entirely as dimethyl phosphoric acid on an anion exchange resin (*15*).

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Literature Cited

(1) Assoc. Offic. Agr. Chemists, Washington, D. C., "Official Methods of Analysis," 8th ed., p. 248, Sections 15.26, 15.27, 1955.
(2) Casida, J. E., *Biochem. J.* **60,** 487 (1955).
(3) Casida, J. E., *Science* **122,** 597 (1955).
(4) Casida, J. E., Gatterdam, P. E., Getzin, L. W., Chapman, R. K., J. AGR. FOOD CHEM. **4,** 236 (1956).
(5) Claborn, H. V., U. S. Dept. Agr. Bull. **ARS-33-25** (July 1956).
(6) Dahm, P. A., Fountaine, F. C., Pankaskie, J. E., *Science* **112,** 254 (1950).
(7) Dahm, P. A., Fountaine, F. C., Pankaskie, J. E., Smith, R. C., Atkeson, F. W., *J. Dairy Sci.* **33,** 747 (1950).
(8) Dahm, P. A. Jacobson, N. L., J. AGR. FOOD CHEM. **4,** 150 (1956).
(9) Gatterdam, P. E., Casida, J. E., Dept. of Entomology, University of Wisconsin, 1957, unpublished results.
(10) Kodama, J. K., Anderson, H. H., Dunlap, M. K., Hine, C. H., *A.M.A. Arch. Ind. Health* **11,** 487 (1955).
(11) Kodama, J. K., Morse, M. S., Anderson, H. H., Dunlap, M. K., Hine, C. H., *Arch. Ind. Hyg. Occupational Med.* **9,** 45 (1954).
(12) Kodama, J. K., Morse, M. S., Hine, C. H., *Federation Proc.* **12,** 337 (1953).
(13) March, R. B., Metcalf, R. L., Fukuto, T. R., Gunther, F. A., *J. Econ. Entomol.* **49,** 679 (1956).
(14) Pankaskie, J. E., Fountaine, F. C., Dahm, P. A., *Ibid.*, **45,** 51 (1952).
(15) Plapp, F. W., Casida, J. E., *Anal. Chem.* **30,** 1622 (1958).
(16) Robbins, W. E., Hopkins, T. L., Eddy, G. W., J. AGR. FOOD CHEM. **5,** 509 (1957).
(17) Robbins, W. E., Hopkins, T. L., Eddy, G. W., *J. Econ. Entomol.* **49,** 801 (1956).
(18) Treon, J. F., *et al.*, The Kettering Laboratory, Univ. of Cincinnati, Cincinnati, Ohio, March 4, March 15, 1957, unpublished report.

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ANIMAL METABOLISM OF INSECTICIDES

Bovine Metabolism of Organophosphorus Insecticides. Metabolic Fate of *O,O*-Dimethyl *O*-(2,4,5-trichlorophenyl) Phosphorothioate in Rats and a Cow

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O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate is susceptible to hydrolysis at either the methyl-phosphate or phenyl-phosphate bond. Both sites of hydrolysis have been demonstrated with alkali and bovine rumen juice and in rats, houseflies, and a cow. The oxygen analog of this insecticide undergoes similar hydrolic cleavage. The excretory metabolites of O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothioate and three derivatives were established for rats. A slower detoxification and excretion of the insecticide metabolites occurred with the cow compared to rats, but the same metabolic pathway was demonstrated for each.

SEVERAL ORGANOPHOSPHATE INSECTICIDES are effective chemotherapeutic agents for cattle grubs following oral, subcutaneous, or epidermal administration (*17, 18*). Robbins, Hopkins, and Eddy have published on the distribution and metabolic fate of two of these, Diazinon and Dipterex, in lactating cows (*24, 25*) and have presented a preliminary report on the fate of a third, *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phos phorothioate (Trolene), in a calf (*23*). Trolene administered orally at 100 mg. per kg. not only kills larvae present in the backs of cattle, but also completely prevents the encystment of new larvae (*17, 26*). At the recommended dose, the insecticide rarely produces symptoms and these are of a mild transient nature (*22*).

In this study, Trolene was administered orally to rats and a lactating cow at 100 mg. per kg., and the distribution and metabolic pathway were determined.

Methods and Results

Chemical Studies

Radioactive Syntheses. Phosphorus-32 trichloride was obtained through neutron irradiation of phosphorus trichloride or by chlorination (*10*) of red phosphorus-32 from AEC service irradiation at Oak Ridge, Tenn. (both irradiations at 7×10^{11} neutrons per sq. cm. per second flux for 4 weeks). The phosphorus trichloride was converted to thiophosphoryl trichloride (*14*) and then to dimethyl thiophosphoryl chloride (*9*). *O,O*-Dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate was formed by reaction in acetone of the dimethyl thiophosphoryl chloride with equimolar 2,4,5-trichlorophenol (purified sample from Hooker Electrochemical Co.) in the presence of equimolar sodium carbonate (*9*). The product was dissolved in hexane, washed three times with 10% sodium carbonate, and further purified by partition chromatography on a Celite–*iso*-octane–methanol column (*2*). The purified organophosphate represented a 20% yield from phosphorus trichloride and was identical in infrared spectrum to known *O,O*-dimethyl *O*-(2,4,5-trichlorophenyl) phosphorothioate.

