In Vivo Fate of the Insecticide Zectran in Spruce Budworm, Tobacco Budworm, and Housefly Larvae

R. B. Roberts, R. P. Miskus, C. K. Duckles, and T. T. Sakai

The insecticide Zectran (4-dimethylamino-3,5-xylyl methylcarbamate) was tested on spruce budworm, tobacco budworm, and housefly larvae. It is highly toxic to spruce budworm larvae, but considerably less toxic to the other two larval insects. Differences were found in the location of activity and rate of metabolism of Zectran among the three insects studied. After topical treatment Zectran was metabolized to at least nine metabolites in the spruce budworm and tobacco budworm and 10 in the housefly. The identity of four of the major metabolites was established by cochromatography and the use of radioactive Zectran labeled in the carbonyl or dimethyl-C^{14}-amino positions. These four metabolites were found in all three insects. In order of occurrence and in decreasing concentration they were: 4-methylamino-3,5-xylyl methylcarbamate, 4-amino-3,5-xylyl methylcarbamate, 4-methylformamido-3,5-xylyl methylcarbamate, and 4-formamido-3,5-xylyl methylcarbamate. One additional metabolite was tentatively identified as 4-dimethylamino-3,5-xylyl-*N*-hydroxymethyl carbamate. Hydrolysis of Zectran was not observed, as indicated by a lack of $C^{14}O_2$ evolution. Oxidation of Zectran on the surface of the spruce budworm cuticle may be a major source of the metabolites found in the homogenates. The rate of cuticular penetration was much the same for all three insects until the third hour.

The carbamate insecticide Zectran (4-dimethylamino-3,5-xylyl methylcarbamate) is highly toxic to the western form of spruce budworm larvae, *Choristoneura occidentalis* Freeman (Freeman *et al.*, 1967), a major forest defoliator. Laboratory tests showed that after topical application an LD_{50} for this insect was 1 μg. per gram of body weight (Miskus *et al.*, 1968). At that toxicity level, Zectran displayed some selectivity, in that other insects were relatively unaffected. The dosage of Zectran had to be increased at least 10-fold to be equally toxic to tobacco budworm larvae, *Heliothis virescens* (Fab.), and housefly larvae, *Musca domestica* L.

Our studies failed to show the presence of cholinesterase (CHE) inhibition by Zectran using the Ellman method of detection (Ellman *et al.*, 1966). However, the typical visual symptoms of CHE inhibition were observed in addition to regurgitation, defecation, and large loss of hemolymph.

Of some importance was the fact that we were not able to synergize Zectran (Lyon, 1967) in the spruce budworm with most of the common synergists having the methylenedioxy ring system (Wilkinson *et al.*, 1966). Numerous other compounds suspected of having synergistic activity were also tested, including compounds known to inhibit oxidative enzymes. The lack of response to synergists and the inability to show CHE inhibition suggested that Zectran has a different mode of action in spruce budworm.

The rate of penetration and the metabolic fate of Zectran in spruce budworm were compared with those in tobacco budworm and housefly larvae. This information on the comparative differences in toxicity of Zectran to the three insects might aid in understanding its mode of action in spruce budworm larvae.

Information is sparse on the in vivo fate of carbamate insecticides and no information is available on the in vivo fate of Zectran in immature insects. The metabolism of Zectran in adult houseflies (Tsukamoto and Casida, 1967) and rat liver microsomal preparations (Oonnithan and Casida, 1966) has been reported. Zayed *et al.* (1966) established the fate of carbaryl in the adult cotton leafworm and Andrawes and Dorough (1967) in the bollworm. The metabolic fate of other carbamate insecticides on various adult insects was reported by Dorough *et al.* (1963), Dorough and Casida (1964), Metcalf *et al.* (1967), Shrivastava (1967), and Tsukamoto and Casida (1967).

METHODS AND MATERIALS

Carbonyl-C^{14} and dimethyl-C^{14}-amino Zectran, both 99% pure, were used throughout our studies. Carbonyl-C^{14} Zectran (New England Nuclear, Chicago, Ill.) had a specific activity of 6.3 mc. per mmole and was adjusted at 55,000 d.p.m. per μl. at a concentration of 0.07 μg. of Zectran per μl. The dimethyl-C^{14}-amino-labeled Zectran had a specific activity of 0.6 mc. per mmole at 25,000 d.p.m. per μl. or 4.4 μg. of Zectran per μl. This material was synthesized by Melvin Look, U. S. Forest Service, Berkeley, Calif.

