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Insect Metabolism of a Phenyl Epoxygeranyl Ether Juvenoid and Related Compounds[1]

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1-(4′-Ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene (the ethyl-epoxide), a potent insect morphogenetic agent, is converted to 6,7-diol and other derivatives in living cockroaches and mealworms. Enzyme preparations of these organisms, and of houseflies and several other insect species, also carry out these hydration and/or oxidation reactions. In addition, housefly microsomes epoxidize the ethyl-epoxide to a diepoxide. The diepoxide and diol are then converted by microsomes to at least six cyclic diols, probably via an epoxy-diol intermediate, the major ones being the *cis*- and *trans*-tetrahydrofuran-diol derivatives. The metabolites formed by these reactions have little or no morphogenetic activity in *Tenebrio* assays. Attempts to find potent inhibitors for housefly epoxide hydratases were unsuccessful. The corresponding ethylphenyl geranyl ether is epoxidized by housefly microsomes, forming the more morphogenetically active ethyl-epoxide, but the major reaction is oxidation on the geranyl moiety to an unidentified olefinic carboxylic acid. The chemical modifications needed for improved stability and morphogenetic activity in this juvenoid series depend on the insect species and strain and the relative activities of their enzymes involved in various inactivation pathways.

1-(4′-Ethylphenoxy)-3,7-dimethyl-6,7-epoxy-*trans*-2-octene (the ethyl-epoxide; R 20458) acts at very low levels to disrupt the development of many pest insect species (1–3). This juvenoid is metabolized in living mice or mammalian liver enzyme systems by epoxide hydration, 2,3-epoxidation, α- and β-oxidation of the ethyl group and ether cleavage (4). Its morphogenetic potency and selectivity are expected to depend, in part, on the relative rates and types of detoxification in various insect species, strains and developmental stages.

The ethyl-epoxide, its diene precursor (ethyl-diene) and its 6,7-diol (ethyl-diol) and 2,3-6,7-diepoxide (ethyl-diepoxide) derivatives (Fig. 1) are used in the present study to examine the types of reactions involved in insect metabolism of substituted-phenyl epoxygeranyl ethers and related compounds. More than 30 ethylphenyl ethers derived from these four compounds by various types or sequences of oxidation, hydrolysis, and addition reactions are available for use in metabolite identification (5). Also available are more than 10 α- and β-hydroxyethylphenyl and acetophenyl ethers within the same series (5). These unlabeled standards are com-

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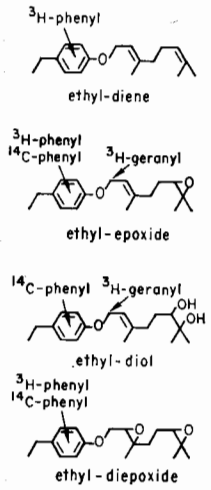

FIG. 1. *Structures for the ethyl-diene, -epoxide, -diol, and -diepoxide showing the positions of* ^{14}*C- or* ^{3}*H-labeling.*

pared with radioactive juvenoid metabolites produced in living cockroaches and mealworms and in enzyme systems of houseflies and three other insect species.[4]

MATERIALS AND METHODS

Chemicals

The radiolabeled compounds (Fig. 1) have the following specific activities: 654 mCi/mmole for the [^{3}H]phenyl compounds; 17 mCi/mmole for the [^{14}C]phenyl compounds; 33 mCi/mmole for the [^{3}H]geranyl compounds (4). Each compound was greater than 98% radiochemically and isomerically pure except the [^{3}H]geranyl compounds which consisted of both *trans-* and *cis-*isomers in a 16:9 ratio; the individual isomers were separated for use in some dual-label experiments (4). Unlabeled authentic standards of possible metabolites and related compounds were prepared by described procedures (5). The structures of all standards and their derivatives were confirmed by at least ir,[5] pmr,[5] and ms.[5] The other standards and the metabolites are referred to by trivial names made up of two parts, the first designating the *para-*substituent of the phenoxy group and the second the nature of the geranyl-derived moiety. Figure 2 shows these two portions of the molecule which, when appropriately combined, serve to designate the various compounds, i.e., α-hydroxyethyldiene, ethyldiepoxide, etc.

Chromatography and Derivatization Studies

The radiolabeled metabolites were fractionated by tlc[5] or column chromatography on silica gel (described later) or both and tentatively identified by tlc cochromatography, usually with confirmation by formation of one or more derivatives followed by cochromatography with the appropriate standard derivatives when available (4, 5).

Tlc utilized silica gel F_{254} chromatoplates (0.25 mm gel thickness, EM Laboratories, Elmsford, NY) and one or several of the BP,[5] CMAP,[5] and TE[5] solvent systems for 1- or 2-dimensional development. Radiolabeled compounds were detected by radioautography, using a coating with a scintillation fluor for the ^{3}H-materials (6), while the unlabeled standards were detected by their quenching of gel fluorescence

[4] For complete description of materials, methods, and results see the following PhD dissertations, University of California, Berkeley, 1973: B. D. Hammock, Chemical and biological studies on aryl geranyl epoxide ether juvenoids; S. Singh, Metabolism and environmental degradation of the juvenoid 1-(4′-ethylphenoxy)-3, 7-dimethyl-6, 7-epoxy-2-octene.