O,O-Dimethyl *O*-(2,4,5-trichlorophenyl) phosphate (the oxygen analog of Trolene) was prepared in 70% yield from phosphorus-32 trichloride by the method of Fukuto and Metcalf (*11*) and purified as above yielding an infrared spectrum identical with the known compound. The compound was also prepared (nonlabeled) by nitric acid oxidation of the corresponding phosphorothioate after the method of Johnson (*13*).

O-Methyl *O*-hydrogen *O*-(2,4,5-trichlorophenyl) phosphorothioate was prepared from radioactive Trolene by alkaline hydrolysis with 2 molar equivalents of alcoholic potassium hydroxide and purified by ion exchange chromatography. The radioactive sample was

Table I. Nature of Hydrolysis Products of Trolene and Derivatives after Exposure to Various Chemical and Biological Systems Based on Ion Exchange Chromatography

Compound	Treatment	Per Cent as Various Hydrolysis Products				
		$(HO)_2P(O)OH$	$(CH_3O)_2P(O)OH$	$(CH_3O)_2P(S)OH$	Phenyl phosphotes	Unidentified
		Alkaline Hydrolysis				
$(CH_3O)_2P(S)O\phi Cl_3$	1 mole KOH	..	...	22	78	0
	2 moles KOH	..	...	36	64	0
	4 moles KOH	..	...	96	4	0
$(CH_3O)_2P(O)O\phi Cl_3$	1 mole KOH	0	51	..	49	0
	2 moles KOH	0	75	..	25	0
	4 moles KOH	0	98	..	2	0
		Metabolites in Rat Urine after Oral Administration of 100 Mg. per Kg.				
$(CH_3O)_2P(S)O\phi Cl_3$	0–12 hr.	0	20	8	72	0
	12–24 hr.	0	42	8	50	0
	24–48 hr.	0	57	26	17	0
	48–168 hr.	0	65	25	10	0
	0–168 hr. total	0	33	11	56	0
$(CH_3O)_2P(O)O\phi Cl_3$	0–168 hr.	0	46	..	54	0
[a]	0–12 hr.	0	28	..	72	0
[a]	12–24 hr.	0	36	..	64	0
[a]	24–48 hr.	0	52	..	44	4
[a]	48–168 hr.	0	58	..	36	6
[a]	0–168 hr. total	0	44	..	53	3
$CH_3O(HO)P(S)O\phi Cl_3$ [b]	0–72 hr.	12	...	..	88	0
$(CH_3O)_2P(O)OH$	0–72 hr.	0	100	..	..	0
		Bovine Rumen Juice in vitro				
$(CH_3O)_2P(S)O\phi Cl_3$	0–24 hr.	0	8	16	76	0
		Housefly Topical in vivo				
$(CH_3O)_2P(S)O\phi Cl_3$	0–10 min.	0	0	98	2	0
	0–60 min.	0	3	89	8	0

[a] Estimated dosage of 250 mg. per kg.
[b] Prepared by hydrolysis of Trolene with 2 molar equivalents of potassium hydroxide in absolute alcohol.

characterized by an infrared spectrum and by chromatography with nonradioactive carrier on an ion exchange resin as described below. The known sample of *O*-methyl *O*-hydrogen *O*-(2,4,5-trichlorophenyl) phosphorothioate as the ethylamine salt (provided by the Dow Chemical Co.) was analyzed and gave the following results:

	$C_9H_{13}Cl_3O_3NPS$				
	Cl	N	P	S	CH_3O
Calculated	30.24	3.98	8.81	9.11	8.82
Found	30.30	3.98	7.48	8.30	8.64

Dimethyl phosphoric acid was recovered as a metabolite of another phosphorus-32-labeled insecticide and purified by ion exchange chromatography. Nonradioactive trisodium phosphorothioate was prepared by reacting, in aqueous medium, thiophosphoryl trichloride with 6 molar equivalents of sodium hydroxide and then precipitating with ethyl alcohol (*19*). This salt (melting point 60° C.) was about 93% pure based on ion exchange chromatography.

Alkaline Hydrolysis. The stability to hydrolysis by alcoholic potassium hydroxide and nature of the products formed were studied with Trolene and its oxygen analog. Organophosphate, 200 μmoles, was dissolved in 95% ethyl alcohol and incubated at 28° C. for 16 hours with 1, 2, or 4 molar equivalents of alcoholic potassium hydroxide. After evaporation of the solvent, the residue was partitioned with water and chloroform. Aliquots of both phases were analyzed for total phosphorus to determine the per cent hydrolysis. The water layer was analyzed by ion exchange chromatography to determine the nature of the hydrolysis products (Table I). Identical experiments were made with dimethyl phosphoric acid, potassium dimethyl phosphorothioate, and the ethylamine salt of *O*-methyl *O*-hydrogen *O*-(2,4,5-trichlorophenyl) phosphorothioate except that the aqueous solutions were not extracted with chloroform prior to ion exchange chromatography.

