Selectivity of Carbaryl–2,3-Methylenedioxynaphthalene Combination

Metabolism of the Synergist in Two Strains of Houseflies and in Mice

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The metabolism of H^3- and C^{14}-labeled 2,3-methylenedioxynaphthalene has been studied in susceptible and resistant houseflies and in white mice. The major metabolites in both the insects and the mammals were identified. Only minor qualitative differences, primarily in the nature of the conjugating sugars, were observed in the metabolic pattern of this compound between houseflies and mice. However, enormous quantitative differences in the oxidative degradation of the synergist account for its selectivity when used in combination with carbaryl. Efficient oxidative degradation of 2,3-methylenedioxynaphthalene in mice caused its complete deactivation in 12 hours. Conversely, the compound was extremely stable in flies and was scarcely degraded in 24 hours.

Hundreds of compounds containing the 1,2-methylenedioxyphenyl (1,3-benzodioxole) moiety have been extensively studied as synergists for the pyrethrins and carbamate insecticides, as reviewed by Beroza and Barthel (1957), Hewlett (1960), and Metcalf (1967). Recently 2,3-methylenedioxynaphthalene (naphtho-[2,3-*d*]-1,3-dioxole), synthesized by Julius Hyman, was shown to be among the most active synergists for carbaryl (1-naphthyl *N*-methylcarbamate) (Metcalf *et al.*, 1966). When this synergist was applied topically to the housefly at 5 to 1 with carbaryl, the LD_{50} was 5 μg. per gram (as carbaryl), yet this combination produced no mortalities or symptoms of discomfort in white mice dosed orally with 750 mg. per kg. of carbaryl together with 3750 mg. per kg. of 2,3-methylenedioxynaphthalene (Sacher, 1967). This synergist-insecticide combination, therefore, represents an outstanding example of insecticide selectivity. The potential usefulness of this combination and the relatively simple chemical structures of both carbaryl and 2,3-methylenedioxynaphthalene make this combination an attractive subject for the present study of the biochemical factors involved in insecticide selectivity.

MATERIALS AND METHODS

Chemicals. The tritiating technique of Yavorsky and Gorin (1962) was used to label 2,3-dihydroxynapthalene by direct contact with tritiating reagent $H_2TPO_4 \cdot BF_3$, as described by Hilton and O'Brien (1964). Approximately 500 mg. of 2,3-dihydroxynaphthalene was tritiated and used without further purification for the coupling reaction with diiodomethane. After several recrystallizations from hexane, a pure sample of 2,3-methylenedioxynaphthalene, m.p. 96° C., was obtained in 43% yield with a specific activity of 6000 c.p.m. per μg. (0.5 mc. per mmole) and a radiochemical purity of 99.7%.

2,3-Methylene-C^{14}-dioxynaphthalene was synthesized from the corresponding dihydroxynaphthalene and C^{14}-labeled diiodomethane. Iodoform C^{14} (New England Chemical Co., 1 mc. per mmole) was reduced with sodium arsenite at 60° C. over 4 hours (Kuwatsuka and Casida, 1965). The resulting diiodomethane was washed with water, taken up in acetone, dried over sodium sulfate, and kept at 0° C. The acetone solution was subsequently added dropwise to a reaction vessel containing 50% excess of 2,3-dihydroxynaphthalene and four-fold excess of K_2CO_3 in *N,N*-dimethylformamide. After refluxing for 2 hours, the product was crystallized from ether at −70° C. (dry ice–acetone), and when recrystallized from hexane was obtained in 32% overall yield, m.p. 96–98° C., with a specific activity of 4000 c.p.m. per μg. (0.31 mc. per mmole). The compound had a radiochemical purity of 99.2%, as checked by TLC with three different solvent systems.

The R_f values for 1-hydroxy-2,3-methylenedioxynaphthalene in TLC with four solvent systems, as well as its color reactions with chromogenic agents, are summarized in Table I.

Houseflies. Most of the metabolic work was carried out on the S_{NAIDM} strain of housefly, *Musca domestica* L. The resistant strains (R_{MIP}, $R_{ronnel\ II}$, and R_{SC}) were used for some specific studies. Typically, 200 2-day-old female flies were treated by the topical application of a 1-μl. drop of w./v. acetone solution of the radiotracer to the pronotum. The flies were kept in 500-ml. glass jars with wide tops covered with cheesecloth, and placed in a 50° C. constant-temperature room for appropriate periods, ranging from 3 to 24 hours. Food (sugar wicks) was provided in those cases in which the experiment lasted longer than 12 hours. The flies were then sacrificed by freezing on dry ice, and subsequently analyzed for penetration and metabolism. Since 2,3-methylenedioxynaphthalene is not toxic to flies, the dosages ranged between 0.5 and 10 μg. per fly (2.5 to 500 μg. per gram), according to specific requirements.