[5] Abbreviations used in the order that they appear are: ir, infrared spectra; pmr, proton magnetic resonance spectra; ms, electron ionization mass spectra; thf, tetrahydrofuran; thp, tetrahydropyran; tlc, thin layer chromatography; BP 10:1 or 4:1, tlc solvent system consisting of benzene-*n*-propanol 10:1 or 4:1 mixture; CMAP, tlc solvent system consisting of chloroform–methylene chloride–ethyl acetate-*n*-propanol 10:10:1:1 mixture; TE, tlc solvent system consisting of carbon tetrachloride–ether 2:1 mixture; lsc, liquid scintillation counting; MCPBA, *m*-chloroperoxybenzoic acid; BBA, *n*-butylboronic acid; $PbAc_4$, lead tetraacetate; Ac_2O-acetic anhydride-pyridine; BSA, bovine serum albumin; NADPH, reduced nicotinamide-adenine dinucleotide phosphate.

under ultraviolet light or by various spray reagents (5). The amount of each individual labeled product was determined by scraping the appropriate gel region into a scintillation vial for lsc.[5]

Functional group tests used to confirm tentative metabolite identifications are based on the following relationships (4, 5); olefins with OsO_4 in pyridine form pyridine-stabilized osmate esters which decompose to diols or cyclic products; olefins form mono- or diepoxides with MCPBA[5] which in some cases cyclize to thf[5] or other derivatives; epoxides cleave to diols or rearrange and other acid-labile compounds are degraded with 0.05 *N* H_2SO_4 in 40% aq. thf; diols form diesters with BBA[5] and cleave to the corresponding aldehydes with $PbAc_4$[5]; benzylic alcohols are oxidized to the corresponding ketones with $PbAc_4$; aldehydes and ketones are converted to semicarbazones with semicarbazide, to the corresponding alcohols with $NaBH_4$, and in addition the aldehydes give the corresponding acids with Tollens' reagent or MCPBA; primary and secondary but not tertiary alcohols are acetylated with Ac_2O[5]; carboxylic acids form methyl esters with diazomethane; phenols partition into 5% aq. KOH but not into 5% aq. $NaHCO_3$ from ether while carboxylic acids partition into the aq. phase under both conditions. All tests were run on each isolated metabolite in order to determine the presence or absence of the above functional groups.

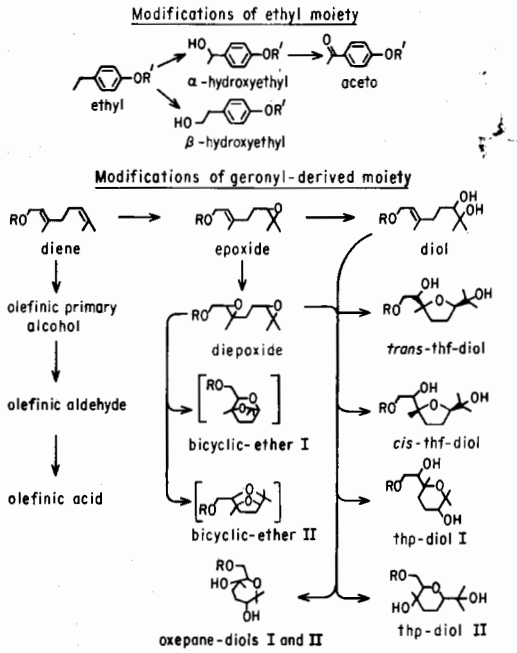

FIG. 2. *Metabolic modifications of the ethyl- and geranyl-derived moieties of the ethyl-diene, -epoxide, -diol, and -diepoxide by the housefly microsome–NADPH system.*

Insects

Males and females of the following species were used: *Periplaneta americana* (L.) (0.8 g, Dow Chemical Co., Walnut Creek, CA); *Tenebrio molitor* L. (last-instar larvae, 213 ± 5 mg/larva; Sure Live Mealworm Co., Los Angeles, CA); *Musca domestica* L. [adults, SCR-susceptible or R_{Baygon}-resistant strain (7), 18 mg, reared in this laboratory]; *Sarcophaga bullata* Parker (adults, 100 mg, Zoecon Corp., Palo Alto, CA); *Manduca sexta* (Johannson) (pre-pupae, 10 g, Zoecon Corp.); *Trichopulsia ni* (Hubner) (last-instar larvae, 300 mg, Chevron Chemical Co., Richmond, CA). Treated insects were maintained on their normal diets at 29°C under constant light conditions. Controls for the *in vivo* studies consisted of insects killed immediately before treatment by immersion in boiling water.

Treatment of Living Insects and Analysis of Metabolites

The feces collected within 24 hr after injection of *P. americana* with the [^{3}H]-phenyl-ethyl-epoxide (0.25 μg) in ethanol (1 μl) were extracted with methanol and then the methanol extracts were subjected directly to tlc.

Tenebrio larvae, anesthetized by immersion in cold water, were injected with the [^{3}H]phenyl - ethyl - epoxide (0.5 μg) in ethanol (0.5 μl). Five replicates of 20 treated larvae were held in glass petri dishes

then the excreted radioactivity was recovered by rinsing the dishes with an ether–ethyl acetate mixture. Each group of larvae was homogenized in a mixture of water (9 ml) and ether (15 ml) and then the homogenate was centrifuged to obtain an organosoluble fraction. This procedure was repeated twice more for complete recovery of the injected ethyl-epoxide. After evaporation of the ether extract to dryness, the metabolites in the residue were partitioned between an equal volume of hexane and acetonitrile for cleanup. After tlc cochromatography of the acetonitrile extract, the individual products were subjected to lsc and derivatization studies. The neutral aq. fraction was obtained by centrifuging the aq. phase and combining it with two additional water extractions of the residue obtained by rehomogenization and centrifugation. Three extractions of the residue with 0.2 *N* aq. HCl then yielded the acidic aq. fraction and the unextractable residue.

