Factors Influencing Organophosphorus Insecticide Resistance in Tobacco Budworms

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The metabolism of GC-6506-sulfone [dimethyl *p*-(methyl-^{14}C-sulfonyl)phenyl phosphate] and ^{14}C-labeled aldrin was studied in tobacco budworms, *Heliothis virescens* (F.), that were resistant and susceptible to organophosphorus insecticides. The results indicated that certain important enzyme systems that could be involved in the detoxification of organophosphorus insecticides, including mixed-function microsomal oxidases, soluble phosphotriesterases, glutathione-dependent alkyl transferases, and glucosidic conjugation mechanisms, were substantially enhanced in resistant insects.

The extensive use of methyl parathion to control cotton pests in certain regions of Texas, Mexico, and Central America has apparently led to the development of resistance to that and other organophosphorus (OP) insecticides in field populations of the tobacco budworm, *Heliothis virescens* (F.) (Adkisson, 1969; Nemec and Adkisson, 1969). This important pest was already highly resistant to organochlorine and carbamate insecticides (Adkisson, 1968) and now the appearance of OP-resistance has forced the curtailment of cotton production in certain areas and threatens others because economic control no longer can be achieved with the present chemicals (Adkisson, 1969).

Preliminary studies with susceptible (S) and OP-resistant (R) strains of tobacco budworms indicated that resistance was related to an increased capacity for detoxification in R insects (Whitten and Bull, 1970). We therefore compared certain mechanisms implicated in the metabolism of insecticides in R and S tobacco budworms and assessed their relative importance in the development of OP-resistance.

EXPERIMENTAL

Chemicals. Most of the studies of metabolism were made with radio-labeled (5 mCi per mmol) GC-6506-sulfone [I; dimethyl *p*-(methyl-^{14}C-sulfonyl)phenyl phosphate] prepared by the chemical oxidation (Wendel and Bull, 1970) of dimethyl *p*-(methyl-^{14}C-thio)phenyl phosphate (Allied GC-6506, supplied by Allied Chemical Co., New York, N.Y.). Radiolabeled preparations of the principal products of detoxification, II [methyl *p*-(methylsulfonyl)phenyl phosphate], III [*p*-(methylsulfonyl)phenol], and IV [*β*-(*p*-(methylsulfonyl)-phenyl)-D-glucoside] were also synthesized (Wendel and Bull, 1970; Bull and Stokes, 1970) from the ^{14}C-labeled GC-6506 for use as analytical standards. The ^{14}C-labeled aldrin (52 mCi per mmol) and all other chemicals were obtained in the best grade available from different commercial sources.

The sulfone derivative of GC-6506 was used because we wanted to simplify the array of metabolites produced and evaluate only those reactions that resulted in detoxification. Aldrin was used because it is a convenient model for determining the activity of the mixed-function microsomal oxidases.

Insects. Larval stages of the tobacco budworm were selected at random from laboratory colonies that were reared on synthetic diets (Vanderzant, 1967) in continuous light at 27°C. Four strains were used, one that was generally susceptible to all insecticides (S), and three different OP-resistant strains from areas of northern Mexico (R_1) and south Texas (R_2, R_3), where methyl parathion has been used extensively in cotton production for several years. (The use of more than one R strain was necessary because the insects tended to lose their resistance after several generations of in-breeding.)

Tests *in vivo*. The relative susceptibility of each strain was assessed by determining the toxicity of topical doses of methyl parathion to third-instar larvae (weight range 25–30 mg) with the procedure described by Whitten and Bull (1970).

Toxicants used for the studies of metabolism were administered orally or injected into the body cavity of individual fifth-instar tobacco budworms with a calibrated, micrometer-driven syringe; the doses caused at most only minimum symptoms of poisoning.

Larvae treated with aqueous solutions of I were held individually without food in ventilated glass containers at 27°C. Then at specified times, the treated insects (two per sample) were homogenized in cold water and acetone (1 to 4, v/v). The excreta, recovered by scrubbing the containers with water and then rinsing them with acetone, was combined with the homogenates, and the solids in the mixture were removed by centrifugation. After removal of the acetone under vacuum, the aqueous portion remaining was partitioned with chloroform (1 to 2.5 v/v), and the two fractions were analyzed separately. Each fraction was radioassayed and then reduced under vacuum to a volume convenient for chromatography. Chloroform extracts were analyzed by thin-layer chromatography (tlc) with solvent mixtures of 4 to 1 ethyl ether and acetonitrile or 6:3:2 chloroform, hexane, and acetic acid; aqueous fractions were analyzed by paper chromatography (pc) with a solvent mixture of 12:8:6 butanol, pyridine, and water. Details of procedures, R_f values, and quantitation were reported earlier (Wendel and Bull, 1970; Bull and Stokes, 1970).