In some experiments, the synergist was introduced to the flies by mixing it with their food. Flies were starved for 4 hours and subsequently fed on a 5% synergist in sugar diet for periods of between 2 and 48 hours. The treated flies were later transferred to untreated sugar, provided with water, and used in metabolic studies or for topical application of insecticides.

Four major fractions were assayed in all the metabolic studies performed: The "external wash" was determined by thoroughly washing the flies with 25-ml. portions of acetone. The "internal extract" was obtained by placing the flies in a Waring blender with 75 ml. of CH_3CN,

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Table I. Chromatographic Properties of 2,3-Methylenedioxynaphthalene and Two of Its Metabolites

Naphthalene Derivative	Color Reactions: Diazonium fluoborate	Color Reactions: Chromotropic acid	R_f Values: A [a]	B [b]	C [c]	D [d]
2,3-Methylenedioxy	No reaction	Purple	0.85	0.72	0.82	0.87
2,3-Dihydroxy	Pink	No reaction	0.59	0.19	0.78	0.83
1-Hydroxy-2,3-methylenedioxy	Orange-pink	Dark purple	0.69	0.26	0.85	0.80

[a] System *A*—ether-benzene 3 to 1. [b] System *B*—benzene. [c] System *C*—hexane-benzene-ethanol 1:1:2. [d] System *D*—butanol-ethanol-water 10:2:3.

homogenizing for 1 minute and filtering through a Buchner funnel. The residue was rehomogenized in 50 ml. of ethanol, and finally with 50 ml. of water. The "holding jars" were washed with 25-ml. portions of CH_3CN, hot methanol, and water. For the determination of the "$C^{14}O_2$ expired" fraction, a metabolism chamber, similar to that described by Metcalf *et al.* (1967), was utilized. The CO_2 expired by the treated flies was passed through a trap provided with a bottom stopcock, and contained a 20-ml. mixture of ethanolamine and 2-methoxyethanol at a 1 to 2 ratio. Aliquots were drawn through the stopcock at ½-, 1-, 2-, 4-, 8-, 16-, and 24-hour intervals and counted in a liquid scintillation counter. The counting solution consisted of a 10-ml. solution of toluene and 2-methoxyethanol (2 to 1 v./v.) containing 0.55% 2,3-diphenyloxazole (PPO) (Jeffry and Alvarez, 1961).

The total recoveries ("expired," "external," "excreted," and "internal") varied between 70 and 85%, although recoveries above 90% were achieved whenever the bodies were combusted (Metcalf *et al.*, 1967) and the "internal" fraction was determined as $C^{14}O_2$.

Analysis of Metabolites. Two column chromatographic systems were utilized in this study. For chromatography on silica gel (Florisil), the procedure described by Dorough and Casida (1964) was followed, with some modification in the eluant system used. The chromatogram was typically developed with the following solvent sequence: 200 ml. of hexane, 200 ml. of 3 to 1 hexane-ether, 200 ml. of 1 to 3 hexane-ether, 100 ml. of ether, 100 ml. of chloroform, 100 ml. of ethyl acetate, 200 ml. of methanol, and 200 ml. of water. Individual fractions of 22 ml. were collected. Measurements of radioactivity were made by liquid scintillation counting of 0.5-ml. aliquots using 10-ml. volumes of standard mixtures of PPO, POPOP, and toluene for nonpolar solvents, or of PPO, POPOP, and naphthalene in dioxane for polar solvents (Packard Instruments Tech. Bull., 1966).

Separation of metabolites was also carried out by ion exchange chromatography in a manner similar to that described by Knaak *et al.* (1965). The extracted metabolites were applied to the top of a 2- × 30-cm. column of DEAE-cellulose, and eluted by using two linear gradients: 0.01*M* tris-HCl buffer, pH 7.5, to 0.05*M* tris-HCl buffer, pH 7.5, with 300 ml. of each eluant, and 0.05*M* tris-HCl buffer, pH 7.5, to 0.1*M* tris-HCl buffer, pH 7.5, with 300 ml. of each eluant. One hundred and fifty 8-ml. fractions were collected and 0.5-ml. aliquots were analyzed for radioactivity by liquid scintillation counting.

Detection of methylenedioxynaphthalene derivatives on TLC was done by iodine vapor, spray with chromotropic acid (Beroza, 1963), spray with diazonium fluoborate (Miskus *et al.*, 1961) for hydroxylated metabolites, and ultraviolet light. Radioautography was performed using Polaroid 3000 ASA film, which was placed directly on the plates for 24 to 72 hours.

White Mice. Male Swiss mice were treated orally with radiotracers in 0.1 ml. of olive oil and placed in individual metabolism cages for the separate collection of urine and feces (Roth *et al.*, 1948). Animals were provided with food and water, and kept at 80° F. for 48 hours. Urine samples were collected at 12-hour intervals. In the experiments where respiratory products were collected, a modified three-neck flask having a stopcock in the bottom was utilized. The procedures employed for the analysis of metabolites were those described in the studies with the housefly.