Metabolism by Insect Homogenates or Fractions

Whole insects or portions thereof were homogenized in sodium phosphate (0.2 *M*) at pH 7.8 for epoxide hydratase assays or at pH 7.4 when both hydratase and oxidase activities were to be assayed. The homogenization medium was sometimes varied, as specified, in the type or pH or in that it contained BSA[5] at 1% (w/v) to enhance the stability of microsomal enzymes. Fly abdomens were conveniently obtained in large numbers by freezing the flies on dry ice, breaking them into various parts by shaking with dry ice and 4-mm glass beads, and sieving to obtain the separated thoraces, wings, and abdomens from which the abdomens were easily picked out. The oxidase activity of microsomes prepared in this manner was very similar to that obtained with microsomes prepared from freshly clipped abdomens (7) or by the Morello procedure (8) based on using each of these methods in the present studies. The homogenates were subjected in sequence to the following steps: filtration through cheesecloth; centrifugation at 2000*g* for 5 min to sediment nuclei and debris; centrifugation at 12,100*g* for 15 min to sediment the mitochondrial fraction; filtration through glass wool; centrifugation at 102,000*g* for 60 min to sediment the microsomal fraction. All sedimented fractions were washed once by resuspension in fresh buffer and recentrifugation. Protein was determined by the method of Lowry *et al.* (9).

Epoxide hydratase assays. The [^{3}H]-phenyl-ethyl-epoxide (20 nmoles) in ethanol (10 μl) was added to the microsomal preparation (5 or 10 whole fly equivalents; ca. 0.3 or 0.6 mg protein) in 1 ml of pH 7.8 phosphate buffer. After incubation (30°C, 1 hr), the amounts of the ethyl-epoxide and -diol were determined by extracting them into ether, tlc cochromatography, and lsc. This procedure provides recovery values of >98% for each of the ethyl-epoxide and -diol and the percentage of ethyl-diol formed is reproducible within 1% for replicates of the same enzyme experiment. Epoxide hydratase activity is given as the percentage of the ethyl-epoxide hydrated to the ethyl-diol, after correction for nonenzymatic reactions as determined with a heat-denatured (100°C, 10 min) control.

In similar assays, the [^{3}H]phenyl-ethyl-diepoxide (10 nmoles) in ethanol (5 μl) was added to the microsomal preparation (five whole fly equivalents) in 1 ml of 0.1 *M* KCl containing 1% BSA and incubated as above. The reaction mixtures were lyophilized, extracted with methanol, and the recovered products were cochromatographed by tlc in BP 5:1 with a mixture of the 2,3,6,7-tetrahydroxy derivative of the ethyl-diene (the ethyl-tetraol) and the acid-cleavage products of the unlabeled ethyl-diepoxide; this mixture of acid-cleavage products contains the ethyl-thf-diols, the

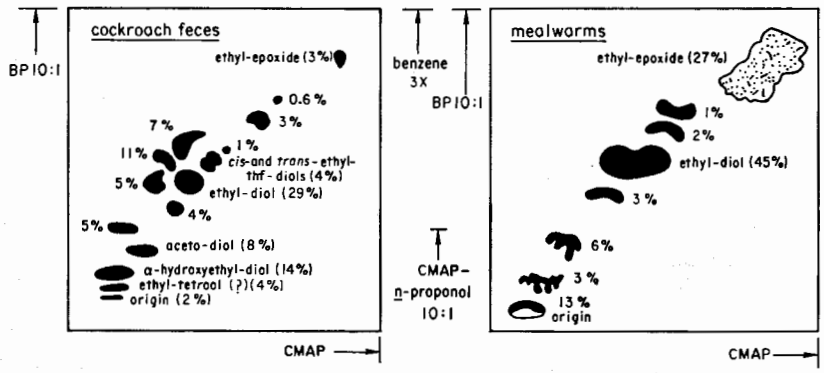

FIG. 3. *Tlc patterns of radioactive organosoluble metabolites in adult female* Periplaneta americana *feces and* Tenebrio molitor *last-instar larvae injected with the [^3H]phenyl-ethyl-epoxide. The quantitative values are the averages of those obtained on analyses of 0- 24-hr feces from three treated cockroaches and at 1, 3, and 6 hr after injection of mealworms.*

ethyl-thp[5]-diols, the ethyl-oxepane-diols, and the ethyl-bicyclic ethers (see Fig. 2) (5). The percentage of ethyl-diepoxide metabolism is corrected for nonenzymatic reactions as before.

In determining the pH optimum, the reaction mixtures (1 ml) contained the microsomal suspension (five whole fly equivalents), substrate, 0.05 *M* KCl, 0.1 *M* phosphate, borate, or Tris buffer at the appropriate pH, and 0.5% BSA.

Compounds assayed as possible inhibitors of epoxide hydratase activity were added in ethanol (10 μl) or water (up to 50 μl) to the complete incubation mixture 5 min prior to introduction of the [^3H]phenyl-ethyl-epoxide and subsequent assay as before.

