charge on the sulfonium salt may be expected to increase the affinity for cholinesterase by coulombic attraction to the negatively charged anionic site. However, as with the quaternary ammonium salts, position isomerism is of overriding importance, as is shown in Table IV. In both the thioether and amine series, the most active quaternary compounds are those with the charged atom in the meta-position of the ring. The attraction of the ammonium nitrogen to the anionic site is considerably stronger than that of the sulfonium sulfur. It is of particular interest to compare the affinities of the sulfonium compounds in Table IV with the uncharged methylthioethers of Table I. It is apparent that quaternization decreases the affinity of the ortho-isomer (XLV) to about 0.05, increases the affinity of the meta-isomer (XLVII) by about 10 times, and increases the affinity of the para-isomer (XLIX) by about 3 times. These affects are qualitatively similar to those observed with quaternization of the uncharged dimethylaminophenyl *N*-methyl carbamates (*8*): The ortho-isomer (XLVI) decreased to about 0.2, meta-isomer (XLVIII) increased by about 130-fold, and para-isomer (L) increased about 68-fold.

As has been observed with the quaternary ammonium carbamates (*5*), the presence of a formal positive charge in the sulfonium carbamates effectively destroys the contact toxicity to *Musca* and *Culex*, presumably because of the inability of the charged molecules to penetrate into the nerve synapse.

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INSECTICIDE METABOLISM

Thin-Layer Chromatography and Cholinesterase Detection of Several Phosphorothiono Insecticides and Their Oxygen Analogs

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Partition thin-layer chromatography techniques have been developed to separate and detect phosphorothionate, phosphorodithioate, and phosphoramidothioate insecticides and their corresponding oxygen analogs. Cellulose layers on chromatoplates are coated with polar and apolar stationary phases and developed with immiscible mobile phase solvents. Different chromatographic systems reverse the order of mobility of the compounds and their oxons. Chromogenic agents detect as little as 0.1 to 0.5 μg. of the compounds tested. A cholinesterase spray method on the intact cellulose layers detects anticholinesterases at the nanogram level or below. The weak cholinesterase inhibitors are also detected by prior conversion to their oxons by suitable oxidation techniques.

ORGANOPHOSPHORUS insecticides containing the thiono sulfur group (P=S) are known to be converted to their corresponding oxygen analogs or oxons (P=O) in biological systems, the latter having greatly enhanced cholinesterase-inhibiting properties (*6*, *11*). Since the oxons are the major anticholinesterase metabolites of this class of compounds and are thought to be primarily responsible for the toxic action, considerable interest has centered on formation and accumulation of these metabolites in animals and plants. The oxons are generally much more susceptible to hydrolytic degradation than their parent compounds and, probably for that reason, do not usually accumulate to any great extent in biological systems. Sensitive techniques of separation and detection therefore greatly simplify the study of these metabolites. Paper chromatography, combined with direct cholinesterase detection, to detect inhibitors was first reported by Cook (*1*), who studied activation products of several organophosphorus pesticides. The method was later used to identify anticholinesterases produced in mammals and insects (*7*, *8*, *12*).

The present study was concerned with developing thin-layer chromatographic (TLC) methods for separating several phosphorothionate, $(RO)_2P(S)OX$, phosphorodithioate, $(RO)_2P(S)SX$, and phosphoramidothioate, $(RO)(RNH)P(S)OX$, insecticides from their corresponding oxons and for detecting the inhibitors on the intact plates by the cholinesterase detection method. The advantages of thin-layer chromatography over paper systems are much greater sensitivity and resolution of mixtures, rapid separation, and the ability to separate greater quantities of material, which make this technique preferable in a study of the metabolism of organophosphorus compounds in insects and other organisms.

Several workers have developed TLC methods for organophosphorus insec-

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ticides, but these have not included data on the mobility of the oxons of phosphorothiono insecticides having little or no systemic activity in plants which are included in this study (*3*, *13*). Separations were done on activated silica gel layers which in preliminary studies failed to separate the paired analogs satisfactorily. For that reason, techniques of partition thin-layer chromatography were developed in which cellulose layers were coated with polar and apolar immobile phases as is practiced in paper chromatography. Cellulose adheres more strongly to glass plates than does silica gel and is easily coated with a variety of compounds. The technique proved entirely satisfactory and permitted transfer of paper partition systems to TLC with greatly improved results. A method for detecting the oxons directly on the intact layers by the cholinesterase technique has also been developed.

