Biochemical Pharmacology, Vol. 19, pp. 1719–1727. Pergamon Press. 1970. Printed in Great Britain

MICROSOMAL EPOXIDATION OF ALDRIN IN LEPIDOPTEROUS LARVAE

R. L. WILLIAMSON and M. S. SCHECHTER

Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md. 20705, U. S. A.

(*Received* 15 *January* 1969; *accepted* 3 *October* 1969)

Abstract—Epoxidation of aldrin occurred in microsomal preparations from a number of species of lepidopterous larvae. The epoxidation process required NADPH and oxygen and was inhibited by carbon monoxide and 2-(diethylamino)ethyl 2,2-diphenylvalerate hydrochloride (SKF-525-A). The presence of the hemoprotein P-450 in larval microsomes was determined by difference spectra, and the characteristic peak at 450 nm that was produced by the addition of carbon monoxide to the reduced microsomal preparation appears to arise from a substance that is present in the reduced microsomes having an absorbance at 407 nm.

THE CONVERSION of unsaturated cyclodiene insecticides to their corresponding epoxides is a microsomal process requiring the cofactors NADPH and oxygen and occurs in several species of insects.[1–7] Moreover, the intrinsic toxicity of these insecticides seems to result from this metabolic conversion and from the relatively high stability of the epoxides to further metabolism and excretion.[8] The formation *in vitro* of the epoxides is inhibited with the methylenedioxyphenyl derivatives (e.g. sesamex)[5, 6] and 2-(diethylamino)ethyl 2,2-diphenylvalerate hydrochloride (SKF-525-A).[3]

The discovery of cytochrome P-450 and its implication as the terminal oxidase component in mammalian microsomes[9] have now been extended to include microsomes of adult insects.[5] However, little attention has been given to studies of microsomes from immature insects. We therefore report the epoxidation of aldrin and the detection of P-450 in microsomal preparations from several economically important species of lepidopterous larvae.

MATERIALS AND METHODS

Insect sources and rearing procedures. Eggs of several species were obtained from the following sources: *Heliothis zea* (Boddie) corn earworm (M. J. Lukefahr, Brownsville, Tex.; M. M. Bozik, Beltsville, Md.; A. H. Baumhover, Oxford, N. C.; Nutrilite Products, Inc.,* Buena Park, Calif.); *Heliothis virescens* (F.) tobacco budworm (M. J. Lukefahr and D. A. Wolfenbarger, Brownsville, Tex.; A. H. Baumhover, Oxford, N. C.); and *Pectinophora gossypiella* (Saunders) pink bollworm (M. J. Lukefahr, Brownsville, Tex.). Newly hatched larvae from these eggs were reared on a fortified wheat germ diet.[10] Two other insect species were obtained as larvae already on medium as follows: *Ostrinia nubilalis* (Hubner) European corn borer (T. A.

* The use of trade or proprietary names does not necessarily imply the endorsement of these products by the U. S. Department of Agriculture.

Brindley, Ankeny, Iowa), and *Argyrotaenia velutinana* (Walker) red-banded leaf roller (M. L. Cleveland, Vincennes, Ind.). All larvae were reared normally with a long-day photoperiod (LD 16:8) at 24°. Pink bollworm and European corn borer larvae were induced to diapause by rearing them with a short-day photoperiod (LD 10:14) at 24°.[11]

Preparation of microsomes. All preparative steps and differential centrifugation were done at 0 to 4°. Microsomes were prepared from late instar larvae just before pupation. Because of the heavily scleritized head capsules, the larvae were decapitated and ground in a mortar with a 0·1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7·0 to 7·4. The suspension was mechanically homogenized in a Potter–Elvehjem glass homogenizer equipped with a Teflon pestle. Differential centrifugation was done in a Beckman Model L-2 ultracentrifuge as follows: the homogenate (unfiltered) was centrifuged at 12,000 *g* for 10 min, and the supernatant was filtered through glass wool; the filtrate (hereafter referred to as the S + M fraction) was centrifuged at 105,000 *g* for 60 min, and the reddish-brown microsomal pellet was carefully resuspended by hand in the appropriate phosphate buffer with a small Potter–Elvehjem glass homogenizer. Microsomal protein was determined by the biuret method[12] with recrystallized bovine serum albumin Fraction V as the standard.

