figure 1). The phosphonates were the most rapidly hydrolyzed, although the vinyl phosphate was 65% hydrolyzed within 3 days.

Human blood plasma yielded a very rapid in vitro degradation of the two phosphonates compared with the vinyl phosphate (Figure 1). Such efficient in vitro enzymatic cleavage of the phosphonate bond agrees with the in vivo mammalian observations (Table III). This susceptibility of the 2,2,2-trichloro-1-hydroxyethyl phosphonate to decomposition by plasma esterases appears to be a major factor in its low relative toxicity to mammals. The sensitivity of these serum esterases to pH change is indicated by a 3.5% hydrolysis of L13/59 at pH 4 during a 4-hour incubation period with 8.5% hydrolysis at pH 5, 42.5% at pH 6, and 60% at pH 7.

Chemical Stability

Alkaline hydrolysis rates for the three compounds are shown in Figure 2. L13/59 and acetyl L13/59 gave similar initial hydrolysis rates, but L13/59 reverted to a more stable and chloroform-soluble material which then hydrolyzed at the rate of the vinyl derivative. At pH 11.6, a small portion of the L13/59 cleaves at the phosphonate bond, but the major portion undergoes a dehydrochlorination, rearrangement, and subsequent hydrolysis of the vinyl phosphate formed. With acetyl L13/59 the phosphonate cleavage was the primary reaction rather than deacetylation, as the hydrolysis curve did not revert to the characteristics of the vinyl derivative.

Supporting evidence for the concurrent dehydrochlorination and phosphonate cleavage on incubation of L13/59 in bicarbonate solution was obtained by demonstrating one material capable of reducing permanganate and another material (other than L13/59), which gave a positive Fujiwara reaction after extraction into pentane. Both chloral (*8*) and the alkaline degradation product of L13/59 were extractable into pentane from a pH 11.6 carbonate solution but not from distilled water. Absorbance–wave length curves (325 to 675 mμ) for the alkali degradation product of L13/59 were identical to those for chloral exposed to the same alkaline treatment and quite distinct from trichloroethanol curves. [These curves did not show the 675-mμ peak for chloral reported by Giang, Barthel, and Hall (*22*), but a slightly different modification of the Fujiwara method was utilized].

The phosphonates were more stable in acid than the vinyl phosphate, L13/59 being the more stable of the phosphonates (Table I). The trichloro-containing part of the acid-degraded L13/59 was extractable with pentane, gave a negative Fujiwara reaction before oxidation, but when oxidized with dichromate yielded an absorption–wave length curve identical with that for trichloroethanol.

Dichromate oxidation of L13/59 formed formaldehyde and a material yielding a positive Fujiwara reaction. Formaldehyde [as determined with chromotropic acid (*12*)] was also formed from dimethyl phosphite and dimethyl 2,2-dichlorovinyl phosphate by methoxyl oxidation and as such contributed little as an analytical method for metabolism studies. Standard curves for L13/59 chloral, and trichloroethanol as determined by dichromate oxidation and the Fujiwara reaction are shown in Figure 4. This colorimetric determination following dichromate oxidation was of value in studying the metabolism of L13/59 and derivatives.