Liquid samples of radioactive materials were counted in a Packard Tri-Carb liquid scintillation spectrometer, Model 3003. The scintillation fluid consisted of the following scintillation grade compounds: 1000 ml. of dioxane, 12 grams of PPO (2,5-diphenyloxazole), 0.6 gram of dimethyl POPOP {1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene}, 60 grams of naphthalene, and 200 ml. of ethylene glycol monomethyl ether (Bruno and Christian, 1961).

The spruce budworm was reared and maintained in our laboratory on a diet modified from that described by McMorran (1965). The tobacco budworm was reared on a

Pacific Southwest Forest and Range Experiment Station, Forest Service, U. S. Department of Agriculture, Berkeley, Calif. 94701

pinto bean–brewer's yeast–agar diet formulated by W. E. Allison and L. V. Hyder, Dow Chemical Co., Walnut Creek, Calif. Housefly larvae were reared on CSMA fly larval media supplied by Ralston-Purina Co., St. Louis, Mo. Three larvae were normally used per test and a minimum of four replicates for each time period, except for the 6-hour study of tobacco budworm, where only two replicates were performed because of lack of larvae. Where variability was large more replicates were added.

Radioactive Zectran was applied topically in 1-μl. quantities with a calibrated syringe and microburet, Model SB2 (Micro-Metric Instruments Co., Cleveland, Ohio). During and after insecticide application, the larvae were held in scintillation vials. At selected time intervals, radioactive Zectran remaining outside was removed by washing the larvae twice with about 2 ml. of acetone. The washings were evaporated by a stream of cool air to about 1-ml. volume. Aliquots of this were used for scintillation counting and for chromatography.

The washed larvae were then homogenized in 20 ml. of hexane in a Potter-Elvehjem homogenizer and extracted twice with equal volumes of acetonitrile. An aliquot was removed from both the acetonitrile and hexane fractions to determine the quantity of radioactivity. The remaining acetonitrile fraction was heated slightly and evaporated by a stream of cool air to about 0.1 ml. The remaining hexane fraction was discarded because less than 1% of radioactivity was present. The reduced volumes from both external rinse and the homogenization were spotted on 2 × 8 inch glass plates coated with a 250-micron thick layer of silica gel G (Kensington Scientific, Berkeley, Calif.).

Resolution of Zectran and its metabolites was carried out using two solvent systems: acetonitrile-hexane (4 to 1) and ether-hexane-ethanol 77:20:3, by volume (Abdel-Wahab and Casida, 1967). The latter system proved best for the degradation products of Zectran found in this study. Known nonradioactive compounds were cochromatographed with Zectran and its metabolites to help identify unknown products. Two chromogenic systems based on the ninhydrin and ferric chloride–ferricyanide reactions (Krishna *et al.*, 1962) were used to locate the nonradioactive compounds. The latter detecting system was the most sensitive but was less specific.

Radioactive compounds on the chromatoplates were detected by a Tracerlab strip scanner and by radioautography using No-Screen Duplitized x-ray film. After radioautography, the radioactive areas on the chromatoplates were scraped into scintillation vials and counted to ascertain the amount of radioactivity in each metabolite. The use of both carbonyl-C^{14} and dimethyl-C^{14}-amino-labeled Zectran aided in identification of the metabolites. $C^{14}O_2$ was trapped in 1 ml. of monoethanolamine in scintillation vials, according to a slight modification of the procedure described by Saba and Di Luzio (1966).

RESULTS AND DISCUSSION

Nine metabolites of Zectran were detected in the larvae of the two budworm species and 10 metabolites in the housefly larvae. The major metabolites were similar to those found as a result of photodecomposition of Zectran on bean plants (Abdel-Wahab *et al.*, 1966), and when incubated with rat liver (Oonnithan and Casida, 1966) and adult housefly microsomal preparations (Tsukamoto and Casida, 1967). In the order of decreasing concentration the major metabolites were: 4-methylamino-3,5-xylyl methylcarbamate (MA), 4-amino-3,5-xylyl methylcarbamate (A), 4-methylformamido-3,5-xylyl methylcarbamate (MF), and 4-formamido-3,5-xylyl methylcarbamate (F). The pathway for the formation of these metabolites appears to be the same in spruce budworm, tobacco budworm, and housefly larvae as proposed by Abdel-Wahab and Casida (1967).