Incubation and extraction. The usual incubation medium contained 0·2 ml of the microsomal suspension and the following components to a final volume of 3 ml: KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH 7·4; NADPH, 10^{-3} M; bovine serum albumin, Fraction V, 8 mg; aldrin, 5×10^{-5} M. Before the addition of substrate, the mixture was equilibrated for 5 min. Aldrin was added in 100 μl absolute ethanol to initiate the reaction. All incubations were done at 30° in 25-ml Erlenmeyer flasks placed in a Dubnoff metabolic shaking incubator. For the kinetic studies, SKF-525-A was added to the incubation medium in 5 μl of ethanol.

Carbon monoxide inhibition was accomplished with duplicate flasks supplied with carbon monoxide–oxygen mixtures in ratios of 1:1 or 2:1 (the total gas flow rate was 100 ml/min). The incubation medium, previously saturated with the carbon monoxide–oxygen mixture, was equilibrated for 5 min after addition of the microsomal suspension. Aldrin was injected directly into the mixture through rubber septa covering the necks of the flasks, and the mixture incubated for 20 min.

The reaction was stopped by the addition of 2 ml of acetone. The mixture, together with 1 ml acetone wash, was transferred to 15-ml stoppered centrifuge tubes. The mixture was extracted twice with 2 ml of hexane, and the combined extracts were dried over anhydrous sodium sulfate. In experiments utilizing SKF-525-A, the extracts were washed with 4 ml of 0·1 N HCl to fully remove this compound from the hexane solution before drying.

Analyses. The dried hexane extracts were analyzed with a MicroTek model 220 gas chromatograph equipped with a 10 mc Ni^{63} electron capture detector. The following conditions were used: 100 cm × 3·5 mm i.d. glass column, containing 10% silicone rubber, UC W-98 on Diatoport S, 60/80 mesh; prepurified nitrogen carrier gas with a flow rate (column outlet) of 100 ml per min; detector potential, 50 V; temperatures—column, 180° (isothermal); injection port, 200°; detector, 265°; sample volumes, 1 to 5 μl. Formation of dieldrin was measured by integration of peak areas obtained with a 1 mV recorder (Texas Instruments, Inc.) equipped with a Disc integrator.

Spectral measurements. Difference spectra were obtained for the microsomal preparations with a spectrophotometer designed for analysis of dense light-scattering samples.[13] A Corning 5330 filter was placed between the sample and light source. Noise interference was reduced by averaging the spectra obtained from 4 consecutive sweeps of the sample.

Chemicals. The following chemicals were purchased: NADPH (Calbiochem); bovine serum albumin, Fraction V (Sigma Chemical Co.); SKF-525-A, 2-(diethylamino)ethyl 2,2-diphenylvalerate hydrochloride (Smith, Kline & French Laboratories); hexane, redistilled in glass (Burdick & Jackson Laboratories, Inc.); and carbon monoxide, high purity grade (Fisher Scientific Co.). Recrystallized aldrin and dieldrin were supplied by P. A. Giang of our laboratories. All other chemicals were of A.R. grade.

RESULTS

Inhibition of aldrin epoxidation by the darkened S + M fraction. The S + M fractions (12,000 *g* filtered supernatant) of lepidopterous larvae darkened rapidly on exposure to air which interfered with the epoxidation of aldrin. However, the addition of 1-phenyl-2-thiourea or ascorbic acid to the S + M fraction prevented this melanization reaction. Table 1 shows that as the concentration of the S + M fraction

TABLE 1. ALDRIN EPOXIDATION WITH S + M AND MICROSOMAL FRACTIONS*

Enzyme source (ml)	Dieldrin formation (10^{-9} moles)
S + M (no phenylthiourea)	
0·5 = 1 larvae	14·8
1·0 = 2 larvae	11·5
1·5 = 3 larvae	7·2
S + M (plus phenylthiourea)	
0·5 = 1 larva	20·1
1·0 = 2 larvae	24·8
1·5 = 3 larvae	26·4
Microsomal fraction (no phenylthiourea)	
0·2 = 2 larvae	22·3

* Incubations at 30° for 60 min. Reaction mixture: S + M or microsomal fraction (*H. zea* from Lukefahr); KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH 7·4; NADPH, 10^{-3} M; saturated phenylthiourea, if added, 0·1 ml; aldrin, 5×10^{-5} M; final volume, 3 ml. S + M (2 larvae/ml) and microsomes (4·01 mg protein/0·2 ml) prepared in 0·1 M phosphate buffer, pH 7·4, with bovine serum albumin 1% (w/v).

was increased, the degree of darkening also increased with a subsequent decrease in the formation of dieldrin. When the phenythiourea was incorporated into the incubation medium or a microsomal preparation was used, activity increased greatly.