RESULTS AND DISCUSSION

Metabolism of 2,3-Methylenedioxynaphthalene in Houseflies. Stability of the Compound in vivo. A preliminary metabolic study with H^3-labeled 2,3-methylenedioxynaphthalene indicated that 6 hours after topical treatment, the majority of this compound had penetrated into the flies, but more than 85% of it was unchanged. This suggests that the high synergistic activity of this compound is a result of its exceptional in vivo stability.

In another experiment, S_{NAIDM} flies were fed for 24 hours on a sugar diet containing 5% 2,3-methylenedioxynaphthalene and subsequently treated with carbaryl alone. Compared to LD_{50} of >500 μg. per gram for unsynergized carbaryl, the LD_{50} values recorded at 1, 2, 3, and 6 days after feeding were 11.5, 10.5, 9.0, and 7.0 μg. per gram, respectively. It is quite obvious that the LD_{50} for carbaryl against the flies did not change appreciably for as long as 6 days after the administration of the synergist and were quite similar to the values obtained by joint topical application (LD_{50} = 5.0 μg. per gram). The slow decrease in LD_{50} value over the 6-day period is due to the increased susceptibility of houseflies with age (El-Aziz *et al.*, 1968).

Another way to measure the stability of this compound was to follow the rate of its in vivo conversion to water-soluble metabolites. The results of this investigation, with four different strains of houseflies, are presented in Table II. In all cases, flies were treated topically with synergist at 1 μg. per fly and kept at a constant temperature for various lengths of time, until they were sacrificed, washed, extracted, and the internal water-soluble and fecal radioactivity counted and compared. There are large differences between the four strains in their ability to detoxify 2,3-methylenedioxy-

Table II. Rate of Formation of Water-Soluble Metabolites in Four Strains of Houseflies Treated Topically with H^3-Labeled 2,3-Methylenedioxynaphthalene

	Water-Soluble Metabolites, % of Applied Dose			
	Time, Hours			
Strain	1.5	3.5	6.5	20
R_{MIP}	13.5	28.0	35.0	43.0
$R_{ronnel\ II}$	6.0	13.0	23.0	35.0
R_{SC}	1.0	5.5	8.3	12.0
S_{NAIDM}	0	4.0	6.7	9.5

naphthalene. The organophosphorus-resistant R_{SC} strain behaved similarly to the susceptible S_{NAIDM} strain. Thus, it appears that resistance to OP compounds in the R_{SC} strain involve primarily hydrolytic pathways, and the flies were not selected for oxidative reactions which are responsible for the degradation of aromatic compounds, including 2,3-methylenedioxynaphthalene. The carbamate-resistant R_{MIP} strain is more efficient in detoxifying the synergist, as was expected from the oxidative pathways of carbamate detoxification (Leeling and Casida, 1966). The $R_{ronnel\ II}$ strain has been shown by Metcalf *et al.* (1967) to be resistant not only to carbamates alone, but also to carbamate–piperonyl butoxide combinations. Apparently the high selection pressure with a variety of insecticides induced the selection and development of active microsomal enzymes, and as a result, the flies are able to detoxify aromatic compounds rapidly.

These results are consistent with the finding (Metcalf *et al.*, 1967) that this compound will synergize carbaryl when applied jointly at a ratio of 1 to 1000 (2,3-methylenedioxynaphthalene to carbaryl), and can be explained only by the unusual resistance of the synergist to detoxication.

Cleavage of the Methylenedioxynaphthyl Ring and Its Metabolism to CO_2. Casida *et al.* (1966) studied the scission of the methylene-C^{14}-dioxyphenyl moiety in six compounds and showed that, although only 6 to 20% of the applied dosage was recovered from the housefly as $C^{14}O_2$ within 24 hours, as much as 76% of $C^{14}O_2$ was evolved by the mouse during the same period. They proposed an oxidative pathway for the ring cleavage, based on identification of C^{14}-formate and the *o*-dihydroxyphenyl derivatives. Since the cleavage of the methylenedioxynaphthyl ring brings about the deactivation of the synergist (Wilkinson *et al.*, 1966), it was of interest to measure the rate and extent of this process in resistant and susceptible strains of the housefly and to compare them with mice.

Table III presents the results of the absorption, metabolism, and excretion of 2,3-methylene-C^{14}-dioxynaphthalene in S- and R- strains of houseflies over a 24-hour period. There is no significant difference in absorption with the strains evaluated, suggesting that the resistance to carbamate-synergist combination found in the $R_{ronnel\ II}$ strain is not the result of decreased cuticular absorption. Metcalf *et al.* (1967) found similar penetration patterns in their study on metabolism of carbamates in a variety of resistant and susceptible housefly strains.