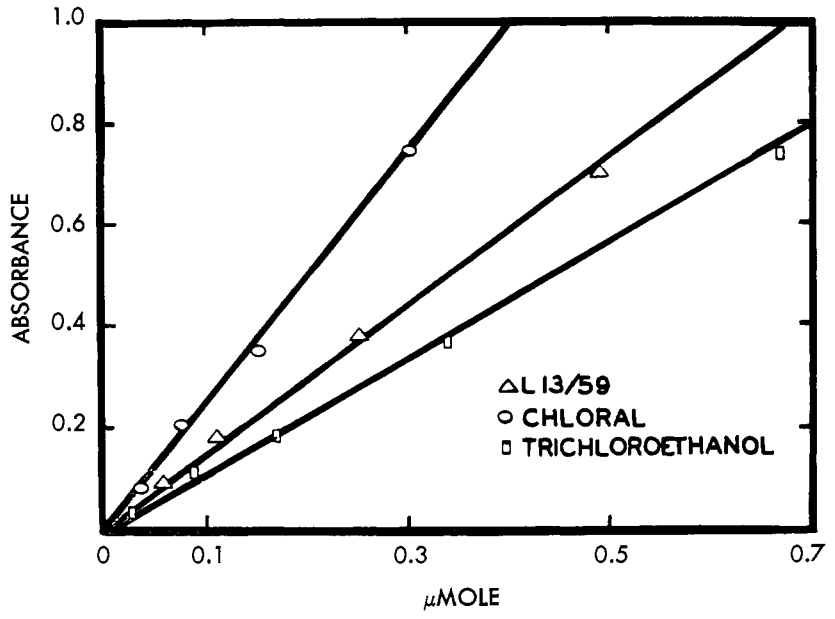

Figure 4. Standard curve for L13/59, trichloroethanol, and chloral as determined by a modified pyridine-alkali Fujiwara test

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PLANT METABOLISM OF INSECTICIDES

Metabolism of the Systemic Insecticide *O,O*-Diethyl *S*-Ethylthiomethyl Phosphorodithioate (Thimet) in Plants

J. S. BOWMAN and J. E. CASIDA

Department of Entomology, University of Wisconsin, Madison, Wis.

O,O-diethyl *S*-ethylthiomethyl phosphorodithioate (Thimet) is metabolized by plants to form very potent anticholinesterase agents. When used as a systemic insecticide for seed treatment of cotton, the metabolites within the plant consist of O,O-diethyl *S*-ethylsulfinylmethyl phosphorodithioate, O,O-diethyl *S*-ethylsulfonylmethyl phosphorodithioate, O,O-diethyl *S*-ethylsulfinylmethyl phosphorothiolate, and O,O-diethyl *S*-ethylsulfonylmethyl phosphorothiolate. The last of these metabolites is the most active cholinesterase inhibitor and provides a method of residue analysis. Cotton seeds treated with Thimet on charcoal at concentrations as high as 32 pounds of Thimet per 100 pounds of seed showed less than 0.03 p. p. m. of Thimet or metabolites in the seeds maturing from the treated plants. The residual persistence following soil and foliage application was studied with six vegetable crops and radioactive Thimet.

MANY *O,O*-DIALKYL *S*-ALKYLTHIOALKYL phosphates and thiophosphates are very active contact and systemic insecticides (*1, 3, 9, 12, 17*). The metabolism in plants of demeton (mixture of *O,O*-diethyl *S*-ethylthioethyl phosphorothiolate and *O,O*-diethyl *O*-ethylthioethyl phosphorothioate) has been studied extensively (*4–7, 10, 11, 13, 15, 16, 18*). Fukuto and coworkers (*6, 7*) have shown clearly that, in plants, these isomers are oxidized to their corresponding sulfoxides and sulfones. *O,O*-diethyl *S*-ethylthiomethyl phosphorodithioate (American Cyanamid 3911 or Thimet) was the most active systemic insecticide of a large series of phosphorodithioates (*3*). Thimet has shown economic promise in the treatment of cotton seed for protection of young plants against early season pests and as a systemic insecticide for control of chewing and sucking insects following soil or foliage application (*3*). Registration has been received recently from the Food and Drug Administration for the use of Thimet as a cotton seed treatment.

This study concerns the nature and rate of plant metabolism of Thimet in relation to the persistence of toxic residues.

Methods

Synthesis of Radioactive *O,O*-Diethyl *S*-Ethylthiomethyl Phosphorodithioate. Phosphorus-32 pentasulfide was prepared from 1.25 grams of red phosphorus-32 (service irradiation at Atomic Energy Commission, Oak Ridge, Tenn.), 3.22 grams of sulfur, and 0.54 gram of carrier phosphorus pentasulfide. The mixture was reacted in a nitrogen atmosphere by slowly raising the temperature to 270° C. and maintaining it at 270° to 300° for 4 hours. *O,O*-diethyl phosphorodithioic acid was formed by dropwise addition of 5.75 ml. of absolute ethyl alcohol to the phosphorus pentasulfide dissolved in 6 ml. of toluene and the reaction mixture was held at 90° to 95° C. for 1 hour. The final toluene solution was added dropwise to a mixture of 2.64 grams of potassium hydroxide, 8.28 ml. of absolute ethyl alcohol, and 4.14 ml. of benzene at 25° to 35° and the reaction mixture held at 80° C. for 1 hour. Potassium diethyl phosphorodithioate was recov-