Requirements for epoxidation of aldrin. The requirements *in vitro* for epoxidation of aldrin are shown in Table 2. Only a fraction of the epoxide was produced under anaerobic conditions, indicating a requirement for oxygen. The additional requirement for the cofactor NADPH places these larval epoxidases in the same category as the drug-metabolizing oxidative enzymes.[14]

The addition of EDTA or nicotinamide had no significant effect on activity, but

TABLE 2. MICROSOMAL REQUIREMENTS FOR ALDRIN EPOXIDATION*

Variables	Dieldrin formation (10^{-9} moles/mg protein)
None (standard mixture)	8·9
$-O_2$	0·5
−NADPH	0
+ L-Ascorbic acid, 2×10^{-3} M	8·9
+ EDTA, 10^{-3} M	8·9
+ Nicotinamide, 5×10^{-3} M	8·8
+ KCN, 5×10^{-3} M	7·5
− BSA	4·0

* Incubations at 30° for 60 min. Reaction mixture, except as indicated: microsomal suspension, 0·2 ml (*H. zea* from Baumhover); KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH 7·4; NADPH, 10^{-3} M; bovine serum albumin (BSA), 8 mg; aldrin, 5×10^{-5} M; final volume, 3 ml.

the inclusion of potassium cyanide reduced the formation of dieldrin about 15 per cent. The incorporation of bovine serum albumin (BSA) was not an abolute requirement for activity.[5] However, formation of dieldrin with larval microsomes was reduced about 50 per cent by its absence. Enzyme activity was not improved by increasing the amount of BSA in the incubation medium above 8 mg. In addition, microsomes prepared with phosphate buffer plus BSA and incubated in a mixture without additional BSA showed the same activity as microsomes prepared only with phosphate buffer but incubated in a mixture containing BSA.

Rate of aldrin epoxidation by larval microsomes. The rate of aldrin epoxidation by microsomes from larvae of *H. virescens* is shown in Fig. 1. The rate was nearly linear

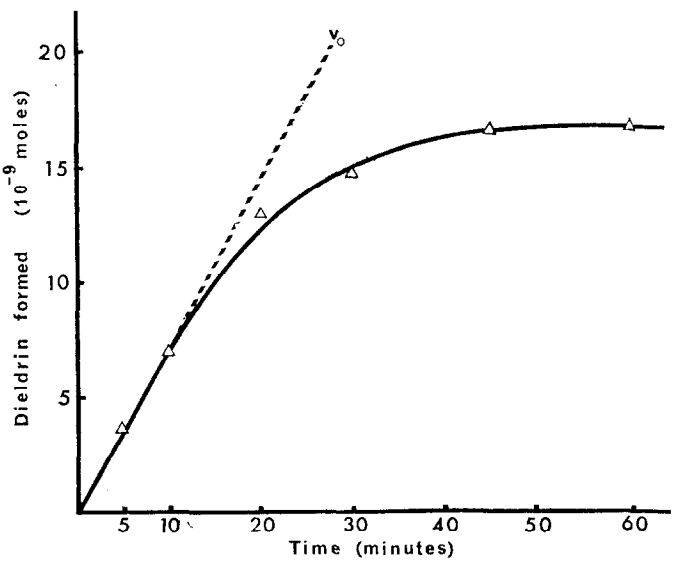

FIG. 1. Rate of aldrin epoxidation. Incubations at 30°. Reaction mixture: microsomal suspension, 0·2 ml (*H. virescens* from Baumhover, 3·85 mg protein); KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH 7·4; NADPH, 10^{-3} M; bovine serum albumin, 8 mg; aldrin, 5×10^{-5} M; final volume, 3 ml. V_0 = initial velocity.

for 20 min and reached a maximum after 40 min. After similar curves were obtained for each insect species, the initial rate of dieldrin formation could be determined from the tangent to the curve at zero time. Table 3 compares the rates, expressed as specific activity (10^{-9} moles of dieldrin/min/mg of microsomal protein), for the various larval species. The highest activity was observed with microsomal preparations from *H. zea* and *H. virescens*, but within these groups microsomal activity varied considerably. The microsomal activity was not significantly different among diapausing and non-diapausing larvae of the European corn borer and the pink bollworm.