The four metabolites were identified by cochromatography and the use of two C^{14}-labeled Zectran molecules. The use of the dimethyl-C^{14}-amino Zectran confirmed the results obtained by cochromatography, since the metabolites having one or both of the methyl-C^{14} groups showed smaller amounts or no radioactivity.

The first metabolite detected was MA, followed by A, with MF and F appearing almost concurrently. The MF may have appeared before the F metabolite (Figure 1). Compounds I through V (Figure 1) were not identified. Compound III may be the *N*-hydroxymethyl product of Zectran (4-dimethylamino-3,5-xylyl-*N*-hydroxymethylcarbamate), for it cochromatographed with and had the same R_f value (0.48) as the standard for that compound in the ether-hexane-ethanol solvent. The hydrolysis product of dimethyl-C^{14}-amino Zectran, 4-dimethylamino-3,5-xylenol, was not detected by radioautography. Furthermore, if hydrolysis of carbonyl-C^{14} Zectran occurred, $C^{14}O_2$ would be one of the products after the carbamic acid portion was further degraded to $C^{14}O_2$ and methylamine. Little $C^{14}O_2$ was recovered, except at the

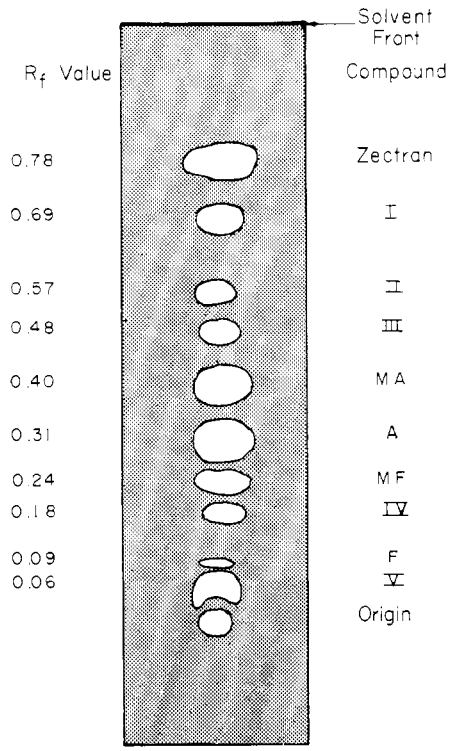

Figure 1. Radioautographic pattern of carbonyl-labeled Zectran and radioactive metabolites extracted from spruce budworm, tobacco budworm, and housefly homogenates

Ether-hexane-ethanol solvent system

sixteenth hour (Table I). In most cases recovery of applied radioactivity approached 100%, particularly during the early periods of poisoning (Table I).

The following compounds were cochromatographed and eliminated as possible metabolites: 4-methylamino-3,5-xylenol, 4-dimethylamino-3,5-xylyl dimethylcarbamate, and 4-methylamino-3,5-xylyl dimethylcarbamate. Compounds IV and V, and more specifically V, may be a ring-hydroxylated or a hydroxymethyl product of Zectran. These compounds would probably be more polar, and therefore move less in the ether-hexane-ethanol solvent. Ring hydroxylation and oxidation of methylcarbamate groups to hydroxymethyl carbamates occur in other insects with carbaryl, Baygon, Banol, Matacil, and UC 10854 (Dorough and Casida, 1964; Metcalf *et al.*, 1967; Oonnithan and Casida, 1966; Shrivastava, 1967; Tsukamoto and Casida, 1967).

Compounds remaining at the origin of the chromatoplate after solvent separation contained up to 15% of the total radioactivity, depending on the time of homogenization after Zectran application. Nothing is known of their identity except that they are considerably more polar than the identified metabolites. They may be amino acid or glucosidic conjugates.