Table I. Phosphorothiono Insecticides and Their Corresponding Oxons Separated by Thin-Layer Chromatography

Common or Trade Name	Chemical Name
Co-Ral	*O,O*-Diethyl *O*-(3-chloro-4-methyl-7-coumarinyl) phosphorothionate[a]
Coroxon	*O,O*-Diethyl *O*-(3-chloro-4-methyl-7-coumarinyl) phosphate[a]
Narlene	*O*-4-*tert*-Butyl-2-chlorophenyl *O*-methyl methylphosphoramidothionate[b]
Ruelene	*O*-4-*tert*-Butyl-2-chlorophenyl *O*-methyl methylphosphoramidate[b]
Guthion	*O,O*-Dimethyl *S*-4-oxo-1,2,3-benzotriaz-3(4*H*)-ylmethyl phosphorodithioate[a]
Gutoxon	*O,O*-Dimethyl *S*-4-oxo-1,2,3-benzotriaz-3(4*H*)-ylmethyl phosphorothiolate[a]
Malathion	*O,O*-Dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorodithioate[c]
Malaoxon	*O,O*-Dimethyl *S*-(1,2-dicarboethoxyethyl) phosphorothiolate[c]
Methyl parathion	*O,O*-Dimethyl *O*-*p*-nitrophenyl phosphorothionate[d]
Methyl paraoxon	*O,O*-Dimethyl *O*-*p*-nitrophenyl phosphate[e]
Parathion	*O,O*-Diethyl *O*-*p*-nitrophenyl phosphorothionate[c]
Paraoxon	*O,O*-Diethyl *O*-*p*-nitrophenyl phosphate[c]
Ronnel	*O,O*-Dimethyl *O*-2,4,5-trichlorophenyl phosphorothionate[b]
Ronoxon	*O,O*-Dimethyl *O*-2,4,5-trichlorophenyl phosphate[b]

[a] Chemagro Corp. [b] Dow Chemical Co. [c] American Cyanamid Co. [d] Monsanto Chemical Co. [e] Oxidation on chromatoplate.

Materials and Methods

Apparatus. Thin-layer applicator with fixed thickness 250-micron gate, plastic mounting board, and 8- × 8- and 2- × 8- inch glass plates (Desaga-Brinkman, Westbury, N. Y., and Research Specialties, Richmond, Calif.). The narrow plates are especially useful for rapid testing of chromatographic systems, but the 8 × 8 plates give the most uniform results, so are always used in the final procedure.

Stainless steel Thomas-Mitchell chromatography tanks and trough assemblies (*10*) (A. H. Thomas Co., Philadelphia, Pa.) are preferred, since they require much less mobile phase solvent than conventional glass tanks.

A stainless steel Thomas-Mitchell dipping tank is used (No. 3680D, A. H. Thomas Co.) to coat the cellulose thin-layer plates with the stationary phases.

Ultraviolet light sources are General Electric G15T8 germicidal lamp for short-wave ultraviolet (254 mμ) and F 15T8 BLB black light for long-wave ultraviolet radiation (366 mμ).

Reagents. MN cellulose powder 300G according to Stahl with 10% $CaSO_4$ binder (Desaga-Brinkman).

Immobile phases are *N,N*-dimethylformamide, Eastman practical grade; and mineral oil, heavy white petrolatum liquid U.S.P.

Mobile phases are 2,2,4-trimethylpentane, Eastman practical grade; benzene, glass-distilled over sodium; and acetonitrile, Matheson Coleman & Bell, practical grade. Different brands and grades of acetonitrile may give variable R_f values, but the ratio of acetonitrile to water can be changed to obtain the optimum resolution.