The rate of excretion of 2,3-methylenedioxynaphthalene is extremely slow, reaching 24-hour totals of 4.7

Table III. Absorption, Metabolism, and Excretion of 2,3-Methylene-C^{14}-dioxynaphthalene by Susceptible and Resistant Houseflies

Fly Strain	Applied Dose		% of Applied Dose, 24 Hours		
	C.p.m./fly	μg./fly	Absorbed	$C^{14}O_2$	Excreta
S_{NAIDM}	4750	~1.0	91.6	17.2	4.6
S_{NAIDM}	4790	~1.0	94.0	17.8	4.8
$R_{ronnel\ II}$	5061	~1.0	94.0	27.0	8.3
$R_{ronnel\ II}$	4930	~1.0	94.0	26.7	8.3
$R_{ronnel\ II}$	8970	~2.0	90.1	16.8	5.1
$R_{ronnel\ II}$	9078	~2.0	89.4	16.2	5.3
Pretreated 1.5 hour with 50 μg. piperonyl butoxide					
S_{NAIDM}	4807	~1.0	92.3	10.2	3.2

and 8.3% of applied dose for S- and R-flies, respectively. These figures are substantially lower than those reported for the 24-hour fecal excretion of other groups of pesticides, which range between 25 and 50% for carbamates (Metcalf *et al.*, 1967) and more than 50% for methyl parathion and Sumithion (Hollingworth *et al.*, 1967).

The R_{ronnel} strain is much more efficient in attacking the dioxole ring, yielding about 50% more $C^{14}O_2$ than the S_{NAIDM} flies.

A striking difference was found when the R_{ronnel} strain was treated at two dosage levels of 1 and 2 μg. per fly. The lower dose produced relatively larger amounts of $C^{14}O_2$. The amount of $C^{14}O_2$ evolved at the two dosages is surprisingly similar, being on the average 1340 and 1490 c.p.m. per fly, respectively. The most probable explanation of the phenomenon is that the synergist is a good inhibitor of the microsomal enzyme which participates in the oxidative cleavage of the methylenedioxy ring; thus, the compound may synergize itself. This process becomes more pronounced at high levels of synergist. In view of the fact that this compound was shown to have such marked in vivo stability, it is suggested that "autosynergism" is likely to occur.

Figure 1 shows the linear rate of production of $C^{14}O_2$ from 2,3-methylene-C^{14}-dioxynaphthalene by S_{NAIDM} and $R_{ronnel\ II}$ female houseflies. These rates are consistent with the 24-hour "total" $C^{14}O_2$ data shown in Table III.

Methylenedioxyphenyl synergists such as piperonyl butoxide have a profound effect in retarding the detoxification of carbamate insecticides (Metcalf *et al.*, 1967) and of other foreign compounds such as naphthalene (Philleo *et al.*, 1965) in both susceptible and

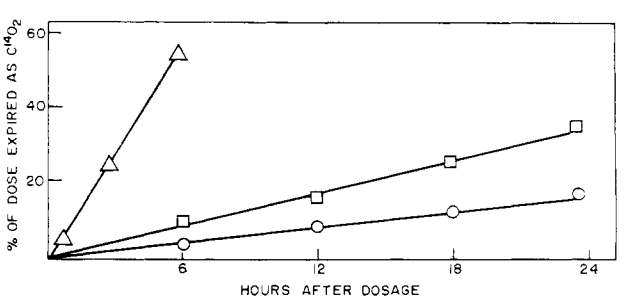

Figure 1. Rate of $C^{14}O_2$ evolution from susceptible and resistant flies and mice dosed with 2,3-methylene-C^{14}-dioxynaphthalene

△—White mice □—Resistant flies ○—Susceptible flies

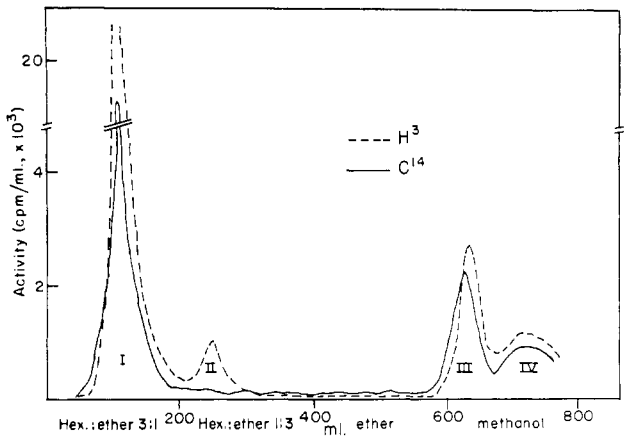

Figure 2. Column chromatography of H^3 (ring-labeled) and C^{14} (methylene-labeled) 2,3-methylenedioxynaphthalene and its metabolites extracted from S_{NAIDM} houseflies 24 hours after application with 5 μg. per fly

resistant houseflies. This effect was studied by pretreating the houseflies with 50 μg. of piperonyl butoxide applied to the ventral abdomen so as not to influence the subsequent application of C^{14}-labeled methylenedioxynaphthalene, 1.5 hours later, on the dorsal prothorax. As expected, the output of $C^{14}O_2$ over the 24-hour period was sharply reduced to 57% of the amount produced without piperonyl butoxide treatment. The fecal excretion was also substantially lowered, totaling 3.2%, as opposed to 4.8% of the applied dose found in the control experiment. The results obtained are in good agreement with previous studies on the effects of synergists on detoxification of foreign compounds in houseflies (Metcalf *et al.*, 1967).