Trolene and its oxygen analog were susceptible to alkaline hydrolysis at two sites on the molecule. Under milder conditions (equimolar potassium hydroxide and organophosphate in alcohol) the phosphorus-oxygen-methyl bond was predominantly cleaved while with 4 molar equivalents of potassium hydroxide the phosphorus-oxygen-phenyl bond was the main site of hydrolysis. Under these alkaline conditions all primary hydrolysis products appeared to be stable as no phosphoric, phosphorothioic, or monoalkyl phosphoric or phosphorothioic acids were formed. Further, dimethyl phosphoric, dimethyl phosphorothioic, and *O*-methyl *O*-(2,4,5-trichlorophenyl) phosphorothioic acids were stable to hydrolysis by ethanolic potassium hydroxide.

Chromatography. Partition chromatography was used for separation and purification of Trolene and its oxygen analog. With a Celite–*iso*-octane–methanol column (*2*) of 3.6 × 26 cm., Trolene was eluted after about 160 ml. of eluate and the oxygen analog after about 220 ml. Complete separation of the two materials was obtained.

Ion exchange chromatography by the procedure of Plapp and Casida (*21*) allowed separation of the following possible hydrolytic products of Trolene: phosphoric, phosphorothioic, methyl phosphoric, dimethyl phosphoric, dimethyl phosphorothioic, *O*-methyl *O*-(2,4,5-trichlorophenyl) phosphoric, and the corresponding phenyl phosphorothioic acids.

Metabolism of Trolene and Derivatives in Rats

Radioactive Trolene and several derivatives were administered orally to white rats at dosages indicated in Table I. Trolene and its oxygen analog were administered in corn oil solutions and the phosphoric acid derivatives in aqueous solution. Rats were also treated with Trolene by subcutaneous injection of the compound in corn oil. In any given experiment, equal numbers of 200-

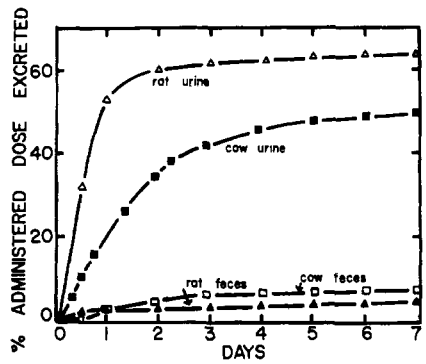

Figure 1. Excretion curves for Trolene metabolites from rats and a cow after oral administration at 100 mg. per kg.

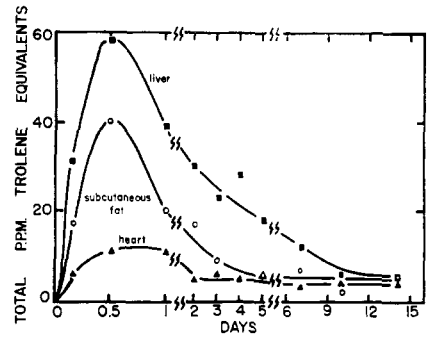

Figure 2. Persistence of Trolene and metabolites in several rat tissues after oral administration at 100 mg. per kg.

to 300-gram male and female rats were used. Treated animals were held in metabolism cages which permitted separate collection of urine and feces (7). The rate of excretion was measured, and the urine samples were held at 4° C. until analyzed by ion exchange chromatography. The excretion curves for Trolene from rats are shown in Figure 1.

When radioactive Trolene was administered orally to rats at 100 mg. per kg., and the rats were sacrificed at different time intervals after treatment, the highest levels of Trolene and derivatives in all tissues were reached after 12 hours with an equilibration of radioactivity within the rats after about 12 days (Figure 2). The tissues in order of decreasing persistence of Trolene and metabolites were: liver, kidney, spleen, subcutaneous and mesenteric fat, heart, and brain. The residues persisting 7 days after oral administration were similar to Trolene and its oxygen analog (Table II). Skin, even when freed of subcutaneous fat by scraping, was extremely high in persisting residues of both compounds. Tissue residues were not fractionated because of insufficient radioactivity.

Trolene and its oxygen analog were hydrolyzed in rats at two sites on the molecule, and the primary hydrolysis products were rapidly excreted. Excretion in the urine and feces accounted for up to 70% of the administered dose, and both organophosphates were excreted in the urine completely as hydrolyzed metabolites. Ion exchange chromatography showed that the first urine samples contained the highest percentage of phenyl phosphoric and phosphorothioic acids and that the percentage decreased progressively with time while the per cent of dimethyl phosphoric acids increased correspondingly (Table I). Of the phosphorus-oxygen-phenyl metabolites in the urine following Trolene treatment about 40% were 2,4,5-trichlorophenyl phosphorothioic acids and 60% were 2,4,5-trichlorophenyl phosphoric acids. The proportion of phenyl phosphoric metabolites was about the same with Trolene administered orally or subcutaneously at 100 mg. per kg. and with the oxygen analog given orally at 100 or 250 mg. per kg.