Chromogenic Sprays. Bromophenol blue–silver nitrate (*4*). $AgNO_3$, 1.0% in acetone-water (75:25); bromophenol blue, 0.4% in acetone. Prepare spray solution by mixing 9 parts of the first with 1 part of the second solution. Citric acid, 0.01% in distilled water.

Alkaline Silver Nitrate. Aqueous $AgNO_3$ 10% stock solution (1 part), concentrated ammonium hydroxide (1 part), methanol (8 parts). Discard solution after one week.

Alcoholic potassium hydroxide, 5% in ethanol.

Cholinesterase Detection. Enzyme-indicator solution. Mix 10 ml. of Bacto horse serum (Difco Laboratories, Detroit) or human serum, 30 ml. of distilled water, 1 ml. of 0.1*N* NaOH, and 4 ml. of 1.2% bromothymol blue in 0.1*N* NaOH.

Substrate solution, 2% acetylcholine bromide or chloride in distilled water. Both spray solutions may be used one week or more if refrigerated. Warm both solutions to 37° C. before spraying on plates.

N-Bromosuccinimide. Dissolve 0.1 gram of *N*-bromosuccinimide in 100 ml. of acetone. Dilute 5 ml. of this stock to 100 ml. with acetone for the spray solution.

Insecticide Solutions. Dissolve the compounds in glass-distilled acetone for spotting on the thin-layer plates (listed in Table I).

Preparing Adsorbent Layer. The glass plates are washed in hot water and detergent and rinsed thoroughly with tap water, distilled water, and finally with acetone. The surface is wiped with 95% ethyl alcohol on absorbent tissue as a final cleaning step after the plates are mounted on the plastic board. Cellulose layers crack or peel if the glass plates are not absolutely clean. The cellulose powder (10.5 grams) is ground for 1 minute with 35 ml. of distilled water in a porcelain mortar. An additional 25 ml. of water are immediately added and grinding continues for another minute, until the mixture is a smooth slurry. The plates are then coated with the cellulose, allowed to dry at room temperature for 30 minutes, and finally oven-dried for 15 minutes at 105° C.

Washing Cellulose Layers. The troughs in the developing tank are filled to a depth of 1 cm. with a 50% (v./v.) solution of acetone and distilled water. The plates are inserted and developed to within 1 inch of the top. Washing should be done in the same direction as the layer application and be started from the side first coated by the applicator. The compounds should be spotted at that margin, as described later. Therefore, imperfections sometimes produced at the top margin of the layers due to unevenness of the glass plates will not affect the results of separation. The plates are dried at room temperature, then at 105° C. for 15 minutes, and stored in a desiccator. The washing removes impurities in the cellulose layer which would otherwise react with several chromogenic reagents to give a "curtain" at the solvent front and obscure any compounds migrating to this area.

Chromatographic Systems. The nonaqueous or normal phase system employs various concentrations of dimethylformamide as a stationary phase on cellulose and 2,2,4-trimethylpentane alone or in mixture with benzene as the mobile phase (Table II). Since the dimethylformamide is volatile, the plates must be placed in the mobile phase directly after the acetone evaporates from the coating. For that reason the compounds are spotted before coating the plates. The mobile phase (25 ml.) is placed in each trough and (50) ml. in the bottom of the developing tank to saturate the atmosphere. The insecticide solutions are spotted at a line 3 cm. from the edge of the plate, held carefully at this edge, and immersed in the stationary phase contained in the dipping tank until the solvent just touches the starting line. The plates are immediately removed and, as soon as the solvent dries (about 10 seconds), are inverted and placed in the trough containing the mobile phase with the upper edge of each plate leaning against the side of the tank. The tank is covered and sealed with masking tape and the mobile phase allowed to ascend at least 10 cm. from the starting line. The plates are then removed and dried for assay with the chromogenic agents or by the cholinesterase method.