Identification of Metabolites. The separation by column chromatography of H^3- and C^{14}-labeled 2,3-methylenedioxynaphthalene and metabolites recovered from topically treated houseflies 24 hours after dosage is presented in Figure 2. Most of the parent compound was recovered unchanged and is represented by peak number I (86.6% of the recovered radioactivity). This metabolite was concentrated and cochromatographed on TLC with an authentic sample of 2,3-methylenedioxynaphthalene. R_f values, color reaction with chromotropic acid, and infrared spectrum showed that this substance was the parent compound. Mixing the compound extracted from the column with carbaryl at a 5-to-1 ratio gave an LD_{50} to flies of 5.3 μg. per gram, in agreement with the value of 5.0 μg. per gram (Metcalf *et al.*, 1966).

Table I summarizes R_f values for some of the possible metabolites of 2,3-methylenedioxynaphthalene with four different solvent systems and, in addition, the colors produced by these compounds with different chromogenic sprays.

Since the rate of cleavage of the methylenedioxynaphthyl ring was extremely slow and did not exceed 10% in 12 hours, close similarities were expected in metabolic studies performed with C^{14}- and H^3-labeled methylenedioxynaphthalene. Any degradation step involving ring hydroxylation without breakage of the methylenedioxy moiety should appear in both experiments, regardless of which part of the molecule contained the radioactive isotope. Only the H^3-labeled material would indicate those metabolites arising from the dihydroxynaphthalene moiety, after cleavage of the five-membered ring and liberation of CO_2.

Figure 2 shows the close similarity of two metabolic studies, one with C^{14}- and the other with H^3-labeled synergist. Column chromatography of the nonpolar metabolites from houseflies gave almost identical results with both labels. The only difference appears to be in peak number II, which represents 1.2% of the recovered nonpolar radioactivity and is absent from the C^{14} run.

Both H^3- and C^{14}-labeled synergist gave two peaks in methanol (Figure 2, peaks III and IV). When III was chromatographed on TLC in butanol–ethanol–water 10:2:3, it showed one metabolite (R_f = 0.65) labeled with both C^{14} and H^3, and an additional compound (R_f = 0.46) which was labeled only with H^3. When these metabolites were subjected to acid hydrolysis (1*N* HCl at 100° C. for 15 minutes and subsequent ether extraction) and then analyzed on TLC with the same solvent system, the predominant compound had an R_f value of 0.80, identical with 1-hydroxy-2,3-methylenedioxynaphthalene. In addition, this metabolite gave similar reactions with chromotropic acid and diazonium fluoborate as the reference compound (see Table I). These findings suggest that the major part of the methanol peak III is a 1-hydroxy-2,3-methylenedioxynaphthalene conjugate. The other compound, which represents about 30% of the radioactivity of the tritium-labeled methanol peak, is a conjugate of 2,3-dihydroxynaphthalene.

Peak IV was also chromatographed in the butanol-ethanol-water system, and gave an R_f value of 0.33 before hydrolysis. This metabolite was labeled with both isotopes and reacted positively with chromotropic acid, indicating that the methylenedioxynaphthyl ring was still intact. To determine the nature of the conjugating agent, the methanol extract containing IV was evaporated under reduced pressure, and the residue was redissolved in a small amount of water. One milligram of β-glucosidase (Sigma Chem. 5 units per mg.) in 1 ml. of phosphate buffer (1/15*M*, pH 6.8) was added and the mixture was incubated for 30 minutes at 37° C. The aqueous solution was subsequently extracted with an equal amount of chloroform and the two fractions were counted to determine the distribution of radioactivity. This procedure changed the partition coefficient from 125 to 1.8, indicating that the product of enzymic hydrolysis is less polar than the initial substrate. Since the enzyme is highly specific for β-glucosides, it was concluded that the hydroxyl group of metabolite IV was conjugated with β-glucose.

Ion Exchange Chromatography of Aqueous Metabolites. To compare the metabolism of this compound by the mouse with its metabolism by insects, the water-soluble metabolites from houseflies and mouse urine were chromatographed on an ion-exchange resin. Figure 3 shows the results of chromatography on diethylaminoethyl-cellulose (DEAE-cellulose) of the aqueous C^{14}- and H^3-labeled metabolites extracted from S_{NAIDM} houseflies 18 hours after topical application. The water-soluble metabolites represent only 4 and 21.2% of the recovered C^{14} and H^3, respectively. The remaining radioactivity, which was partitioned into chloroform,