Extracts of larvae (one per sample) treated with solutions of ^{14}C-labeled or nonradioactive aldrin in polyethylene glycol and ethanol (3 to 1, v/v) were processed the same as those treated with I, except that after centrifugation the water and acetone extracts were partitioned three times with hexane (1 to 1, v/v). The organic fractions were pooled, radioassayed, and then analyzed either by tlc, on glass plates coated (0.25 mm thick) with silica gel, with a solvent mixture (1 to 1, v/v) of carbon tetrachloride and hexane, or with gas chromatography (gc) as described.

Tests *in vitro*. Intact larvae or specific tissues were homogenized briefly in 0.1 *M* phosphate buffer (pH 7.4) at 4°C with a tissue grinder or a glass tube-Teflon pestle homogenizer and then centrifuged at 10,000 × *g* for 15 min at 0°C. The fractions were separated, and the particulate portion was resuspended in buffer to make the original volume. For preparations of the microsomal and soluble subcellular

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Table I. Metabolism of GC-6506-Sulfone (I) *in vivo*[a]

Strain and method of treatment	% of dose as indicated compound				
	I	II	III	IV	Unk A
	Treated with I (1.5 μg)				
S-Injection	54.7	20.2	13.4	10.1	1.6
S-Oral	61.2	7.3	24.9	6.0	0.6
R_1-Injection	14.7	32.9	13.8	33.8	4.8
R_1-Oral	24.0	21.6	18.2	32.1	4.1
	Treated with I (1.5 μg) + TBTP (50 μg)				
S-Injection	67.0	13.9	8.8	10.1	0.2
S-Oral	74.4	10.0	11.1	4.4	0.1
R_1-Injection	40.0	22.5	10.2	26.4	0.9
R_1-Oral	54.8	19.8	15.1	9.1	1.2
	Treated with I (1.5 μg) + sesamex (50 μg)				
S-Injection	70.1	7.7	7.6	13.7	0.9
S-Oral	65.4	5.4	19.9	8.9	0.4
R_1-Injection	34.8	18.1	13.5	30.2	3.4
R_1-Oral	39.7	13.6	20.1	23.0	3.6

[a] Insects analyzed 1 hr after injection and 2 hr after oral treatment. The data are averages of ten or more experiments.

constituents, a 10,000 × *g* preparation was made first and then centrifuged again at 105,000 × *g* for 60 min at 0°C. The supernatant (soluble fraction) was decanted, and the particulate portion (microsomes) was resuspended in buffer. All preparations were maintained at 4°C until tested. In addition, a small amount of 1-phenyl-2-thiourea (PTU) was added to each tissue extract to suppress melanization and thus minimize possible interference with certain oxidation processes (Williamson and Schechter, 1970). Also bovine serum albumin (BSA) was included in microsomal preparations to protect them from possible endogenous inhibitors (Ray, 1967; Tsukamoto and Casida, 1967; Krieger and Wilkinson, 1970).

The *in vitro* studies of the metabolism of I were done with a mixture that included a 10,000 × *g* supernatant preparation (containing five larval equivalents) made either with homogenates of whole fifth-instar larvae or certain of their tissues, 7.5 μg of I, 0.1 ml of a saturated solution of PTU, and the specified concentrations of cofactors or inhibitors in a final volume of 5 ml of 0.1 *M* phosphate buffer (pH 7.4). (Preliminary tests established that the pellet formed by centrifuging homogenates at 10,000 × *g* caused very little degradation of I, either with or without cofactors. Thus, only the 10,000 × *g* supernatant or its subfractions were used for subsequent testing.) Samples were incubated in open flasks for 2 hr at 30°C; then the reaction was stopped by the addition of acetone, and the mixture was analyzed as described for the *in vivo* tests.

In vitro studies of the metabolism of aldrin were done with incubation mixtures that included two larval equivalents of microsomal preparations, 3 μ*M* NADPH, 20 μg of aldrin, 0.1 ml of a saturated solution of PTU, and 0.2% BSA in a final volume of 3 ml of 0.1 *M* phosphate buffer (pH 7.4). After incubation in open flasks for 1 hr at 30°C, the reaction was stopped with acetone, and the samples were analyzed a described for the *in vivo* tests.