The aqueous or reverse phase system

Table II. Nonaqueous (Normal Phase) System of TLC of Phosphorothiono Insecticides and Their Oxons

Compound	Immobile Phase, % DMF in Acetone[a]	Mobile Phase, % Benzene in TMP[a]	R_f	Lower Limits of Detection: Chromogen, μg.			ChE inhibition, nanograms
Co-Ral	20	0	0.49	II[b]		0.5	[c]
Coroxon			0.21	II		0.5	0.1
Narlene	20	0	0.35	II		0.1	...
Ruelene			0.15	II		0.1	0.25
Guthion	20	25	0.27	I[d]		0.1	...
Gutoxon			0.10	I		0.1	0.025
Malathion	15	15	0.52	I		0.1	...
Malaoxon			0.23	I		0.1	10.0
Methyl parathion	20	25	0.47	II	III[e]	0.1	
Methyl paraoxon			0.24	II	III	0.1	2
Parathion	20	0	0.45	II	III	0.2	...
Paraoxon			0.25	II	III	0.1	0.5
Ronnel	20	0	0.64	II		0.1	...
Ronoxon			0.31	II		0.5	0.025

[a] Solutions % v./v. DMF = dimethylformamide; TMP = trimethylpentane.
[b] II = alkaline $AgNO_3$.
[c] Sulfur analogs can be detected only after oxidation with NBS or bromine.
[d] I = Bromophenol blue–$AgNO_3$.
[e] III = alcoholic KOH.

has a stationary phase of mineral oil dissolved in ethyl ether to coat the cellulose layer and a mobile phase of acetonitrile and water in various ratios (Table III). The procedure for this system differs from the nonaqueous system as follows. Plates are coated with the stationary phase prior to insecticide spotting, the spotting line is 2 cm. from the bottom edge, the chromatographic tank is lined on three sides with filter paper, and the top cover is left unsealed by tape.

Chromogenic Sprays. SILVER NITRATE–BROMOPHENOL BLUE (*4*). This reagent detects only the sulfur-containing phosphate esters and is sensitive for the thiono, thiol, and dithio compounds. The chromatoplates are dried at 50° C. for 15 minutes, sprayed with the reagent, and reheated at 50° C. for 10 minutes. The cooled plates are then carefully immersed in 0.01% aqueous citric acid until the blue spots appear.

AMMONIACAL SILVER NITRATE. The chromatoplate is exposed to the short-wave ultraviolet for 10 minutes, sprayed with the reagent, dried at 75° C. for 15 minutes, and then exposed again to the same light until the spots appear as gray or brown areas on a tan background. This reagent detects many of the organophosphorus insecticides. Co-Ral and coroxon may be detected as fluorescing spots by viewing under the long-wave ultraviolet even before spraying with the silver nitrate solution.

ALCOHOLIC POTASSIUM HYDROXIDE. This solution is used to detect parathion, methyl parathion, and their corresponding oxons (*9*). The plates are sprayed and then heated at 100° C. to give yellow spots. Visibility is increased by viewing under long-wave ultraviolet light.

Cholinesterase Detection. This method was adapted for TLC from that reported by Cook (*1*) and modified by Getz (*5*) for spraying the enzyme and substrate on paper chromatograms.

The dried chromatoplates are placed horizontally and sprayed with the enzyme-indicator solution from an all-glass spray bottle so that the cellulose layer is moist but not wet enough to drip. The plate is allowed to incubate for 20 minutes at room temperature without being moved. During this period the cellulose layer dries and is then sprayed lightly with the substrate solution. The entire surface should be sprayed with each solution or else the solution and the spots will creep toward the dry area. Areas of inhibition, indicating the presence of anticholinesterases, become visible within 2 minutes and appear as bright blue spots on a yellow background in 20 to 30 minutes. These remain visible for 2 to 24 hours or more, depending on the quantity spotted and the activity of the inhibitory compound.

Phosphorothiono compounds can be detected by the cholinesterase method after they are converted to their oxons on the developed chromatoplates by spraying with *N*-bromosuccinimide (*2*) or by exposure to bromine vapor (*5*). The dried chromatoplates are sprayed with a fresh solution of *N*-bromosuccinimide in acetone and then dried at 60° C. for 15 minutes in a forced air oven to eliminate interference by the oxidizing agent.