TABLE 3. MICROSOMAL EPOXIDASE ACTIVITY IN SEVERAL SPECIES OF LEPIDOPTEROUS LARVAE*

Species and source	Dieldrin formation (10^{-9} moles/min/mg protein)
Heliothis zea, pH 7·4	
Brownsville, Tex.	0·33
Beltsville, Md.	0·12
Nutrilite Products Inc., Calif.	0·11
Heliothis virescens, pH 7·4	
Brownsville, Tex. (field collected)	1·09
Brownsville, Tex. (laboratory strain)	0·25
Oxford, N. C.	0·18
Ostrinia nubilalis, pH 7·2	
Ankeny, Iowa	
a. Nondiapause	0·05
b. Diapause	0·05
Argyrotaenia velutinana, pH 7·0	
Vincennes, Ind.	0·04
Pectinophora gossypiella, pH 7·4	
Brownsville, Tex.	
a. Nondiapause	0·02
b. Diapause	0·02

* Incubations at 30°. Reaction mixture: microsomal suspension, 0·2 ml; KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH as indicated; NADPH 10^{-3} M; bovine serum albumin, 8 mg; aldrin, 5×10^{-5} M; final volume, 3 ml. Specific activity determined from initial velocities.

Inhibition of aldrin epoxidation by carbon monoxide. Epoxidation of aldrin with microsomes from larvae of *H. zea* (from Lukefahr) was inhibited 58 per cent by a 1:1 mixture of carbon monoxide and oxygen. With a 2:1 mixture, inhibition increased to 85 per cent. Light reversibility[5] of this inhibition was not attempted.

K_m determination and the effect of SKF-525-A. The effect of substrate concentration on the microsomal epoxidase activity and the inhibition by SKF-525-A are shown in Fig. 2. A K_m of $6{\cdot}03 \times 10^{-6}$ M for aldrin was obtained from the Lineweaver–Burk reciprocal plot with microsomes from larvae of *H. virescens*. Similarly, a K_m of $6{\cdot}66 \times 10^{-6}$ M was obtained with microsomes from larvae of *H. zea*, which suggests an enzymatic as well as a morphological resemblance between these two closely related species. Since a curvilinear reciprocal plot was obtained with both concentrations of SKF-525-A, it is difficult to interpret whether the inhibition was competitive or noncompetitive.

Incubation of larval microsomes with dieldrin. Throughout the investigation, dieldrin was the only metabolite of aldrin detected. To check the possibility of the formation