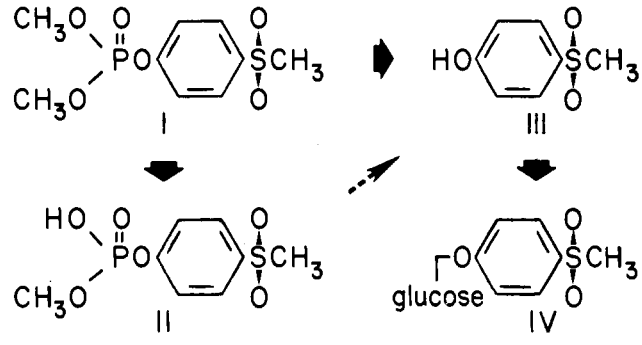
Analytical. Radioassays of extracts or radioactive areas from chromatograms were made by liquid scintillation at ambient temperature; when necessary, data were corrected for quenching by internal standardization.

The gc analyses were done with an instrument equipped with a tritium foil electron-capture detector. A U-shaped glass column (183-cm × 4-mm i.d.), packed with a mixture of 6% QF-1 fluorosilicone and 4% SE-30 silicone gum rubber on 90 to 100-mesh Anakrom ABS (Analabs Inc., Hamden, Conn.), was used at temperatures of 190°C for the oven, 200°C for the injection port, and 205°C for the detector. A mixture of 95% argon and 5% methane was used as the carrier gas at a flow rate of 85 ml per/min.

RESULTS

The 72-hr LD_{50} values for topical applications of methyl parathion on third-instar larvae of strains S, R_1, R_2, and R_3 were 5, 340, 54, and 85 μg per g of live weight, respectively.

Metabolism of I. The results of comparative studies of the metabolism of I *in vivo* by R_1 and S fifth-instar tobacco budworms reaffirmed previous evidence (Bull and Stokes, 1970) that the initial detoxification was accomplished by disruption of the arylphosphate and a methylphosphate linkage of the molecule and that the substituted phenol (III) formed during metabolism was rapidly conjugated as the glucoside (IV).



The extent of the detoxification by both pathways was substantially greater in R_1 insects (Table I) regardless of the method of treatment. In either strain, injected doses were metabolized faster than were those administered orally. Proportionally more *O*-demethylation occurred when the dose was placed in the body cavity. There, immediate contact with the fat bodies would be expected, and the evidence suggests that the enzymes mediating that reaction were more abundant in those tissues than in the digestive tract. By both methods of treatment, the concentration ratio of IV to III was greater in R_1 than in S larvae. This indirect evidence implies that the enzyme systems associated with the conjugation process also were more active in the R_1 insects. An enhanced capability for conjugation could be an important factor contributing to the resistance of tobacco budworms to certain insecticides. For example, compounds such as trichlorfon (Bull and Ridgway, 1969) or the toxic hydroxylated metabolites of dicrotophos (Bull and Lindquist, 1964) and other compounds are detoxified to some extent by these insects through direct incorporation into glucosidic conjugates.

The coadministration of *S,S,S*-tributyl phosphorotrithioate (TBTP) with I caused a reduction of the concentrations of products that were formed by degradation of the arylphosphate linkage (Table I) in both S and R_1 insects, and the inhibition was relatively greater in larvae that were treated orally. TBTP also inhibited the *O*-demethylation of I in larvae that were treated by injection. The inclusion of sesamex in treatments with I caused a definite inhibition of *O*-demethylation, particularly in larvae treated by injection, but had no significant effect on the mechanisms associated with arylphosphate cleavage.

Preliminary tests with the *in vitro* preparations established that the degradation of I by the 10,000 × *g* supernatant from different homogenates was stimulated by the addition of re-

duced glutathione (GSH) but was not affected by other cofactors, including NADPH, NADP, and NADH. Further, microsomal preparations made from the 10,000 × *g* supernatants caused only an insignificant degradation of I, whether fortified or not with NADPH or GSH; more than 95% of the total degradation was caused by soluble enzymes associated with the 105,000 × *g* supernatant. Thus, for convenience, subsequent tests were made with the 10,000 × *g* supernatant.