When dimethyl phosphoric acid was fed to rats, the compound was excreted per se. Phosphorothioic acid never appeared as a metabolite of Trolene. When *O*-methyl *O*-(2,4,5-trichlorophenyl) phosphorothioic acid was fed, about 12% of the radioactivity excreted was phosphoric acid and the remainder was almost completely oxidized to the corresponding phenyl phosphoric acid.

Table II. Total Parts per Million of Trolene Derivatives 7 Days after Oral Administration of Trolene and Its Oxygen Analog to Rats at 100 Mg. per Kg.[a]

Sample	Trolene	Oxygen Analog
Subcutaneous fat	6.8 ± 2.7	1.7 ± 0.6
Mesenteric fat	2.7 ± 0.6	1.7 ± 0.5
Liver	5.6 ± 2.1	10.4 ± 1.9
Spleen	0.8 ± 0.3	2.9 ± 0.7
Kidney	5.1 ± 2.0	5.4 ± 1.2
Brain	2.9 ± 1.7	5.6 ± 1.5
Skin	53.4 ± 6.0	86.0 ± 17.7

[a] P.p.m. calculated on basis of total radioactivity. Variation expressed as standard error of the mean.

Metabolism of Trolene in a Cow

A 700-kg. Holstein cow in late lactation was treated with 100 mg. per kg. of Trolene (70 grams with 1.60×10^9 total counts per minute). The insecticide was mixed with ground grain and placed in five 4-ounce gelatin capsules which were administered to the cow within 1 hour. The cow was held 7 days in a metabolism stall which allowed semiquantitative collection of excretory products. Records of total milk, urine, and fecal production were kept. Samples of blood, milk, urine, and feces were taken at various times after treatment and held at 4° C. until analyzed. After 7 days, the cow was slaughtered, and tissue samples were taken for analysis.

A 3-day-old bull Holstein calf was fed on the milk of the treated cow at the rate of 4 pounds twice a day for the first 3 days and then 6 pounds twice a day for the remainder of the test period. The calf thus obtained a portion of each milk sample taken from the radioactive cow. Frequent blood samples were taken from the calf, and they never showed more than 1.5 p.p.m. of total Trolene and metabolites, nor any depression in cholinesterase level. The calf was not sacrificed, as insufficient radioactivity was present for satisfactory tissue analysis.

Blood Cholinesterase and Toxicology. The cholinesterase activity of whole blood, plasma, and red blood cells was determined manometrically (6). A progressive cholinesterase depression resulted to about 40 to 50% of pretreatment activity after 3 days (Figure 3). The plasma cholinesterases were more greatly depressed than the corpuscle cholinesterases but the low level of plasma activity made these determinations more variable. No symptoms of phosphate poisoning were observed in the treated animal nor were any gross pathological abnormalities observed when the cow was slaughtered.

Fractionation Procedures for Trolene and Metabolites. Trolene and its oxygen analog were separated from hydrolysis products by extraction into Skellysolve B from aqueous solutions. Cleanup of the Skellysolve B extract from biological materials was effected by extracting four times with acetonitrile, adding an equal volume of water to the acetonitrile, and extracting the organophosphates back into Skellysolve B. The nature of the Skellysolve B solubles was then ascertained by Celite column chromatography.

Blood. The parts per million of Trolene and derivatives in the whole blood based on radioactivity are shown

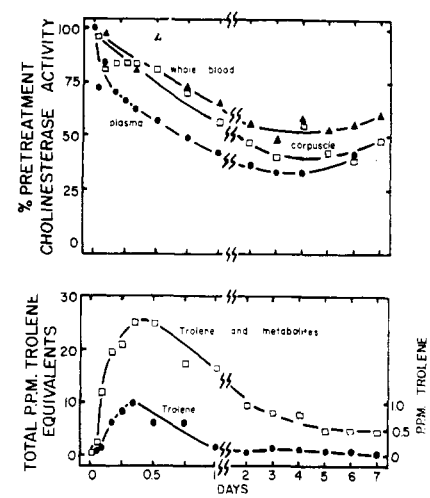

Figure 3. Cholinesterase depression and Trolene derivatives in blood of a cow after oral administration at 100 mg. per kg.

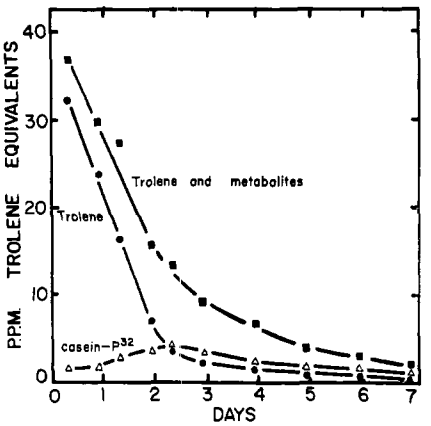

Figure 4. Secretion of Trolene and derivatives in milk of a cow after oral administration at 100 mg. per kg.

in Figure 3. The peak level of 25 p.p.m. of total Trolene equivalents was reached after 8 to 12 hours followed by a progressive decline to about 5 p.p.m. after 7 days. The Trolene level (Skellysolve B solubles) peaked at 8 hours with 1 p.p.m. and dropped to 0.12 p.p.m. after 24 hours and 0.05 p.p.m. after 7 days.