Within 2 to 3 minutes after application on spruce budworm, 50% of the Zectran was oxidized; 26% appeared as metabolite MA, 2.5% as metabolite A, the remaining 21% as unidentified products (Figure 2). The housefly larvae did not metabolize Zectran as rapidly as the two budworm species—after 2 to 3 minutes only 11.2% of the

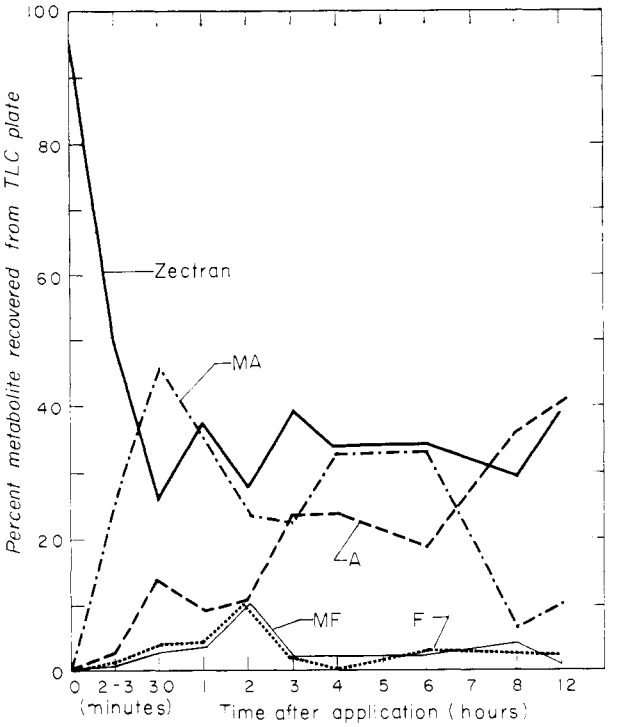

Figure 2. Degradation of carbonyl-labeled Zectran and rate of formation of four identified metabolites by spruce budworm larvae

Table I. Recovery of Radioactivity of Carbonyl-Labeled Zectran at Various Time Intervals in Fractions of Larval Homogenates

Time, Hours	No. of Samples	Av.[a] Activity in Different Fractions				Av. Total Recovery
		Wash	Acetonitrile	Hexane	$C^{14}O_2$	
			SPRUCE BUDWORM			
2–3 min.	8	91.4	17.0	0.32	0	104.3
0.5	9	76.5	20.8	0.64	0	96.7
1	19	63.0	26.0	2.7	0	88.1
2	17	56.5	29.7	1.5	0.5	85.5
3	17	61.0	37.4	1.1	[b]	92.8
4	23	56.7	40.3	2.5	[b]	89.1
5	4	64.0	23.4	0.37	[b]	90.6
6	15	53.9	39.9	8.6	[b]	81.1
8	5	55.1	42.3	0.76	[b]	96.1
12	6	43.0	51.9	1.2	[b]	96.2
16	12	47.2	42.4	1.5	5.2	85.0
			TOBACCO BUDWORM			
2–3 min.	4	86.6	7.9	0.56	0	95.0
1	6	78.7	14.6	0.65	0.25	94.0
3	6	55.9	27.2	2.8	4.7	89.8
6	2	52.9	27.8	1.65	1.2	83.5
			HOUSEFLY			
2–3 min.	5	84.5	10.6	0.28	[b]	93.8
1	5	62.0	21.2	0.91	[b]	84.4
3	8	55.7	27.4	0.89	0.1	82.8
6	7	53.5	23.2	0.97	0.21	77.8
8	5	54.1	9.0	1.90	0.6	65.4
16	8	55.6	7.4	0.66	1.2	64.3

[a] Average of number of samples for respective time period.
[b] Not counted.

radioactivity occurred as the MA metabolite and 2.1% as the A metabolite (Figure 3). There was less unmetabolized Zectran on spruce budworm after 2 to 3 minutes than on tobacco budworm or housefly larvae. Furthermore, the smallest (27.3%) amount of Zectran and the highest (45.7%) amount of MA metabolite were found in spruce budworm homogenates. This pattern was not found in the housefly larvae or tobacco budworm. Instead of showing a variable pattern of formation of metabolites like the spruce budworm, the tobacco budworm and housefly larvae showed a "normal" metabolism of Zectran. Over the total time period the other two insects showed a decrease in the concentration of Zectran; in the spruce budworm the concentration of Zectran remained almost constant after the initial few minutes. The amount of Zectran that had penetrated the spruce budworm was about 15.5% of the total amount applied or about 0.01 μg.