Assays of enzyme preparations fortified with NADPH. The radiolabeled ethyl-diene, -epoxide, -diepoxide, or -diol was added in ethanol (10 μl) to the enzyme preparation (1 ml) in the presence or absence of NADPH.[5] The amounts of substrate and NADPH were 18 and 4000 nmoles, respectively, in studies with houseflies and 100 and 500 nmoles, respectively, with the other insects. The incubated mixtures (30°C, 1 hr) were saturated with $(NH_4)_2SO_4$, extracted with peroxide-free ether, the ether was dried over Na_2SO_4, and the ether-soluble components were analyzed by tlc and lsc. This extraction procedure gave an identical metabolite pattern, qualitatively and quantitatively, to that obtained by extraction with chloroform or ether–ethanol mixture or lyophilization of the reaction mixture and extraction of the residue with methanol. The enzyme preparations assayed were: the microsomal fraction from abdomens or abdomens plus thoraces of *M. domestica* (10 fly equivalents, ca. 0.6 mg protein); microsome plus soluble fractions of *S. bullata* abdomens (five fly equivalents), *M. sexta* gut (17 mg protein) and *M. sexta* fat body (33 mg protein); 600*g* supernatant fractions of *P. americana* midgut (two midgut equivalents).

Larger amounts of metabolites were prepared by incubating the housefly microsome–NADPH system with the *trans*-ethyl-epoxide (mixed with tracer levels of *trans*-pure [^{14}C]phenyl- and [^3H]geranyl compounds) or with the [^3H]phenyl-ethyl-diene, in each case using 6 μmoles of substrate divided equally among 20 incubation flasks. The incubated mixtures were subjected to Soxhlet extraction with pentane recovering 95% and 98% of the radioactivity in the case of the epoxide and diene, respectively, partitioning the pentane with acetonitrile, column chromatography of both the acetonitrile and pentane frac-

TABLE 1

Metabolism of Various Concentrations of the Ethyl-Diene, Ethyl-Epoxide, and Ethyl-Diol by the Housefly Microsome–NADPH System

Substrate Conc.	% Metabolism		
	Ethyl-diene	Ethyl-epoxide	Ethyl-diol
No NADPH			
4 μM	6	56	4
4 mM NADPH			
4 μM	93	96	57
19 μM	92	84	50
38 μM	82	69	36
190 μM	80	72	13
380 μM	66	67	23
760 μM	43	19	5

tions on silica gel developed with hexane–ether and ether–methanol gradients, and finally tlc in BP 10:1 and 4:1 mixtures. The acetonitrile partitioning step reduces the effect of interfering materials on subsequent chromatography. This sequence of chromatographic steps permits purification of individual metabolites of the ethyl-diene which are not resolved by normal tlc techniques.

For comparative purposes, the distribution of mixed-function oxidase activity in housefly homogenate fractions was determined by assaying the *N*- and *O*-methyl hydroxylation reactions of *N*,*N*-dimethyl *p*-nitrophenyl carbamate [DpNC] (10) and [^{14}C]methyl-*p*-nitroanisole [pNA] (11).

RESULTS

Metabolism of the Ethyl-Epoxide in Living Insects

Periplaneta americana. The methanol extract of cockroach feces (0–24 hr, containing >75% of the injected radioactivity) contains the ethyl-diol as the major identified metabolite (Fig. 3), the identification being based on cochromatography with appropriate compounds as the original material and after derivatization (Ac_2O, $PbAc_4$). Tentative identification of the other metabolites (Fig. 3) is based only on cochromatography, evidence which is not conclusive particularly for the ethyl-tetraol which is not firmly established as a metabolite in any of the other insect systems examined. A portion of the metabolites (13%) contains acidic functional groups since they move from the origin on tlc except when treated on the chromatoplate with NaOH prior to development.

Tenebrio molitor. Tritium from injected [^{3}H]ethyl-epoxide is eliminated from the larvae to the extent of 16, 29, 49, and 63% after 1, 3, 6, and 24 hr, respectively. Ether extraction recovers almost all of the injected radioactivity at 0 time whereas at 1, 3, 6, and 24 hr the recoveries are 51, 33, 27, and 14%, respectively, the rest appearing almost entirely in the neutral aq. fraction; very little is in the acidic aq. fraction or is unextractable. Partitioning of the ether-soluble metabolites between hexane and acetonitrile minimizes but does not overcome the interference of lipids in tlc cochromatography studies. Most of the labeled material in the hexane fraction gives the same R_f as the ethyl-epoxide although trace amounts of products are found at higher tlc positions as well as some at the origin. Lipids and other interfering material in the acetonitrile fraction obscure the tlc region of the ethyl-epoxide and of two unidentified metabolites just above the origin (Fig. 3) but the only major organosoluble metabolite (10–24% of the injected dose) is identified as the ethyl-diol (cochromatography without derivatization and after reaction with BBA and Ac_2O). The ether-soluble metabolites from all time intervals show similar partition characteristics between equal volumes of acetonitrile and hexane (74 $\pm$ 3% in the acetonitrile) while such values for the authentic ethyl-diene, -epoxide, and -diol are 29, 76, and 97%, respectively.

TABLE 2

Metabolites of the [^3H]Phenyl-Ethyl-Diene Formed by Enzyme–NADPH Systems of Four Insect Species

Compound	Tlc R_f[a]	Enzyme source				
		M. dom. SCR Th+abd	*S. bull.* Abd	*P. amer.* Midgut	*P. amer.* Fat body	*M. sexta* Gut
Amount of individual metabolite, % of recovered radioactivity[b]						
Ethyl-diene	0.95	8[c]	88	98	84	64
α-Hydroxyethyl-diene	0.80	tr		0.4	9	tr
β-Hydroxyethyl-diene	0.78				1	0.5
Aceto-diene	0.82		1		0.9	0.5
Olefinic primary alcohol	0.60	5				
Olefinic aldehyde	0.40	3				
Olefinic carboxylic acid	0.41	32				
Hydroxylated olefinic carboxylic acid	0.32	2				
Number of unknowns						
Major		4[c]	3	0	2	3
Minor		>5	0	4	4	1

[a] Development 6 cm with BP 4:1 and then 16 cm with BP 10:1.