Milk. One hundred-milliliter samples of whole milk were mixed with 0.5 gram of potassium nitrate, 100 ml. of 95% ethyl alcohol, and 150 ml. of Skellysolve B (*8*), and were extracted for 30 minutes in a mechanical shaker. The Skellysolve B layer was separated by centrifugation and the casein by precipitation with dilute nitric acid (*3*). About 2.2% of the total radioactivity administered to the cow was secreted in the milk within 7 days. A milk sample taken 8 hours after treatment contained 37 p.p.m. of Trolene equivalents of which 32 p.p.m. were Skellysolve B extractable. This extractable radioactivity declined rapidly but still constituted 0.4 p.p.m. after 7 days (Figure 4). Greater than 95% of the radioactivity in a Skellysolve B extract from a pooled 0- to 48-hour milk sample was Trolene based on chromatography and bioassay with houseflies. Radioactive casein in the milk reached a maximum of 4 p.p.m. of Trolene equivalents 54 hours after treatment.

Tissues. A series of 17 tissues was fractionated to separate Trolene and its oxygen analog from hydrolysis products and unextractable residues appearing in the protein fraction. The following procedure was used:

One hundred-gram tissue samples were macerated with 100 ml. of water in a Waring Blendor, and the brei was adjusted to pH 5 with trichloroacetic acid. The sample was extracted for 30 minutes in a mechanical shaker with 150 ml. of Skellysolve B and poured through cheesecloth to remove the precipitated proteins. Centrifugation separated the Skellysolve B and aqueous phases. The aqueous phase was recombined with the precipitated proteins, 15 grams of trichloroacetic acid was added, and the sample was held overnight. The aqueous phase was again separated from the protein residue by pouring through cheesecloth. The residue was washed twice with 10% trichloroacetic acid, twice with 95% ethyl alcohol, once with 3 to 7 ethyl alcohol–ether, and twice with ether.

The parts per million of Trolene equivalents in each fraction of the various tissues are given in Table III. The total Trolene equivalents varied from 6 p.p.m. in the spinal cord to 80 p.p.m. in the kidney. The highest levels of Trolene (Skellysolve B–soluble radioactivity) appeared in the fat, kidneys, and lungs. Chromatography of Skellysolve B extracts of subcutaneous and mesenteric fat with known Trolene and its oxygen analog showed that better than 95% of the radioactivity present chromatographed as Trolene. Upon fractionation of the brain phospholipides after the method of Harrow (*12*), radioactivity was found in the lecithin-cephalin and sphinogomyelin fractions.

Urine. Trolene metabolites in the urine accounted for about 49% of the administered dose (Figure 1), but this per cent excretion figure can be considered as minimal as the metabolism stall utilized was not optimal for quantitative collection. The peak level of radioactivity occurred from 18 to 32 hours after treatment with about 1800 p.p.m. of Trolene equivalents in the urine.

At the time the cow was treated, the ion exchange separation of hydrolysis products was not adequately developed for use as with the rat experiments. However, a large fraction of the radioactivity in the urine could be extracted from acidified urine with chloroform. After cleanup on an ion exchange resin, comparison of infrared spectra of the chloroform-extractable metabolite(s) with spectra of known Trolene derivatives indicated that this fraction was predominantly *O*-methyl *O*-hydrogen *O*-(2,4,5-trichlorophenyl) phosphorothioate.

Feces. One hundred-gram fecal samples were macerated with 100 ml. of water and 100 ml. of Skellysolve B in a Waring Blendor. The supernatant was poured through cheesecloth, and the aqueous and Skellysolve B layers were separated by centrifugation. About 7% of the administered dose was excreted in the feces with about one seventh of this being Skellysolve B soluble. Chromatography showed this fraction to be Trolene. Trolene per se reached a maximum level of 24 p.p.m. in the feces at 24 hours and diminished to 2.3 p.p.m. after 70 hours and 0.1 p.p.m. after 120 hours.

Other Considerations

Bovine Rumen Fluid. The stability of Trolene on incubation with stagnating bovine rumen juice was studied by a described procedure (*1*) and the hydrolysis products were characterized by ion exchange chromatography (Table I). No conversion to the oxygen analog was demonstrated under these conditions. The primary site of hydrolysis of Trolene by rumen fluid was at the phosphorus-oxygen-methyl group to yield phenyl phosphoric acids. Some dimethyl phosphoric acid as well as dimethyl phosphorothioic acid was formed indicating the possible occurrence of oxidative metabolism as well as hydrolysis.

Houseflies. Acetone solutions of Trolene and various derivatives were assayed for toxicity to flies following topical

Table III. Parts per Million of Trolene and Derivatives 7 Days after Oral Administration to a Cow at 100 Mg. per Kg.