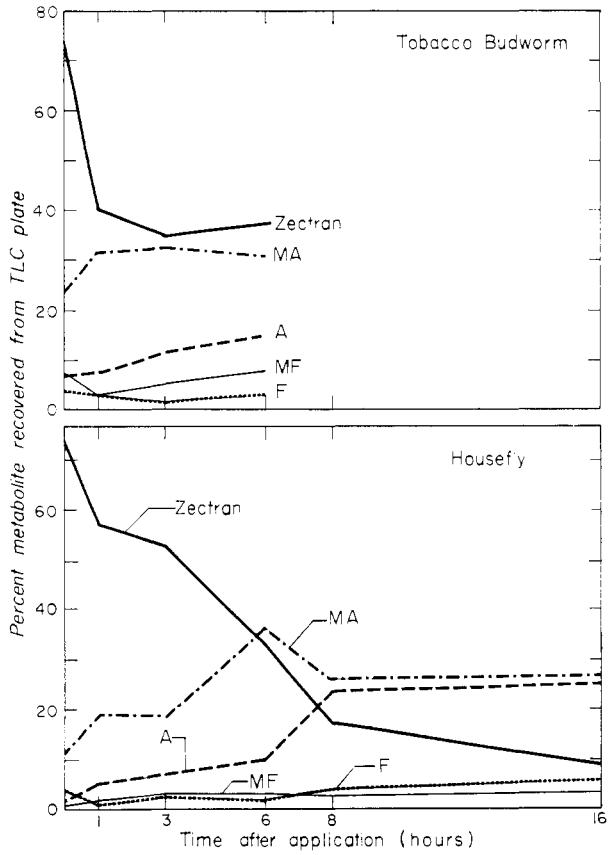

Figure 3. Degradation of carbonyl-labeled Zectran and rate of formation of four identified metabolites by housefly and tobacco budworm larvae

The rate of penetration of radioactive Zectran and metabolites through the integument of the three insects was almost parallel until the third hour, when it began to decrease slightly in the tobacco budworm and housefly larvae (Figure 4). But, after the third hour, changes in penetration were only slight for the tobacco budworm, while the decrease continued for the housefly. Small changes were observed in the spruce budworm from the third to the sixth hour, when the penetration of radioactive materials began to increase again until the twelfth hour. Then penetration began to decrease, the point at which many of the larvae were approaching death.

Zectran is subject to photodecomposition (Abdel-Wahab and Casida, 1967; Crosby *et al.*, 1965). In addition, 75% of the Zectran on TLC plates disappeared (sublimed) after 24 hours. Moore (1965) also found that Zectran will disappear from exposed surfaces. This characteristic of Zectran perhaps explains why we had difficulty recovering all radioactivity applied to TLC plates. Since Zectran is known to photooxidize on exposed surfaces (Abdel-Wahab and Casida, 1967), we examined the acetone washings of

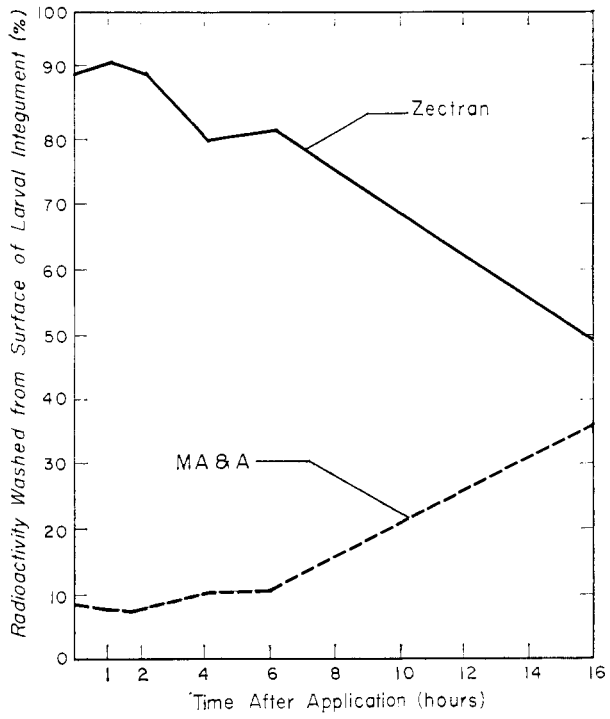

Figure 5. Degradation of carbonyl-labeled Zectran and rate of formation of metabolites MA and A as formed on surface of spruce budworm cuticle