Bromine oxidizes the sulfur analogs to their oxons more efficiently and therefore is preferred. The dried chromatoplates are exposed to bromine vapors in a glass tank for 30 seconds. The remaining bromine on the plate is removed by placing the plates in an oven with circulating air at 60° C. for 15 minutes.

Often it is desirable to use both a chromogenic agent and the cholinesterase method on separate areas of the same chromatoplate to compare R_f values of standards and unknowns. This is easily accomplished by dividing the plate into sections by scraping narrow bands through the cellulose layer to prevent spreading of spray solutions. The plate is then covered with foil or plastic wrap except for the area to be analyzed.

Table III. Aqueous (Reverse Phase) System of TLC of Phosphorothiono Insecticides and Their Oxons

Compound	Immobile Phase,[a] % Mineral Oil in Ethyl Ether	Mobile Phase[a] % CH_3CN in H_2O	R_f	Lower Limits of Detection: Chromogen, μg.			ChE inhibition, nanograms
Co-Ral	10	30	0.11	II[b]		0.5	...[c]
Coroxon			0.64	II		0.5	0.01
Narlene	10	40	0.21	II		0.1	...
Ruelene			0.75	II		0.1	0.1
Guthion	10	20	0.26	I[d]		0.1	...
Gutoxon			0.87	I		0.1	0.001
Malathion	15	20	0.08	I		0.2	...
Malaoxon			0.89	I		0.1	2.0
Methyl parathion	10	20	0.09	II	III[e]	0.1	...
Methyl paraoxon			0.86	II	III	0.1	0.5
Parathion	10	40	0.26	II	III	0.1	...
Paraoxon			0.85	II	III	0.1	0.025
Ronnel	10	50	0.11	II		0.1	...
Ronoxon			0.67	II		0.5	0.025

[a] Solutions % v./v.
[b] II = alkaline $AgNO_3$.
[c] Sulfur analogs can be detected only after oxidation with NBS or bromine.
[d] I = bromophenol blue.
[e] III = alcoholic KOH.

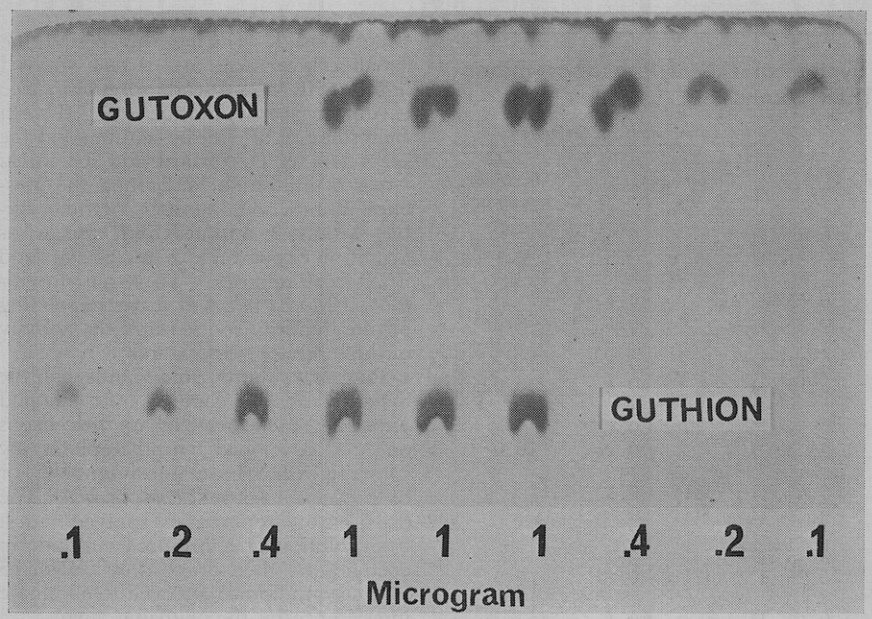

Figure 1. Detection of several concentrations of Guthion and Gutoxon with bromophenol blue–$AgNO_3$ reagent after separation by aqueous system

Results and Discussion

The R_f values and detection limits for seven organophosphorus insecticides and their oxygen analogs by different systems of TLC technique are shown in Tables I and II. In the nonaqueous or normal phase system, which employs an immobile phase of dimethylformamide and mobile phase of 2,2,4-trimethylpentane, the mobility of the sulfur analogs is greater than that of the more polar oxons. Benzene was added to the mobile phase to improve the separation of Guthion, malathion, and methyl parathion from their corresponding oxons (Table II). Mobilities of the paired analogs can be changed to satisfy specific situations by varying the percentage of the stationary phase and the ratio of the two solvents in the mobile phase.