Sample	Skelly B Soluble	Water Soluble	Residue	Total
Subcutaneous fat	18.0 ± 5.0	19.1 ± 1.0	8.2 ± 5.1	44.3 ± 7.7
Mesenteric fat	8.9 ± 2.5	13.2 ± 2.7	1.0 ± 0.2	23.1 ± 3.5
Loin muscle	4.2[a]	10.8[a]	3.2[a]	18.2 ± 1.1
Neck muscle	1.9 ± 1.0	2.0 ± 1.0	3.6 ± 0.8	7.5 ± 1.0
Liver	1.8 ± 0.4	23.3 ± 1.1	6.8 ± 1.1	31.9 ± 6.7
Spleen	1.0 ± 0.4	10.3 ± 0.5	0.3 ± 0.5	11.6 ± 2.1
Kidney	9.4 ± 3.9	48.7 ± 7.4	21.9 ± 3.9	80.0 ± 16.5
Lung	9.2 ± 1.5	28.4 ± 2.8	8.0 ± 1.5	45.6 ± 6.7
Heart	1.7 ± 1.2	18.2 ± 1.6	5.5 ± 2.0	25.4 ± 2.8
Brain	1.9[b]	10.9[b]	1.0[b]	13.8 ± 1.2
Spinal cord	2.2[b]	3.7[b]	0.3[b]	6.2 ± 0.8
Tongue	0.7 ± 0.1	5.8 ± 1.0	3.2 ± 0.9	9.7 ± 0.1
Hide	2.0 ± 1.5	3.9 ± 2.1	3.7 ± 0.6	9.6 ± 1.5
Diaphragm	4.9 ± 0.7	1.6 ± 0.8	4.8 ± 0.3	11.3 ± 2.2
Rumen wall	2.8 ± 1.3	3.6 ± 1.5	1.6 ± 0.2	8.0 ± 0.7
Rumen contents	1.6 ± 0.8	5.8 ± 0.9	0.9 ± 0.4	8.3 ± 2.8
Small intestine and contents	2.9 ± 1.0	28.9 ± 1.1	0.8 ± 0.2	32.6 ± 7.7
Bone (rib)				14.4 ± 0.9
Ovary				27.7 ± 1.4

[a] Two replicates. [b] One replicate. All others, 3 replicates. Variation expressed as standard error of the mean.

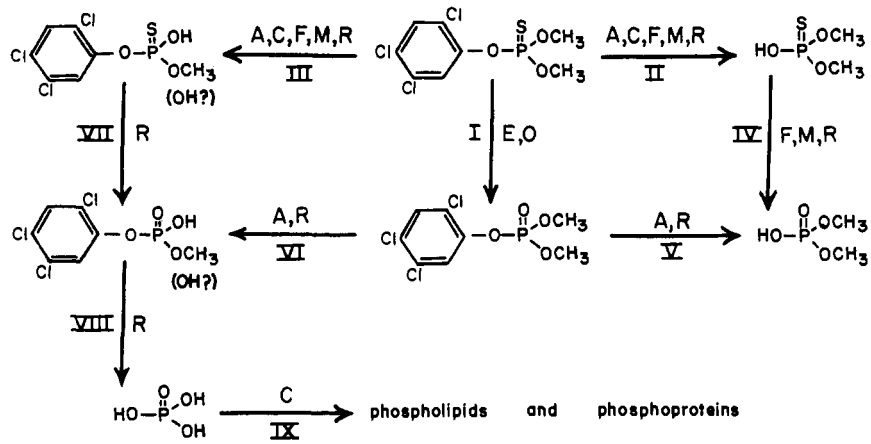

Figure 5. Proposed metabolic pathway for Trolene and its oxygen analog

A. Alkaline hydrolysis
C. Cow
E. Enzyme inhibition
F. Housefly
M. Bovine rumen fluid
O. Chemical oxidation
R. Rats

application (*15*). The microgram-per-gram LD_{50} values were found to be 1.5 for Trolene, 2.0 for the oxygen analog, and over 1000 for *O*-methyl *O*-hydrogen *O*-(2,4,5-trichlorophenyl) phosphorothioate.

Houseflies treated topically with LD_{50} dosages of Trolene and the oxygen analog showed similar time-mortality curves and similar in vivo brain cholinesterase depression and recovery curves (*20*). The oxygen analog of Trolene is about 2000 times as potent as inhibitor of fly brain cholinesterase in vitro as Trolene (*16*).

Two groups of about 1000 adult houseflies (*Musca domestica* L.) were sprayed with an acetone solution of radioactive Trolene. One group was sacrificed after 10 minutes when the flies were hyperactive and the other group after 1 hour when most of the flies were paralyzed. The nonhydrolyzed radioactivity was recovered completely as Trolene with no evidence for the presence of the oxygen analog. Characterization of the hydrolysis products by ion exchange chromatography showed that the primary site of hydrolysis was at the phosphorus-oxygen-phenyl bond (Table I).