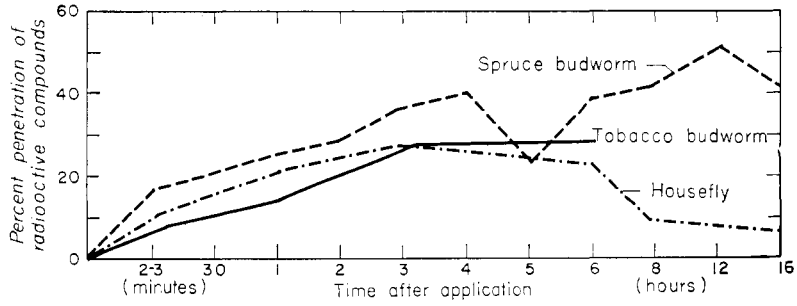

Figure 4. Rate of penetration of topically applied carbonyl-labeled Zectran in spruce budworm, tobacco budworm, and housefly larvae

the larval cuticles for oxidized products of Zectran. At almost any time period at least two metabolites were present in the acetone wash from the spruce budworm cuticle (Figure 5). A similar pattern was observed in the washings from the cuticle of housefly larvae. These oxidized products probably penetrated the integument and were extracted as metabolites from the homogenates. The rate of formation of Zectran metabolites on the cuticle is similar to that found in the homogenate (Figure 2). Possibly these larvae do not metabolize Zectran. The degradation process (photooxidation) on the surface of the integument may be the major mechanism for producing the metabolites; therefore, the use of the word metabolite, in the strict sense, may be questionable.

ACKNOWLEDGMENT

We thank R. L. Lyon, U. S. Forest Service, Berkeley, Calif., for supplying spruce budworms and for other technical assistance; J. E. Casida, University of California, Berkeley, for supplying housefly larvae and chemicals; and William Allison, Dow Chemical Co., Walnut Creek, Calif., for supplying tobacco budworms. We also gratefully acknowledge the technical assistance provided by L. R. White and J. A. Arvold.

LITERATURE CITED

Abdel-Wahab, A. M., Casida, J. E., J. AGR. FOOD CHEM. **15,** 479 (1967).
Abdel-Wahab, A. M., Kuhr, R. J., Casida, J. E., J. AGR. FOOD CHEM. **14,** 290 (1966).
Andrawes, N. R., Dorough, H. W., *J. Econ. Entomol.* **60,** 452 (1967).
Bruno, G. A., Christian, J. E., *Anal. Chem.* **33,** 1216 (1961).
Crosby, D. G., Leitis, E., Winterlin, W. L., J. AGR. FOOD CHEM. **13,** 204 (1965).
Dorough, H. W., Casida, J. E., J. AGR. FOOD CHEM. **12,** 294 (1964).
Dorough, H. W., Leeling, N. C., Casida, J. E., *Science* **140,** 170 (1963).
Ellman, G. L., Courtney, K. D., Andres, V., Jr., Featherstone, R. N., *Biochem. Pharmacol.* **7,** 88 (1966).
Freeman, T. N., Stehr, G. W., Harvey, G. T., Campbell, I. M., *Can. Entomol.* **99,** 499 (1967).
Krishna, J. G., Dorough, H. W., Casida, J. E., J. AGR. FOOD CHEM. **10,** 462 (1962).
Lyon, R. L., U. S. Department of Agriculture, Forest Service, Berkeley, Calif., personal communication, 1967.
McMorran, Arlene, *Can. Entomol.* **97,** 58 (1965).
Metcalf, R. L., Osman, M. R., Fukuto, T. R., *J. Econ. Entomol.* **60,** 445 (1967).
Miskus, R. P., Look, M., Andrews, T. L., Lyon, R. L., J. AGR. FOOD CHEM. **16,** 605 (1968).
Moore, A. D., Proceedings of 1965 Annual Western Forest Pest Committee, Dec. 8, 1965, Vancouver, B.C., 1965.
Oonnithan, E. S., Casida, J. E., *Bull. Exptl. Contam. Toxicol.* **1,** 59 (1966).
Saba, T. M., Di Luzio, N. R., *J. Lipid Res.* **7,** 506 (1966).
Shrivastava, S. P., Ph.D. dissertation, University of California, Berkeley, Calif., 1967.
Tsukamoto, M., Casida, J. E., *Nature* **213,** 49 (1967).
Wilkinson, C., Metcalf, R. L., Fukuto, R. T., J. AGR. FOOD CHEM. **14,** 73 (1966).
Zayed, S. M. A. D., Hassan, A., Hussein, T. N., *Biochem. Pharmacol.* **15,** 2057 (1966).

Received for review June 3, 1968. Accepted September 16, 1968. Mention of trade names and commercial enterprises or products is solely for necessary information. No endorsement by the U. S. Department of Agriculture is implied.