Comparative studies made with 10,000 × *g* preparations of whole fifth-instar larvae or certain of their tissues also demonstrated a greater level of detoxifying activity in the R insects (Table II). With either R or S insects, the overall rate of degradation was considerably slower than that observed in the *in vivo* tests; the *O*-demethylation and conjugation processes were especially diminished *in vitro*. However, this was not unexpected because of the problems, such as dilution and disruption of coordinated enzyme systems, that are inherent in the technique. The data are useful because they provided good evidence that *O*-demethylation was achieved, at least in part, by a GSH-dependent alkyl transferase. That this enzyme was indeed more abundant in fat body than gut tissue was indicated by the relatively greater increase in activity caused by the addition of GSH. In addition, these results provided evidence that there was a significant level of activity in an enzyme system (phosphotriesterase) that catalyzed the hydrolysis of the arylphosphate linkage; this enzyme system was not affected by the addition of GSH. The addition of TBTP to reaction mixtures inhibited the degradation of I by 27.3%, and virtually all of the effect was caused by interference with the cleavage of the arylphosphate linkage. The addition of sesamex caused little inhibition (4.7%).

Metabolism of Aldrin. Comparative studies of the *in vivo* and *in vitro* metabolism of aldrin by S and R_3 fifth-instar tobacco budworms definitely established that the R larvae had higher levels of mixed-function oxidase activity (Table III). The *in vivo* tests demonstrated an approximate two-fold increase in the epoxidation of aldrin to dieldrin at the doses and experimental periods that were used. The coadministration of TBTP or sesamex with oral doses of aldrin caused a marked and approximately equivalent reduction in the production of dieldrin in both strains.

Microsomal preparations were found to be typically inactive in the absence of NADPH. The *in vitro* tests with fortified preparations revealed substantial differences in microsomal oxidase activity between R_3 and S strains which ranged from an approximate four-fold increase in fat body to a 25-fold increase in gut tissue (which had the highest specific activity). In all tests, less than 2% of the dose was found in the aqueous fractions, and only aldrin (R_f 0.57) and dieldrin (R_f 0.10) were detected in organic extracts.

Table II. Metabolism of GC-6506-Sulfone (I) by Larval Homogenates[a]

Strain	Cofactor (0.003 *M*)	Inhibitor (0.001 *M*)	Total degradation, %	Degradation product, % II	III	IV
			Whole larva			
R_2			15.0	1.3	13.1	0.6
R_2	GSH		20.1	4.6	14.9	0.6
R_2		TBTP	10.9	1.0	9.4	0.5
R_2		sesamex	14.3	1.3	12.5	0.5
S			8.9	0.8	7.8	0.3
S	GSH		10.7	2.5	7.9	0.3
			Fat body			
R_2			15.2	1.4	12.8	1.0
R_2	GSH		27.8	14.8	11.9	1.1
S			9.3	1.3	7.2	0.8
S	GSH		12.7	5.1	6.9	0.7
			Gut			
R_2			16.9	0.8	16.1	0.0
R_2	GSH		19.0	2.4	16.6	0.0
S			11.7	1.1	10.6	0.0
S	GSH		13.2	2.9	10.3	0.0

[a] Data are averages of four or more replicates.

Table III. Metabolism of Aldrin *in vivo* and *in vitro* by Microsomal Preparations[a]

Strain	Tissue preparation	% of dose epoxidized: *in vivo* oral injection	*in vitro* (microsomes)	Specific activity (10^{-9} mol dieldrin/hr/mg protein)
R_3	Whole larva		74.6	5.3
S	Whole larva		2.5	0.3
R_3	Gut		40.9	20.2
S	Gut		1.4	0.8
R_3	Fat body		51.1	8.3
S	Fat body		9.1	2.1
		Treated with aldrin alone (5 μg oral, 1.5 μg Injection)		
R_3		76.0	65.0	
S		42.0	28.0	
		Treated with aldrin (5 μg) + TBTP (50 μg)		
R_3		16.0		
S		10.0		
		Treated with aldrin (5 μg) + sesamex (50 μg)		
R_3		14.0		
S		11.0		

[a] Data are averages of ten or more *in vivo* tests or four or more *in vitro* tests. Larvae treated *in vivo* were analyzed after 2 hr.