The aqueous or reverse phase system which consists of a mineral oil stationary phase and a mobile phase of acetonitrile and water (Table III) reversed the order of mobility, with the oxons having larger R_f values than the sulfur analogs.

The partition systems presented were those found to give good separation of each pair with sufficient distance from both the starting point and the solvent front. Experience in actual laboratory practice with biological extracts has proved the techniques to be reproducible and consistent. Because of the variables inherent in TLC, the chemical standards are always spotted on the same plate with the unknowns for direct comparison of R_f values.

The lower limits of detection of compounds wih chromogenic reagents also are presented in Tables II and III, and they varied from about 0.1 to 0.5 μg. A typical example of the sensitivity of Guthion and Gutoxon is shown in Figure 1, representing an increase in sensitivity of about 10 times that obtained with paper chromatography. The ammoniacal silver nitrate reagent detects many of the organophosphorus insecticides and their oxygen analogs either as dark spots on a light background or as negative spots. Exposing the chromatoplates to short-wave ultraviolet light before spraying with alkaline silver nitrate facilitates appearance of the spots in short time on a lighter background.

Anticholinesterase compounds have been detected directly on the intact cellulose layers by spraying first with the cholinesterase-indicator solution and then, after a short time for inhibition, with the substrate acetylcholine bromide. Cholinesterase inhibitors appear as sharp blue spots on a yellow background (Figure 2). The TLC technique greatly increases sensitivity of the method for detecting anticholinesterase compounds, with most oxons visible at the nanogram level or below (Tables II and III). Greater sensitivity was always achieved with the aqueous system than with the nonaqueous system with both chromogenic agents and the cholinesterase method, probably because of the mineral oil stationary phase of the former. Dimethylformamide evaporates when the plates are dried; as a result, the spotted compounds are more susceptible to loss than when they are held in a nonvolatile oil phase. Another improvement over paper chromatography is that the blue spots are stable for longer periods of time.

The weak anticholinesterase phosphorothionate or phosphorodithioate compounds can be detected at the same concentration as their oxons, after separation on the cellulose layers, by oxidation prior to cholinesterase detection. Oxidation prior to separation confirmed the identity of the oxidation products as oxons when compared to authentic standards and can be used to determine R_f values of the latter when standards are not available.

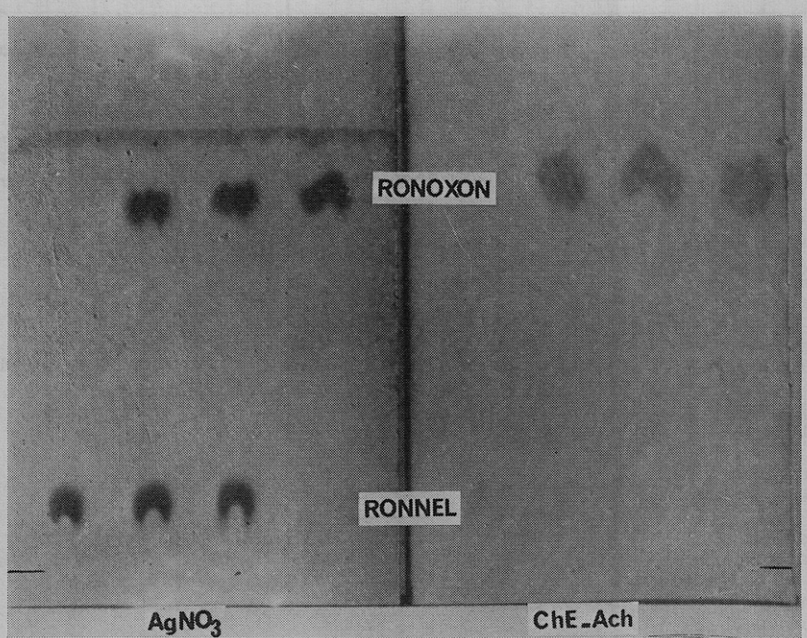

Figure 2. Comparison of cholinesterase spray method and alkaline $AgNO_3$ reagent to detect ronnel and ronoxon after separation by aqueous system