[b] Total radioactivity recoveries are >90% in each case.

[c] Additional metabolites and their percentage recovery values are: ethyl-epoxide, 4%; hydroxyethyl-epoxides, trace; ethyl-diol, 5%; hydroxyethyl-diols, trace; ethyl-thf-diols, 2%. The metabolites are resolved by column chromatography followed by tlc.

Metabolites of the Ethyl-Diene Formed by Insect Enzyme Preparations Fortified with NADPH

The ethyl-diene undergoes little or no metabolism on incubation with housefly microsomes in the absence of NADPH but on cofactor addition it is generally metabolized more rapidly than the ethyl-epoxide or -diol (Table 1). The housefly preparation is much more active than preparations of three other insect species in metabolism of the ethyl-diene, these other preparations acting mostly on the ethyl substituent, oxidizing it at the α- and β-positions (Table 2). The subsequent discussion deals specifically with the housefly metabolites.

Special conditions employed to establish that the ethyl-diene undergoes enzymatic epoxidation to the ethyl-epoxide included extraction with peroxide-free ether or pentane containing 0.001% (w/v) 2,6-di-*t*-butyl-*p*-cresol as an antioxidant and developing the tlc plates in the dark. The yield of ethyl-epoxide was consistently 2–5% in the presence of NADPH but it never exceeded 0.02% in the absence of this cofactor. The ethyl-epoxide metabolite structure was verified by various derivatization techniques including quantitative conversion to the ethyl-*cis*- and -*trans*-thf-diols on OsO_4 oxidation. Further supporting the metabolic 6,7-epoxidation is the finding of a significant amount of ethyl-diol and trace amounts of the α- and β-hydroxyethyl-epoxides and their corresponding diols from metabolism of the ethyl-diene.

Most of the metabolites of the ethyl-diene chromatograph on tlc just above or below the ethyl-diol. The use of column chromatography then tlc allows isolation of at least 14 products sufficiently pure for partial characterization by derivatization studies

TABLE 3

Metabolites of the [^{14}C]Phenyl-Ethyl-Epoxide Formed by Enzyme–NADPH Systems of Four Insect Species

Compound	Tlc R_f[a]	Enzyme source					
		M. dom. Th + abd.		*S. bull.* Abd	*P. amer.*		*M. sexta* Gut
		SCR	R_{Baygon}		Midgut	Fat body	
Amount of individual metabolite, % of recovered radioactivity[b]							
Ethyl-epoxide	0.83	16	4	49	46	35	34
α-Hydroxyethyl-epoxide	0.51			2	0.5	7	3
		18	62				
β-Hydroxyethyl-epoxide	0.48					2	0.7
Aceto-epoxide	0.63	11	19	44	0.4	1	1
Ethyl-diol	0.39	16	1	4	52	43	42
α-Hydroxyethyl-diol	0.25	10	6				
						7	3
β-Hydroxyethyl-diol	0.20	4	2				
Aceto-diol[c]	0.30	5		1		2	1
Ethyl-*cis*-thf-diol	0.47					0.7	2
		8		0.7	0.6		
Ethyl-*trans*-thf-diol	0.43					2	4
Ethyl-diepoxide	0.77	2				0.6	1
Number of unknowns							
Major		0	0	0	0	1	3
Minor		4	4	1	1	1	1

[a] Development 6 cm with BP 4:1 and then 16 cm with BP 10:1.

[b] Total radiocarbon recoveries are >90% in each case.

[c] Includes trace amounts of the ethyl-oxepane-diols I and II (R_f 0.30 and 0.32, respectively) and larger amounts of the ethyl-thp-diols I and II (R_f 0.38 and 0.32, respectively). These R_f values refer to the solvent system given in footnote *a*. A combination of column chromatography and tlc is necessary for tentative identification of these metabolites. Acetate derivatives of each of the ethyl-thp-diol and ethyl-oxepane-diol metabolites cochromatograph with the appropriate standards.

(Table 2). The major product, an olefinic acid with no alcohol functionality, differs from any authentic compound available, including those theoretically formed on: oxidative cleavage of the 2–3, 4–5, or 6–7 bonds of the diene moiety or the α–β bond of the ethyl moiety (compounds 37A, 38A, 20A, and 1H, respectively, ref. 5); oxidation of the *trans*-methyl group at the 7-position or the β-position of the ethyl moiety (compounds 22A and 1E, respectively, ref. 5). Derivatization studies also establish the presence of an olefinic alcohol and an olefinic aldehyde which possibly are the intermediates in enzymatic formation of the olefinic acid. The olefinic aldehyde can be oxidized by Tollens' reagent or MCPBA to a compound with the same R_f value as the olefinic acid (the aldehyde and acid can be separated by multiple developments in TE). A second olefinic carboxylic acid contains a benzylic hydroxyl group, so a portion of the metabolism involves oxidation not only on the geranyl moiety but also on the ethyl group. These two acids give less polar compounds upon reaction with MCPBA suggesting that under these oxidative conditions the carboxyl group attacks the epoxide resulting in cyclization. Thus, the major metabolites of the ethyl-diene cannot be accounted for by the pathways established, as discussed later, for the ethyl-epoxide, -diol, and -diepoxide.