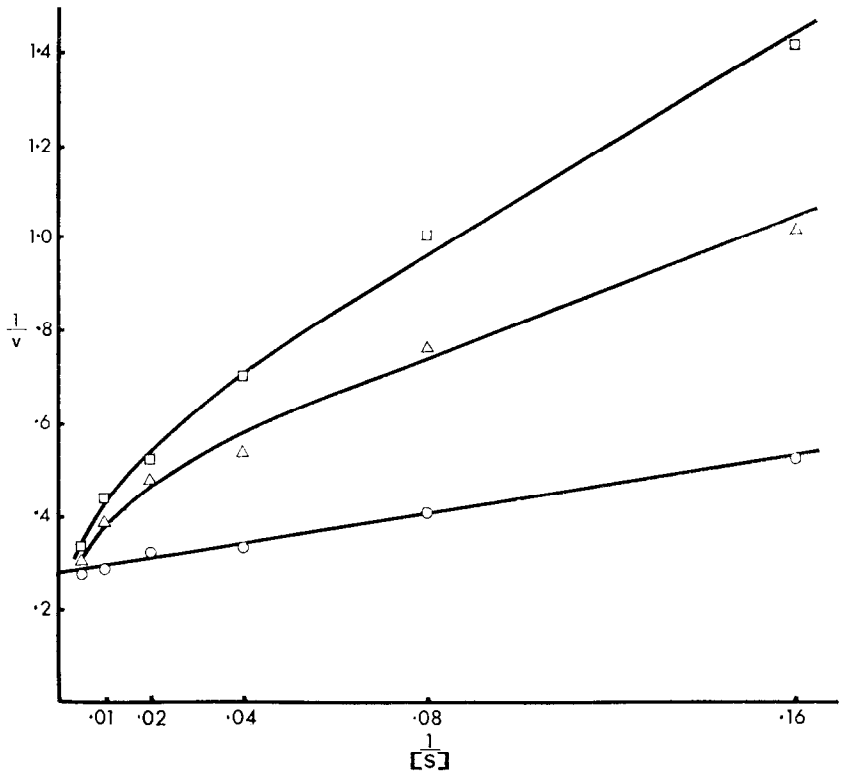

FIG. 2. Reciprocal plots of aldrin epoxidation and inhibition with SKF-525-A. Incubations at 30° for 15 min. Reaction mixture: microsomal suspension, 0·2 ml (*H. virescens* from Lukefahr, 3·79 mg protein); KH_2PO_4–Na_2HPO_4 buffer, 0·1 M, pH 7·4; NADPH, 10^{-3} M; bovine serum albumin, 8 mg; final volume, 3 ml. 1/S = aldrin × 10^{-6} M (added in 100 μl ethanol), v = dieldrin formed (10^{-9} moles/15 min), ○ = no inhibitor (5 μl ethanol), △ = SKF-525-A 5 × 10^{-5} M, □ = SKF-525-A 7·5 × 10^{-5} M; inhibitors added in 5 μl ethanol.

of additional metabolites, microsomes from larvae of *H. virescens* were incubated with dieldrin instead of aldrin. Gas chromatographic analysis showed only a single peak because of dieldrin, and over 97 per cent of the dieldrin was recovered.

Aerobic difference spectra of larval microsomes. Figure 3 shows the difference spectra obtained with microsomal preparations from larvae of *H. zea*. A zero reference absorbance was not assigned to the spectra because of light-scattering effects. Curve A represents the difference spectrum of microsomes reduced with dithionite (after the addition of about 1 mg of sodium dithionite) minus the oxidized microsomes. The spectrum is characterized by an α-band at 557 nm, a β-band at 527 nm, and a prominent γ-band at 424 nm, and it is analogous to the absorption spectrum of reduced cytochrome b_5.[15]

When carbon monoxide was bubbled through the microsomal suspension for 3 min after reduction with sodium dithionite, the difference spectrum (curve B) showed a large band at 450 nm, but the appreciable absorbance caused by dithionite itself around 400 nm interfered with the difference spectrum in this region. However, when the microsomes were reduced with 8 ng of NADPH and carbon monoxide was