DISCUSSION

The results of studies of the metabolism of I by R and S tobacco budworms established that cleavage of the arylphosphate linkage and *O*-demethylation were the key initial detoxification reactions and that both processes were significantly more active in the R insects. However, only tentative conclusions may be drawn concerning the exact nature of these functions. It would appear that degradation of the arylphosphate linkage of I is mediated primarily by a soluble phosphotriesterase. This conclusion is supported by the *in vitro* evidence that the supernatant preparations made with 10,000 × *g* and 105,000 × *g* centrifugal fractions of tissue homogenates had substantial degradative activity while the microsomes had none and by *in vivo* results that demonstrated sesamex, which is a potent microsomal oxidase inhibitor (Casida, 1970), did not inhibit arylphosphate cleavage. Microsomal oxidases have been implicated in the degradation of acid anhydride bonds of OP compounds, and the studies with aldrin clearly established that these enzymes were highly active in the insects tested; however, the absence of this type of activity against I was not unexpected because the compound is a phosphate. Others have reported that microsomal oxidases of mammalian liver and insects degraded phosphorothionates such as parathion and diazinon by attacking the respective arylphosphate or pyrimidinylphosphate linkages of the molecules, but were inactive against the oxygen analogs of those two compounds (Fukunaga, 1967;

Nakatsugawa and Dahm, 1967; Lewis, 1969; Nakatsugawa *et al.*, 1969a,b; Neal, 1967a,b), and also against methyl paraoxon (Hollingworth, 1969). The *in vivo* and *in vitro* degradation of the arylphosphate linkage of I was strongly inhibited by TBTP, which also proved to be an efficient inhibitor of the epoxidation of aldrin. However, TBTP is an inhibitor that has demonstrated cross-synergism (Plapp, 1970); *e.g.*, it enhances the toxicity of insecticides that are degraded primarily by oxidation as well as those that are detoxified by esterases (Plapp and Tong, 1966; Plapp and Valega, 1967). Thus, in the case of I, it would appear that cleavage of the arylphosphate bond was achieved primarily by a phosphotriesterase and that this was the system inhibited by TBTP; however, the present evidence for this conclusion is circumstantial.

The detoxification of I by *O*-demethylation may involve more than one enzyme system. There is little doubt that a GSH-dependent alkyl transferase is involved because the metabolism *in vitro* was stimulated by the addition of GSH and the only product of degradation that increased in concentration was the *O*-demethyl derivative (II). This result agrees with previous reports of the important contribution of GSH-dependent *O*-demethylase systems to the detoxification of dimethyl ester forms of OP insecticides by mammalian liver or insects (Fukami and Shishido, 1966; Fukunaga, 1967; Hutson *et al.*, 1967, 1968; Morello *et al.*, 1968; Hollingworth, 1969, 1970). Other mechanisms cannot be ruled out, however, because *O*-dealkylation of phosphate-class OP insecticides by the NADPH-dependent microsomal oxidases has been demonstrated with preparations of mammalian liver (Donninger *et al.*, 1966; Hutson *et al.*, 1968) and insects (Lewis, 1969). That sesamex inhibited the *in vivo O*-demethylation of I suggests that an oxidative system could be involved, but the apparent inactivity of our microsomal preparations contradicts that conclusion. Lewis (1969) reported that the *O*-deethylation of diazinon and diazoxon by a soluble fraction of a homogenate of the housefly, *Musca domestica* L., was inhibited by TBTP but not by sesamex. In contrast, Dyte and Rowlands (1970) reported that the *in vivo O*-demethylation of fenitrothion and bromophos in *Tribolium castaneum* (Herbst) was markedly inhibited by sesamex and SKF-525A, both of which are oxidative inhibitors. According to previous reports (Hollingworth, 1970; Stenersen, 1969); these two compounds are *O*-demethylated by GSH-dependent alkyl transferases. The issue is further confused by the report of Nolan and O'Brien (1970) on the *O*-deethylation of paraoxon in houseflies, which presented evidence that suggested the mechanism of the reaction could involve hydrolysis by a phosphotriesterase. It is obvious that more information concerning *O*-dealkylation processes is needed.

Thus, the present results and other evidence (Whitten and Bull, 1970; Williamson and Schechter, 1970) clearly indicate that the development of OP resistance in tobacco budworms is coincidental with a substantial increase in their capacity for the biotransformation of a variety of substrates, including OP and other classes of insecticides. An increase in a single mechanism might not account for the dramatic increase in resistance that has been reported in field populations and, indeed, we have shown that soluble phosphotriesterases, GSH-dependent alkyltransferases, microsomal oxidases, and conjugation mechanisms all were enhanced in resistant insects. The relative importance of a given system would depend on the type of insecticidal structure with which the insect was challenged. Since the microsomal oxidases have such a broad substrate specificity, however, a substantial increase in their activity would probably be a major factor in the development of cross-resistance to different types of OP compounds and other classes of insecticides.

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