TABLE 4

Metabolites of the [^{14}C]Phenyl-Ethyl-Diol Formed by Enzyme–NADPH Systems of Four Insect Species

Compound	Enzyme source				
	M. dom. Th + abd	*S. bull.* Abd	*P. amer.* Midgut	*P. amer.* Fat body	*M. sexta* Gut
Amount of individual metabolite, % of recovered radioactivity[a]					
Ethyl-diol	50	96	98	88	71
α-Hydroxyethyl-diol	28				
		0.7	0.3	7	4
β-Hydroxyethyl-diol	6				
Aceto-diol[b]	0.5	2	0.5	2	3
Ethyl-*cis*-thf-diol				0.5	5
	5	1	0.7		
Ethyl-*trans*-thf-diol				0.9	5
Number of unknowns					
Major	1	0	0	0	5
Minor	5	2	2	3	3

[a] Total radioactivity recoveries are >90% in each case.

[b] Includes trace amounts of the ethyl-bicyclic ethers I and II and the ethyl-oxepane-diols I and II and larger amounts of the ethyl-thp-diols I and II. See Table 3 for the chromatographic properties of these compounds.

Metabolites of the Ethyl-Epoxide and -Diol Formed by Insect Enzyme Preparations Fortified with NADPH

The ethyl-epoxide and -diol are stable on incubation in buffer only but they each undergo metabolism on incubation with enzyme preparations, particularly when fortified with NADPH (Table 1).

The major site of metabolic attack on the ethyl-epoxide is either the α-position of the ethyl moiety or the epoxide moiety, depending on the species (Table 3). The dipterous insects are most effective in oxidation of the ethyl substituent relative to epoxide cleavage, with the contrary being true for *P. americana* and *M. sexta*. Products resulting from oxidation of the 2,3-double bond are most significant with *M. domestica* but are also detected with the other insect preparations (Table 3). The most extensive evidence for metabolite characterization comes from the studies with houseflies, as discussed below.

The α-hydroxyethyl-epoxide is poorly resolved from another hydroxylated metabolite of the ethyl-epoxide in the BP solvent system but on multiple developments with the TE solvent system the α-hydroxyethyl compound has a higher R_f value. The lower product has the same R_f value as the β-hydroxyethyl-epoxide; however, this does not constitute identification since other primary or secondary alcohol metabolites cannot be excluded by the available derivatization studies. The α-hydroxyethyl-epoxide is usually present in four to six times the amount of the other hydroxylated metabolite. The aceto-epoxide, ethyl-diol and α-hydroxyethyl-diol are identified by cochromatography and appropriate derivatization tests. All of the metabolites of the ethyl-epoxide, appearing in more than trace levels, with the same or lower R_f values than the ethyl-diol are vicinal diols (BBA reaction). The metabolite chromatographing just below the α-hydroxyethyl-diol is

a triol assumed to be the β-hydroxyethyl-diol; however, derivation studies are not definitive in this case. Two very minor products appear at R_f values just under the ethyl-diol; the R_f value for the upper of these is similar to that of the aceto-diol, the ethyl-thp-diol, or the upper isomer of the ethyl-oxepane-diol. Cochromatography attempts with the four diols indicated above resulted in diffusion of the spots beyond the point of detection. Derivatization studies support the cochromatographic evidence for identification of the ethyl-*cis*- and -*trans*-thf-diols and of the ethyl-diepoxide. One very minor metabolite of the ethyl-epoxide cochromatographs with the ethyl-tetraol and another has the same R_f as the α-hydroxyethyl-tetraol; however, these metabolites are not these tetraols since they do not give appropriate R_f values after reaction with BBA. A minor product formed on decomposition of the ethyl-diol when held in room light on a tlc plate for a short time has a chromatographic position on two-dimensional tlc development slightly below the α-hydroxy-ketone and just above the bicyclic ketal and thf-alcohol (compounds 18A, 33A, and 35A, respectively, ref. 4). This product is also detected in the metabolism mixture but it is uncertain that it is formed metabolically because of its possible presence as an artifact during analysis.

The α-hydroxyethyl-, β-hydroxyethyl-, and aceto-diols are also detected as metabolites of the ethyl-diol along with the same cyclic products formed from the ethyl-epoxide (Table 4). Thus, the ethyl-diol is oxidized at the 2,3-double bond and the α- and β-positions of the ethyl group. The metabolites of the pure ethyl-*trans*-diol, pure ethyl-*cis*-diol, and a mixture of the two isomers show identical R_f values in CMAP and BP 5:1 solvent systems indicating that the apparent number of metabolites formed is not increased by resolution of the *trans*- and *cis*-isomers. Possible formation of the ethyl-*cis*- and -*trans*-thf-diols from the pure ethyl-*cis*-diol was not evaluated since they have similar R_f values to the substrate.

Ether cleavage is not an important metabolic pathway with the ethyl-epoxide or -diol since, in each case, identical metabolite patterns are obtained using the phenyl- and geranyl-labeled preparations. Failure of metabolites to cochromatograph with other standards (5) indicates several other potential pathways are not important in the microsomal degradation of the ethyl-epoxide.