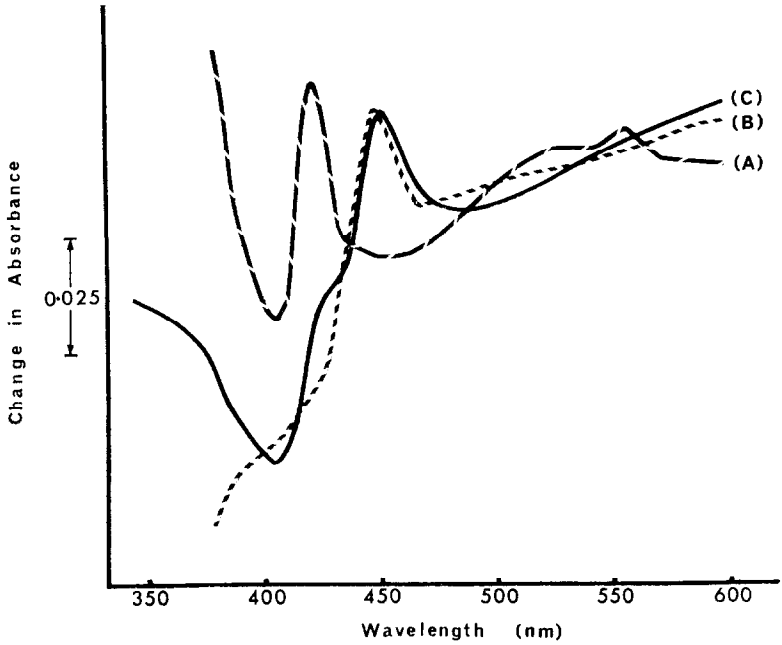

FIG. 3. Aerobic difference spectra of larval microsomes. Microsomes from larvae of *H. zea* (from Lukefahr) were suspended in 4 ml of 0·1 M KH_2PO_4–Na_2HPO_4 buffer, pH 7·4, containing 0·1 ml saturated phenylthiourea (protein concentration, 6·25 mg/0·5 ml); sample size, 0·5 ml; path length, 0·5 cm; samples were reduced with about 1 mg sodium dithionite or 8 ng NADPH. Curve A = dithionite-reduced minus oxidized. Curve B = CO/dithionite-reduced minus dithionite-reduced. Curve C = CO/NADPH-reduced minus NADPH-reduced.

added, the difference spectrum (curve C) clearly showed a minimum at 407 nm, indicating the disappearance of an absorption band in this region. Also, the band at 452 nm shifted very slightly to the right, and its relative intensity became nearly identical to that obtained with dithionite. The shoulder shown at 420 nm was probably caused by the presence of a small amount of the solubilized (P-420)[16] form of cytochrome P-450.

DISCUSSION

The darkening reaction that occurs so rapidly when the S + M fractions of lepidopterous larvae are exposed to air seriously interfered with the epoxidation of aldrin. This melanization process is the result of the activity of the large titer of phenoloxidase[17] that would be expected to be present in larvae just before pupation. Phenoloxidase is similar to the epoxidases in its requirements for NADPH and oxygen, which may explain its apparent competitive inhibition of aldrin epoxidation. The inhibition can be prevented by the incorporation of 1-phenyl-2-thiourea in the incubation medium, as shown in Table 1. Also, inhibition of aldrin epoxidation can be effectively eliminated by using the microsomal fraction since the phenoloxidase and possibly other inhibitors are discarded with the 105,000 *g* supernatant. Since the microsomal pellets were not washed, some darkening did occur over extended periods, particularly with highly concentrated microsomal suspensions. Therefore, phenyl-

thiourea was added to the microsomal suspensions used for the spectral measurements.

The microsomes from lepidopterous larvae were similar to those from mammals and adult insects in their requirements for NADPH and oxygen for the epoxidation of aldrin. The addition of BSA to the homogenizing or incubation medium resulted in greatly enhanced epoxidation of aldrin, whereas nicotinamide was unnecessary for activity.[18] Cyanide failed to stimulate aldrin epoxidation and instead had an inhibitory effect. No explanation can be given for this inhibition since cyanide should inhibit tyrosinase activity,[19] resulting in an increase in the formation of dieldrin.

In pig liver microsomes, an active NADPH-dependent lipid peroxidation system has been found to compete with aldrin epoxidation.[6] Inhibitors of lipid peroxidation, such as EDTA, stimulated dieldrin formation in pig liver but not housefly microsomes. These same investigators found that housefly preparations, in fact, contained an endogenous inhibitor of lipid peroxidation. Likewise, EDTA failed to stimulate aldrin epoxidation with microsomal preparations from lepidopterous larvae (Table 2). Conversely, ascorbic acid, an activator of lipid peroxidation,[20] did not decrease aldrin epoxidation. These results suggest the possible absence of lipid peroxidation enzymes, or the presence of lipid peroxidation inhibitors in microsomes of lepidopterous larvae.

Among larvae of the five species investigated (Table 3), microsomal formation of dieldrin was highest in *H. zea* and *H. virescens*. There was no significant difference between the epoxidase activity of diapausing and nondiapausing larvae. However, considerable intraspecific variation in epoxidase activity occurred, depending on the source of the insect. The extremely high epoxidase activity of a strain of *H. virescens* collected in the field (about four times greater than a laboratory-reared strain from the same area) was of particular interest. These insects were collected from a cotton field in Brownsville, Tex., and were found to be very resistant to DDT and methyl parathion, i.e. 2 pounds of methyl parathion per acre failed to give adequate control.* These results suggest a correlation between epoxidative enzymes and the enzymes responsible for the oxidation of phosphorothionate insecticides. Aldrin epoxidase activity has recently been reported to be greater with microsomal preparations from certain organophosphate-resistant strains of houseflies than from a corresponding susceptible strain.[7]