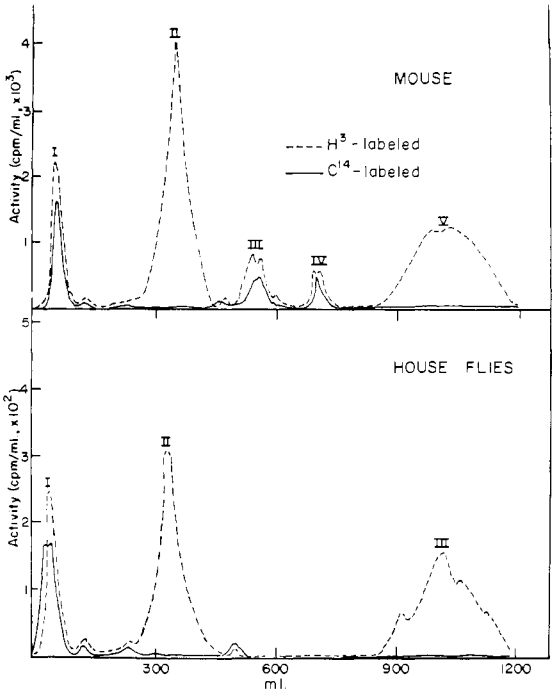

Figure 3. Ion exchange chromatography of water-soluble metabolites of 2,3-methylenedioxynaphthalene from mouse and houseflies

represents about 75% of the counts (some 4% was present in the jar wash and about 2% was recovered from external wash).

In contrast to the three major peaks (I, II, III) found with the H^3-labeled synergist, only one peak (I) was eluted from the ion exchange column in the case of the C^{14}-labeled synergist. It is apparent that peaks II and III (Figure 3) represent conjugated metabolites of the 2,3-naphthalenediol liberated by metabolic degradation of the methylenedioxy moiety.

Metabolite I was hydrolyzed for 30 minutes in 1.0*N* HCl at 100° C. and then partitioned between water and chloroform. About 95% of the radioactivity in the hydrolyzate was extracted into the chloroform. The hydrolyzed compound was tentatively identified as 1-hydroxy-2,3-methylenedioxynaphthalene by cochromatography in TLC with two solvent systems. The conjugate was tentatively identified as a glucoside because it was hydrolyzed by a standard procedure for chemical hydrolysis of glucosidic bonds and by β-glucosidase, and because of similarities in chromatographic behavior to some related standard compounds recently studied by Knaak *et al.* (1967).

Metabolites II and III from the tritium-labeled experiment were eluted from the DEAE-cellulose column in the similar positions to metabolites G (1-naphthyl glucuronide) and I (1-naphthyl sulfate) found by Knaak *et al.* (1967) in their study on carbaryl metabolism. It is, therefore, suggested that metabolites II and III are 2-hydroxy-3-naphthyl glucosiduronic acid and 2-hydroxy-3-naphthyl sulfate, respectively. The latter is very similar in physical properties and chromatographic behavior to 2-hydroxy-1-naphthyl sulfate isolated from urine of rabbits by Boyland and Sims (1957).

Figure 3 indicates the presence of minor metabolites (totaling less than 1% of recovered radioactivity) from both C^{14}- and H^3-labeled compounds. These are probably 2,3-methylenedioxy-7,8-dihydro-7,8-dihydroxynaphthalene derivatives. Dihydrodihydroxy derivatives of naphthalene were isolated by Boyland *et al.* (1964) from rabbits and rats, and by Terriere *et al.* (1961) from houseflies treated with naphthalene and 1-naphthol.

A scheme summarizing the metabolic pathways of 2,3-methylenedioxynaphthalene in resistant and susceptible houseflies and in mice, with percentages of conversion of parent compound to the various metabolites, is found in Figure 4. It seems that resistant flies are capable of carrying out the detoxification reactions (hydroxylation, CO_2 production, and excretion) at rates which are twice as fast as that of susceptible flies. These findings are in agreement with the data presented by Schonbrod *et al.* (1965) on the role of hydroxylation as a factor in resistance in houseflies. This "over-all" two-fold increase in detoxification rate is very important and may account for the larger differences in resistance (or selectivity) found between the different strains. Thus, the total amounts of synergist left in the insects will be 75 *vs.* 55% of applied dose (after 24 hours) for susceptible and resistant strains, respectively. For a given rate of penetration of the compound, a two-fold increase in the rate of a major detoxification process may set up the over-all balance in favor of survival rather than death. These data explain the finding of Metcalf *et al.* (1967) concerning the resistance of the R_{ronnel} strain to a combination of carbamate with synergist.

Metabolism of 2,3-Methylenedioxynaphthalene in White Mice. Figures 1, 3, 4, and 5 summarize the results of the investigation on the metabolism of the 2,3-methylenedioxynaphthalene in mice. The metabolic pathways of the synergist in mammals are compared with those previously found in houseflies.