Metabolites of the Ethyl-Diepoxide in the Housefly Microsome–NADPH System

The ethyl-diepoxide is only slowly degraded on incubation with buffer only whereas it undergoes extensive breakdown in the presence of housefly microsomes, as discussed later. The major metabolites formed by microsomal systems with or without NADPH fortification are the ethyl-*cis*- and -*trans*-thf-diols. There are also minor products tentatively identified by cochromatography and the following additional evidence, where applicable: ethyl-thp-diol I; ethyl-thp-diol II, ethyl-oxepane-diol I, and ethyl-oxepane-diol II each cochromatographed before and after acetylation; one or both of the ethyl-bicyclic ethers I and II. Three minor metabolites of the ethyl-diepoxide when NADPH is added to microsomes chromatograph as expected for the following materials: a monohydroxylated-diepoxide, possibly the α- or β-hydroxyethyl-diepoxide; two aceto-diepoxides, possibly the resolved isomers at the 2,3-position of this compound.

Properties of Insect Epoxide Hydratases Acting on the Ethyl-Epoxide and -Diepoxide.

The ethyl-diol is the only major metabolite formed on incubation of the ethyl-epoxide with homogenates of whole house-

flies or their abdomens or heads plus thoraces in the absence of added cofactors. No ethyl-diol is formed by heat-denatured enzyme (i.e., <2% of the amount formed by normal enzyme preparations). While the highest activity is found in the microsomal fraction (102,000*g* pellet) of abdomen homogenates, epoxide hydratases are also present in other particulate fractions including the mitochondrial fraction (12,100*g* pellet) (Fig. 4); however, a portion of the NADPH-dependent oxidase activity also appears in the mitochondrial fraction (Fig. 4), in confirmation of studies by Hansen (12), so it is not known if the epoxide hydratase activity of the mitochondrial fraction is intrinsically due to mitochondria or to microsomal contamination. In any case, the ethyl-diol constitutes more than 90% of the metabolites formed by each subcellular fraction. The increase in activity of the microsomal oxidases on preparation of the microsomal fraction (Fig. 4) probably results from removal of oxidase inhibitors or competing substrates.

Housefly microsomal epoxide hydratases are active over the broad pH range of 5–10 but activity falls off rapidly at pH values of less than 5.5 or greater than 9.5. Distinct peaks of activity are seen at or near pH 6.8, 8.1, and 8.9, with the first two peaks being most prominent in assays with the ethyl-epoxide and the last peak with the ethyl-diepoxide. The epoxide hydratase activity on the ethyl-epoxide is higher when assayed in phosphate than in borate or Tris buffers, each at the same pH. The hydratases are not inhibited by methylene blue (0.05 μmole) or housefly head homogenate (10 fly equivalents) or on using N_2, CO, or CO_2 as the gas phase replacing air, each of these conditions strongly inhibiting the oxidase activity.

None of the metabolites of the ethyl-epoxide shown in Fig. 2 or Table 3 inhibit the housefly epoxide hydratases when assayed at 10^{-4} *M*. Other compounds that are also inactive as inhibitors under these conditions are: benzyl thiocyanate, chloral, cyclohexene oxide, 2,2-dimethyloxetane, iodoacetic acid, Lethane 384, 1-octene, paraoxon, 1,2-propanediol, semicarbazide ·HCl, NaF, NaCN, styrene oxide, thf, trichloroethanol, and the 6,7-episulfide derivative of the ethyl-diene. The best inhibitors of ethyl-epoxide hydration are the 7-bromo-6-hydroxy derivative of the ethyl-diene (10 μmoles) which halves the enzyme activity and 1,1,1-trichloropropene 2,3-oxide (100 μmoles) which reduces it by 90%. Metyrapone ditartarate (0.2 μmoles) gives a 10–20% enhancement of activity while 2 or 0.1 μmoles causes no change.

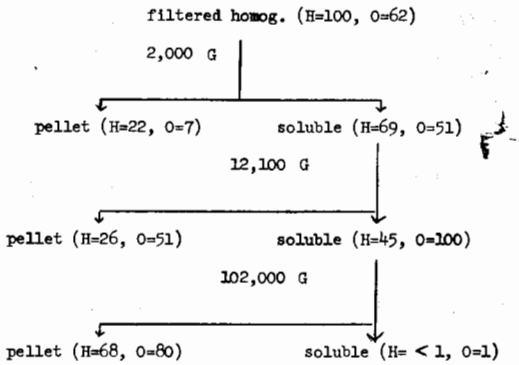

FIG. 4. *Epoxide hydratase and mixed-function oxidase activities of housefly abdomen homogenate fractions.* O = *oxidase, average of assays with pNA and DpNC. The value of 100 is arbitrarily assigned to the activity for the most active fraction.* H = *hydratase, assays with the ethyl-epoxide. The value of 100 represents 16 nmoles of ethyl-diol formed from 20 nmoles of ethyl-epoxide at pH 7.8 for 1 hr at 30°C. These values are the average of results obtained with there homogenates and involve standard deviations of <10% of the reported activities. Identical studies with whole flies and heads plus thoraces gave similar activity distribution patterns.*

Ethyl-diepoxide metabolism by fly microsomes at pH 7 in phosphate buffer gives 18 and 17% ethyl-*trans*- and -*cis*-thf-diols, respectively, 8% ethyl-thp-diols I and II, 1% ethyl-oxepane-diols I and II, trace amounts of the ethyl-bicyclic ethers I and II, and 56% unmetabolized substrate. These products are the same as those formed on incubation of the ethyl-diepoxide with rat liver microsomes or on treatment

with acid (4, 5). The thf-diol isomers vary slightly in their ratio to each other and to other products with buffer pH. These findings and chemical studies on acid hydrolysis of diepoxides (5, 13) indicate that probably the epoxide hydratase initially cleaves one epoxide group to free the 2,3-epoxy-6,7-dihydroxy compound (or less likely the 2,3-dihydroxy-6,7-epoxy compound) for cyclization in the buffer or possibly on the tlc plates during analysis. No ethyl-tetraol is detected as a microsomal metabolite of the ethyl-diepoxide either by tlc cochromatography or by fortification of organic extracts with the authentic ethyl-tetraol and recrystallization.