The curvilinear relationship of SKF-525-A to aldrin epoxidation in larval microsomes (Fig. 2) was similar to that reported for microsomes of houseflies when sesamex was used as an inhibitor.[5] At low concentrations of substrate the trend was towards noncompetitive inhibition, while at higher concentrations competitive kinetics were indicated. This nonlinear effect is not unique to insect microsomes since similar results have been obtained with pig liver microsomes.[6]

The detection of cytochrome b_5 and P-450 has now been extended to microsomes of immature insects and the difference spectra of larval microsomes showing the presence of P-450 help explain the inhibition of aldrin epoxidation by carbon monoxide. The large peak at 450 or 452 nm (Fig. 3, curves B and C) that was seen after the addition of carbon monoxide to the reduced microsomal preparation apparently arose from a substance in the reduced microsomes that had an absorption peak at 407 nm A corrected spectrum of P-450 (with the absorption of cytochrome b_5 cancelled) has been reported with rat liver microsomes,[21] and the subsequent reduction

* D. A. Wolfenbarger, personal communication.

of oxidized P-450 with sodium dithionite showed a distinct band in the region of 407 to 408 nm. When carbon monoxide was added to the reduced sample, the difference spectrum showed the characteristic 450-nm peak and a minimum at 407 to 408 nm. These results agree closely with those we obtained with larval microsomes. However, in our work, the peak at 452 nm, after the addition of carbon monoxide to NADPH-reduced microsomes, was as intense as the band obtained at 450 nm with dithionite as the reductant. This result was in contrast to that reported by other investigators.[5, 15]

Acknowledgements—We wish to thank K. H. Norris for performing the spectrophotometric analyses, S. Akov for her helpful advice and discussions, and M. R. Ditman for her very capable laboratory assistance.

REFERENCES

1. O. Giannotti, R. L. Metcalf and R. B. March, *Ann. ent. Soc. Am.* **49**, 588 (1956).
2. A. S. Perry, A. M. Mattson and A. J. Buckner, *J. econ. Ent.* **51**, 346 (1958).
3. T. Nakatsugawa, M. Ishida and P. A. Dahm, *Biochem. Pharmac.* **14**, 1853 (1965).
4. G. T. Brooks and A. Harrison, *Life Sci.* **5**, 2315 (1966).
5. J. W. Ray, *Biochem. Pharmac.* **16**, 99 (1967).
6. S. E. Lewis, C. F. Wilkinson and J. W. Ray, *Biochem. Pharmac.* **16**, 1195 (1967).
7. R. D. Schonbrod, M. A. Q. Khan, L. C. Terriere and F. W. Plapp, Jr., *Life Sci.* 7, 681 (1968).
8. D. E. Hathway, *Archs envir. Hlth* **11**, 380 (1965).
9. M. Klingenberg, *Archs Biochem. Biophys.* **75**, 376 (1958).
10. P. L. Adkisson, E. S. Vanderzant, D. L. Bull and W. E. Allison, *J. econ. Ent.* **53**, 759 (1960).
11. S. D. Beck, in *Insect Photoperiodism*, p. 135. Academic Press, New York (1968).
12. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
13. K. H. Norris and W. L. Butler, *I.R.E. Trans. biomed. Electron.* **8**, 153 (1961).
14. H. S. Mason, J. C. North and M. Vanneste, *Fedn Proc.* **24**, 1172 (1965).
15. J. Modirzadeh and H. Kamin, *Biochim. biophys. Acta* **99**, 205 (1965).
16. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
17. P. Karlson, *Angew. Chem. (Int. Edn)* **2**, 175 (1963).
18. J. B. Schenkman, J. A. Ball and R. W. Estabrook, *Biochem. Pharmac.* **16**, 1071 (1967).
19. C. R. Dawson and R. J. Magee, in *Methods in Enzymology* (Eds. S. P. Colowick and N. O. Kaplan), vol. II, p. 817. Academic Press, New York (1955).
20. P. Hockstein, K. Nordenbrand and L. Ernster, *Biochem. biophys. Res. Commun.* **14**, 323 (1964).
21. H. Remmer, R. W. Estabrook, J. Schenkman and H. Greim, in *Enzymatic Oxidations of Toxicants* (Ed. E. Hodgson), p. 65. North Carolina State University, Raleigh (1968).