Miscellaneous. Total phosphorus and radioactive measurements were made by previously described methods (*5*). Infrared spectra were run in a Baird spectrophotometer with sodium chloride optics and 10% solutions of the chemicals in chloroform, carbon tetrachloride, and carbon disulfide.

Discussion

Trolene is susceptible to degradation through the pathways shown in Figure 5. There are three initial sites of attack of the molecule. Two of these, phosphorothioate oxidation (Figure 5, I) and hydrolysis of the phosphorus-oxygen-phenyl bond (Figure 5, II), have been previously shown for dialkyl aryl phosphorothioates (*4*). Hydrolytic attack at the alkoxy group to yield a stable monoalkyl aryl phosphodiester has not been previously reported. Both Trolene and its oxygen analog are susceptible to hydrolysis of their phosphorus-oxygen-methyl bond (Figure 5, III, VI) as well as cleavage of the phosphorus-oxygen-phenyl bond (Figure 5, II, V). The relative degree of attack at these three sites (Figure 5, I, II, III) varies with the chemical or biological system and conditions employed.

The metabolic pathway of Trolene in a cow was similar to that found in rats, but with a slower detoxification and excretion (Figure 1). Tissue residues of Trolene equivalents (Trolene plus metabolites) were generally as high after 7 days with the cow as after 1 day with the rats (Figure 2 and Table III). The formation of labeled casein and phospholipides is evidence for the complete degradation of the compound to phosphoric acid via a phenyl phosphate intermediate. Hydrolysis of Trolene by houseflies was predominantly at the phenyl group, under the experimental conditions employed, in contrast to rats where hydrolysis of a methyl group predominated. If this difference in the initial site of hydrolysis of Trolene between insects and mammals proved to be general, it might partially serve to explain the selective toxicity towards insects.

The metabolic pathway of an organophosphate can be partially but not completely elucidated through characterization of excreted hydrolysis products. A primary difficulty is interpreting whether the initial attack was oxidation or hydrolysis. Another difficulty lies in possible degradation of certain unstable hydrolysis products in the sampling and analysis.

The oxygen analog of Trolene was not recovered by chromatography as an in vivo metabolite in flies or in cows (milk fat, body fat, or feces) nor from in vitro incubation with bovine rumen juice. However, indirect evidence for its formation is available from the inhibition of cow blood cholinesterases and fly brain cholinesterases in Trolene-treated animals, despite the very low anticholinesterase activity of Trolene as compared with its oxygen analog.

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Literature Cited

(1) Ahmed, M. K., Nichols, R. E., Casida, J. E., J. Agr. Food Chem. **6,** No. 10, in press.
(2) Bowman, J. S., Casida, J. E., *Ibid.*, **5,** 192 (1957).
(3) Carter, H. E., editor, "Biochemical Preparations," Vol. **I,** p. 22, Wiley, New York, 1949.
(4) Casida, J. E., J. Agr. Food Chem. **4,** 772 (1956).
(5) Casida, J. E., Gatterdam, P. E., Getzin, L. W., Jr., Chapman, R. K., *Ibid.*, **4,** 236 (1956).
(6) Casida, J. E., Gatterdam, P. E., Knaak, J. B., Lance, R. D., Niedermeier, R. P., *Ibid.*, **6,** 658 (1958).
(7) Comar, C. L., "Radioisotopes in Biology and Agriculture," p. 130, McGraw-Hill, New York, 1955.
(8) Dahm, P. A., Fountaine, F. C., Pankaskie, J. E., Smith, R. C., Atkeson, F. W., *J. Dairy Sci.* **33,** 747 (1950).
(9) Fletcher, J. H., Hamilton, J. C., Hechenbleikner, I., Hoegberg, E. I., Sertl, B. J., Cassaday, J. T., *J. Am. Chem. Soc.* **74,** 1856 (1952).
(10) Forbes, M. C., Roswell, C. A., Maxson, R. N., *Inorg. Syntheses* **2,** 145 (1946).
(11) Fukuto, T. R., Metcalf, R. L., J. Agr. Food Chem. **4,** 930 (1956).
(12) Harrow, B., "Textbook of Biochemistry," 5th ed., p. 38, Saunders, Philadelphia, 1950.
(13) Johnson, G. A. (to American Cyanamid Co.), U. S. Patent **2,713,018** (July 12, 1955).
(14) Knotz, F., *Österr. Chemiker-Z.* **50,** 128 (1949).
(15) Krueger, H. R., Casida, J. E., *J. Econ. Entomol.* **50,** 356 (1957).