The epoxide hydratase activity as assayed with the ethyl-epoxide ranges from quite low to very high with various insect preparations. With whole insect homogenates, *Trichoplusia* is more active than *Tenebrio* and *Musca*. There is almost no activity in the microsome plus soluble fraction of either *Sarcophaga* abdomens or thoraces. The microsome plus soluble fraction of *Musca* abdomens is moderately active but the soluble fraction is totally inactive. Moderate epoxide hydratase activity is found in *Manduca* gut and *Periplaneta* midgut or fat body whereas *Periplaneta* haemolymph (up to 20 μl) is not active in metabolizing the ethyl-epoxide.

DISCUSSION

Insect metabolism of the ethyl-diene, -epoxide, -diol, and -diepoxide involves attack at the ethyl moiety of the ethylphenyl group (Fig. 2, upper portion) and various positions of the geranyl-derived moiety (Fig. 2, lower portion). These reactions are most completely defined with housefly enzyme systems but they are also applicable to enzyme systems from other species and probably to living insects. The ethylphenyl moiety is hydroxylated by the housefly microsome-NADPH system yielding the α-hydroxyethyl derivatives which are then oxidized to the aceto compounds. Fly microsomes carry out more extensive β-hydroxylation than rat or mouse microsomes (4) but attack at this site is less favored than α-hydroxylation. The geranyl-derived moiety is metabolized to give both acyclic and cyclic derivatives, the latter probably being formed via epoxy-diol intermediates. Each of these metabolic reactions, with the exception of epoxidation of the ethyl-diene, appears to be a detoxification mechanism based on the results from assaying the various compounds with *T. molitor* pupae (5).

The ethyl-diene is degraded much more rapidly in the housefly microsome–NADPH system than the ethyl-epoxide, -diol, or -diepoxide. Possibly the oxidized 6,7-position in the latter three compounds protects them from the type of attack involved in rapid metabolism of the ethyl-diene. The major pathway for oxidation of the ethyl-diene leads to an olefinic carboxylic acid via primary alcohol and aldehyde intermediates. The most likely sites of oxidation in formation of these metabolites are either the 7-*cis*-methyl or 3-methyl group.

Aliphatic epoxides are detected as metabolites of dienes only when epoxide formation is rapid relative to epoxide cleavage, a situation which exists in the housefly microsome–NADPH system in converting the ethyl-diene to the ethyl-epoxide and this in turn to the ethyl-diepoxide. The demonstration of NADPH-dependent 6,7-epoxidation by microsomal enzymes, although minor in amount, is of interest relative to the morphogenetic activity of dienes and the biosynthesis of the natural juvenile hormones. A strong inhibitor of this reaction might lead to an "anti-juvenile hormone." Enzymatic 2,3-epoxidation is an intermediate step in converting the ethyl-epoxide and the -diol to cyclic products, but not in formation of the 2,3,6,7-tetraol.

Epoxide hydratases acting on the ethyl-epoxide appear in both the membrane-bound and soluble fractions of mouse and

rat liver (4) but they are only in the membrane fraction of houseflies. They are resistant to inhibition by cyclohexene oxide and 1,1,1-trichloropropene 2,3-oxide even though these compounds inhibit certain mammalian epoxide hydratases acting on other substrates (14, 15). These two candidate epoxide hydratase inhibitors even at high levels do not increase the morphogenetic activity of the ethyl-epoxide or its 4-chlorophenoxy analog on *T. molitor* (5), despite the fact that epoxide hydration is the major *in vivo* reaction of the ethyl-epoxide in this species. The most potent inhibitor for housefly epoxide hydratases found in a the present study, the 7-bromo-6-hydroxy derivative of the ethyl-diene, still lacks the desired activity; however, such inhibition may be related in part to the synergism found in *T. molitor* assays between juvenoids containing tertiary chlorines and trisubstituted epoxide juvenoids (16).

Several cyclic metabolites, the major ones being the ethyl-thf-diols, are formed from the ethyl-epoxide, -diol, and -diepoxide. These cyclization reactions do not appear to be stereospecific since approximately equal amounts of the diastereoisomers of the products are formed. The cyclization of epoxy-diol intermediates occurs rapidly so the ethyl-diepoxide is not converted to detectable amounts of the ethyl-tetraol on incubation with rat or mouse (4) or housefly microsomes. A tetraol metabolite is reported for the C_{18}-cecropia hormone (17) with the implication that it forms from the corresponding diepoxide; however, the present studies on the ethyl-epoxide and -diepoxide in enzyme systems and related studies with chemical systems (5, 13) indicate that the metabolite designated as a tetraol may have been a cyclic derivative.

It is difficult to generalize the limiting reaction in insect metabolism of juvenoids, even for a single compound such as the ethyl-epoxide. Although the results are dependent on the assay conditions, it appears that epoxide hydration is more important than oxidative pathways in *T. molitor* whereas the contrary is true with *P. americana* and *M. domestica* (particularly with resistant fly strains of high oxidase activity suggesting that cross resistance is likely to occur). Therefore, the chemical modification of the juvenoid or the optimal synergist needed for improved stability and activity will be dependent on the insect species or strain and the relative activity of the enzymes involved in various pathways of juvenoid inactivation.

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