In vivo Stability of 2,3-Methylenedioxynaphthalene and the Rates of Excretion of Metabolites. In contrast to the marked in vivo stability of the synergist in houseflies, this compound is rapidly metabolized to inactive derivatives in the mammalian system. Compared with the high recoveries (up to 70%) of the parent compound from flies 24 hours after application, no trace of the parent 2,3-methylenedioxynaphthalene could be detected in the mouse after the same period of time.

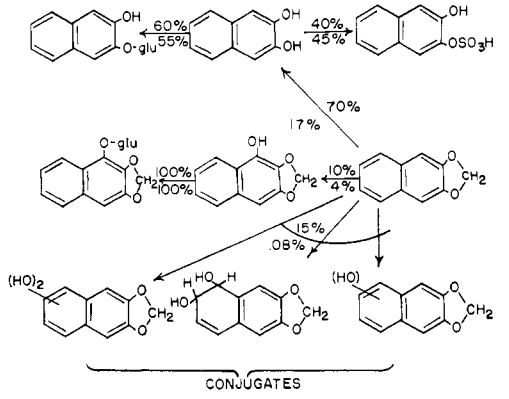

Figure 4. Pathways of 2,3-methylenedioxynaphthalene metabolism in houseflies and mice Per cent conversion of metabolites in mice and houseflies are indicated above and below the arrows, respectively. Glu refers to glucuronides and glucosides in mice and houseflies, respectively

Table IV. Chemical Hydroxylation of 2,3-Methylenedioxynaphthalene in the Udenfriend System

Compound	R_f[a]	C.P.M.[b]	Reaction					Identity[h]
			UV[c]	CA[d]	DFB[e]	QCI[f]	Fe[g]	
I	0.00	100	Light blue	—	Light pink	—	—	
II	0.20	300	Light green	Violet	Pink	Gray	Dark green	5-OH-2,3-MDON
III	0.58	573	Light green	Brown	Pink	Dark violet	Dark green	di-OH-2,3-MDON
IV	0.62	153	Light blue	Violet	—	—	Gray	5,6-di-H-5,6-di-OH-2,3-MDON
V	0.67	90	—	Violet (dark)	Pink-orange	Gray-green	Black	1-OH-2,3-MDON
VI	0.73	160	—	Violet	Pink	Blue	Green	6-OH-2,3-MDON
VII	0.82	9570	Blue	Violet	—	—	—	2,3-MDON

[a] R_f value of metabolite on TLC (silica gel); solvent, ether-benzene 3 to 1. [b] Relative radioactivity recovered in hydroxylated derivatives. [c] Fluorescence under ultraviolet light. [d] Color reaction with chromatropic acid in H_2SO_4. [e] Color reaction with *p*-nitrobenzenediazonium fluoborate + base. [f] Color reaction with 2,6-dibromoquinone-4-chloroimide. [g] Color reaction with ferric chloride. [h] Tentative identification as derivatives of 2,3-methylenedioxynaphthalene (2,3-MDON), as described in text.

Figure 5 shows the rate of appearance of 2,3-methylenedioxynaphthyl metabolites in the urine. No appreciable differences were found in the rate and extent of excretion of metabolites at the three dosage levels (4, 40, and 200 mg. per kg.). Recoveries did not reach 100% for the H^3-labeled compound, and even after 72 hours about 10% of the dose could not be accounted for. This might result from hydrogen exchange between the aqueous medium and the aromatic hydrogens. The fact that even at the highest dose of 200 mg. per kg. the excretion of metabolites was relatively complete suggests that no saturation of the detoxification and excretion mechanisms occurred.

Metabolism of 2,3-Methylene-C^{14}-dioxynaphthalene to $C^{14}O_2$. Casida *et al.* (1966) showed that extensive scission of methylene-C^{14}-dioxyphenyl moiety occurs in mice dosed with a variety of methylenedioxyphenyl synergists. The rate at which 2,3-methylenedioxynaphthalene is metabolized to CO_2 by mice is presented in Figure 1. $C^{14}O_2$ production is extremely rapid, reaching 50% of the applied dose (40 mg. per kg.) 5.5 hours after treatment and 80% in 12 hours. The rate of CO_2 production decreased slightly with the increase in dosage level to 200 mg. per kg., but at 12 hours after treatment the values were similar. These extremely rapid rates of degradation explain the inactivity of 2,3-methylenedioxynaphthalene as a carbamate synergist in mammals. Apparently the synergist is detoxified so rapidly and completely that it is not able to reach the site of action in sufficient concentrations to cause an appreciable effect.

Scission of the methylenedioxynaphthyl ring is the major pathway by which mice detoxify methylenedioxynaphthalene. In addition, other processes simultaneously deactivate and aid in eliminating the compound from the body. As a result, more than 70% of the compound is deactivated as early as 5 hours after treatment, and the whole dose is virtually eliminated 12 hours after dosage.