(16) Ludwig, P. D., Dow Chemical Co., Midland, Mich., personal communication (1957).
(17) McGregor, W. S., Bushland, R. C., *J. Econ. Entomol.* **50,** 246 (1957).
(18) McGregor, W. S., Radeleff, R. D., Bushland, R. C., *J. Econ. Entomol.* **47,** 465 (1954).
(19) Mellor, J. W., "A Comprehensive Treatise on Inorganic and Theoretical Chemistry," Vol. **VIII,** p. 1068, Longmans, London, 1928.
(20) Mengle, D. C., Casida, J. E., University of Wisconsin, Madison, Wis., 1957, unpublished data.
(21) Plapp, F. W., Casida, J. E., *Anal. Chem.* **30,** 1622 (1958).
(22) Radeleff, R. D., Woodard, G. T., *J. Econ. Entomol.* **50,** 249 (1957).
(23) Robbins, W. E., Hopkins, T. L., Eddy, G. W., *Bull. Entomol. Soc. Amer.* **2,** 23 (1956).
(24) Robbins, W. E., Hopkins, T. L., Eddy, G. W., J. AGR. FOOD CHEM. **5,** 509 (1957).
(25) Robbins, W. E., Hopkins, T. L., Eddy, G. W., *J. Econ. Entomol.* **49,** 801 (1956).
(26) Roth, A. R., Eddy, G. W., *Ibid.*, **50,** 244 (1957).

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FUNGICIDE RESIDUES

Determination of Tetrachloro-1,4-benzoquinone (Spergon) Residues on Food Crops

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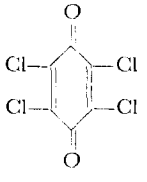
Spergon is dissolved from plant surfaces with benzene. The Spergon in the recovered solvent oxidizes diphenyl-*p*-phenylenediamine to a blue Wurster salt. Extraction of the benzene with an aqueous acetic acid–hydrochloric acid solution removes the Wurster salt to the aqueous acid phase. The absorbance is measured spectrophotometrically at 700 mμ. This method has been applied to broccoli, cauliflower, and lettuce. Recovery data were obtained at the 0.1- and 0.5-p.p.m. level.

THE FUNGICIDAL PROPERTIES of tetrachloro-1,4-benzoquinone (Spergon) were discovered at the General Laboratories of United States Rubber Co. in 1940 (*8*).

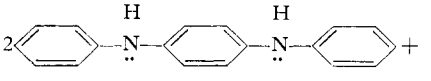


Spergon

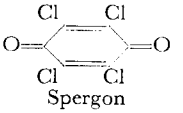
Spergon is used chiefly to protect seeds and bulbs (*1, 4, 6, 7*). It also gives outstanding control of downy mildew of broccoli, cauliflower, and lettuce (*1, 5, 6*). The latter use involves spray or dust application to food crops, which necessitates residue analysis to determine a safe level of use—in the neighborhood of 0.1 p.p.m.

Several good color tests for microdetermination of Spergon are known. For example, Spergon forms a strong yellow color with anhydrous diethylamine, which is the basis for the determination of Spergon on treated seed (*3*). In aqueous sodium hydroxide, Spergon readily converts to the sodium salt of chloranilic acid. Upon acidification, the free chloranilic acid produced is an intense purple color. These color tests are sufficiently sensitive for residue analysis; however, attempts to recover 1-p.p.m. doses of Spergon from broccoli and cauliflower by these tests failed.

Compounds of the same general type as Spergon combine irreversibly with plant constituents. The failure of the above color tests, which utilize the reactive chlorine atoms of Spergon, to produce color indicated that the reactive chlorine atoms of Spergon had been rendered inactive by combination with plant constituents. Next, attempts were made to utilize the oxidative properties of the quinone groups of Spergon to produce a sensitive color, and they were successful. Diphenyl-*p*-phenylenediamine is readily subject to oxidation by mild oxidizing agents such as Spergon (benzoquinones) to produce an intense blue color owing to the formation of a Wurster salt (*9*).

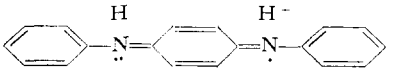


Diphenyl-*p*-phenylenediamine

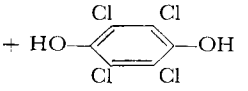


Spergon

(Blue ↕ Color)



Wurster Salt



Tetrachlorohydroquinone

The Wurster salt is a resonating free radical with several possible resonant forms. The two major contributing forms are shown above.

When untreated broccoli or cauliflower was treated with fractions of 1 p.p.m. of Spergon, good recoveries were obtained by use of the diphenyl-*p*-phenylenediamine color test (Table I). The oxidative property of the quinone groups of Spergon was not hindered by the reaction of other parts of the molecule with plant constituents. The bound Spergon remains benzene-soluble and dissolvable from the plant surfaces.

Experimental

Apparatus. Spectrophotometer, Beckman Model DU equipped with 1-cm. matched Corex or silica cells.

Reagents. Diphenyl-*p*-phenylenediamine. Purify by vacuum distillation of crude material. Collect the white crystalline plates and store in a covered jar.

Diphenyl-*p*-phenylenediamine reagent. Dissolve 0.5 gram of purified diphenyl-*p*-phenylenediamine in 50 ml. of benzene. Store in a brown glass bottle. Prepare fresh daily.

Extracting solution. In a suitable acid bottle, place 850 ml. of glacial acetic acid, 100 ml. of distilled water, and 50 ml. of concentrated hydrochloric acid. (Deaerate daily before using by bubbling dry nitrogen gas through the solution for 0.5 hour.)

Processing of Sample. Place a 1000-gram sample of the crop and a volume