ChE assay. Ronnel 2.0 and ronoxon 0.1 nanograms (ronnel not detected)
Alk. $AgNO_3$. Ronnel and ronoxon 1 μg. each

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INSECTICIDE METABOLISM

Conversion of DDT to DDD by Bovine Rumen Fluid, Lake Water, and Reduced Porphyrins

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DDD is often detected as a residue in situations where only DDT has been used, and DDD appears to persist for unusually long periods. Studies with C^{14}-DDT incubated with bovine rumen fluid, lake water, and aqueous solutions of reduced porphyrins showed partial conversion to C^{14}-DDD. Conversion by bovine rumen fluid may explain certain DDD residues in milk, and conversion by lake water could account for the apparent extraordinary persistence of DDD in Clear Lake, Calif., because DDT may be available as a continuing precursor for DDD in these situations. The study with reduced porphyrins indicates a possible mechanism for this conversion in biological systems.

DDD [1,1-bis(*p*-chlorophenyl)-2,2-dichloroethane], also known as TDE, is a widely recognized and commonly used insecticide. Since it is a residual compound, its residues have been found on treated crops and in animals consuming such crops. However, DDD residues have also appeared in locations not treated with DDD and in animals under conditions where contact of the animals with DDD was unlikely or impossible. In 1963, Finley and Pillmore (*5*) reported the presence of DDD in a large number of water, soil, vegetation, and animal samples taken from an area where only DDT [1,1-bis(*p*-chlorophenyl) - 2,2,2-trichloroethane] had been used. Of 300 samples found to contain DDT, 70% also contained DDD. In another area treated with DDT, they found DDD as well as DDT in mule deer. During the first year after treatment with DDT, fat tissues from these deer showed more DDT than DDD, but sampling a year or more later revealed equal or greater amounts of DDD than DDT. DDD also appeared in birds, frogs, fish, and toads in yet a third area treated with DDT. Peterson and Robison in 1964 (*11*) showed that DDD was present in rats fed pure DDT. DDD was also reported to be present in mice by Barker and Morrison (*2*), who found DDD in DDT-treated mice after 2 to 8 days' incubation at room temperature following death.

DDD appears to be formed from DDT in various biological systems. Kallman and Andrews (*9*) demonstrated this conversion by yeast, using radiolabeled material. Allison *et al.* (*1*) found that other microorganisms accomplished the conversion, and Bridges *et al.* (*3*) found DDD in fish and crayfish taken from a pond treated with DDT. Peterson and Robison (*11*) noted DDD formation during incubation of DDT with a rat liver homogenate for 6 days, the part played by putrefaction being unknown. Recently, Castro (*4*) found that DDT was converted to DDD in the presence of ferrous deuteroporphyrin in an anhydrous and anaerobic solution consisting of isopropyl alcohol–acetic acid (1 to 1) under nitrogen saturated with potassium chloride.

In 1961, Heineman and Miller (*7*) reported the insecticide content found in 4000 milk samples collected throughout the United States. In the positive samples, 90% contained DDT, 12% contained DDD, and 43% contained DDE [1,1-bis(*p*-chlorophenyl)-2,2-dichloroethylene]. In 1963, Rollins (*12*) reported that DDD was found in a large number of milk samples collected in California and that DDD was rarely found in hay or other feed. Since milk samples containing DDD always contained DDT, he suggested, without definite experimental evidence, that DDT was degraded to DDD in the rumen of the cow.

The use of DDD to treat Clear Lake, Calif., for control of the Clear Lake gnat, *Chaoborus astictopus* Dyar & Shannon, has resulted in local controversy because of the persistence of the DDD