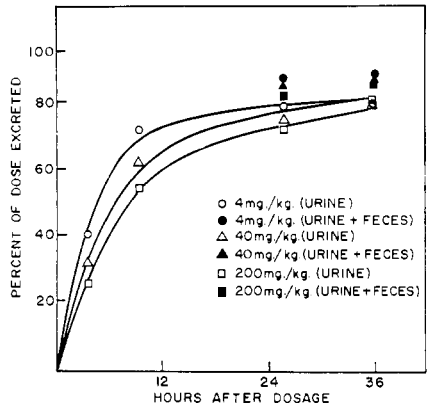

Figure 5. Rate of excretion of radioactivity in the urine and feces of mice dosed orally with 4, 40, and 200 mg. per kg. of 2,3-methylenedioxynaphthalene

Separation and Identification of the Metabolites. As much as 10% of the applied dose was found in the mouse feces. The radioactivity was extracted and analyzed on TLC, before and after acid hydrolysis. Four major compounds were identified in the feces: a small amount of parent compound (5%), free (10%), and conjugated (30%) 2,3-dihydroxynaphthalene, and conjugated 1 - hydroxy - 2,3 - methylenedioxynaphthalene (43%). This is the only place throughout this study in which a free nonconjugated hydroxyl derivative was recovered.

Separation of the polar metabolites from the urine was achieved by ion exchange chromatography. Figure 3 presents a summary of such study with C^{14}- and H^3-labeled synergist on a DEAE-cellulose column with two gradients of tris-HCl buffer, as indicated.

The C^{14}-labeled compounds represent less than 10% of the applied dose, since most of the radioactivity was lost as expired $C^{14}O_2$. On the other hand, up to 80% of the radioactivity from the tritium-labeled synergist was recovered in the urine sample.

Five major peaks (designated as I to V) labeled with tritium and three metabolites labeled with C^{14} (corresponding to I, III, and IV) were eluted from the column. Metabolites II and V represent nearly 70% of the recovered radioactivity, and were tentatively identified as 2-hydroxy-3-naphthyl glucuronic acid and 2-hydroxy-3-naphthyl sulfate. Both metabolites yield 2,3-dihydroxynaphthalene on acid hydrolysis, and their physical and chromatographic properties are similar to known reference compounds. Metabolite II moved toward the cathode, whereas V moved toward the anode upon ionophoresis in 0.05*N* NaOH (on Whatman No. 1 paper and 10 v. per cm. for 2 hours). These results are in agreement with those obtained by Darby *et al.* (1966) for ionophoresis of some naphthol conjugates.

Metabolite I, which is similar to the one recovered from flies treated with either C^{14}- or H^3-labeled synergist, is a conjugate of 1-hydroxy-2,3-methylenedioxynaphthalene. However, this metabolite is probably a glucuronide rather than a glucoside.

The identities of metabolites III and IV, which comprise about 15% of the total recovered radioactivity, and have both C^{14} and H^3 labels, are not known. After

acid hydrolysis, these compounds give a positive reaction with diazonium fluoborate, suggesting a conjugated hydroxyl group. The hydrolyzed compounds had identical R_f values and similar color reactions to compounds produced by chemical hydroxylation utilizing the Udenfriend model system for biological oxidation containing substrate, Fe^{2+}, ascorbate, EDTA, and oxygen (Udenfriend *et al.*, 1954).

Hydroxylation of 2,3-Methylenedioxynaphthalene by the Ascorbic Acid Model Hydroxylating System. The products of hydroxylation of some aromatic hydrocarbons in the rat liver microsomal enzyme system and the ascorbic acid–Fe^{2+}–oxygen model system are similar (Boyland *et al.*, 1964). Work in this laboratory has shown that this in vitro system mimics biological oxidations for a wide variety of insecticides and its employment for guidance in metabolic studies has proved very helpful.

The results of the oxidation of H^3-labeled 2,3-methylenedioxynaphthalene with the Udenfriend system are summarized in Table IV. Six different compounds were recovered as products of this hydroxylating system, even though less than 1.5% of the parent compound was oxidized. With the exception of compound I, all others gave a positive reaction with the chromotropic acid, indicating that the methylenedioxynaphthyl ring was still intact. Four compounds (II, III, V, VI) gave a positive reaction with diazonium fluoborate, suggesting the presence of one or more hydroxyl groups. Compound V was identified as 1-hydroxy-2,3-methylenedioxynaphthalene because of identical color reactions and R_f values with a known sample of this compound. All other compounds were only tentatively identified, as indicated in Table IV, by comparing their characteristic color reactions with those of naphthalene and naphthol derivatives used as reference compounds in Reio's (1958) study on the separation and identification of 450 phenolic derivatives.

Selectivity of Carbaryl 2,3-methylenedioxynaphthalene Combinations. Carbaryl-2,3-methylenedioxynaphthalene combinations are extremely active against houseflies (LD_{50} = 5.0 μg. per gram as carbaryl), yet the same mixture is inactive against white mice at doses higher than 750 mg. per kg. Since carbaryl alone is of very low toxicity to both organisms, basis for selectivity must relate to the behavior of the synergist. A multiplicity of factors may account for the selective action of a certain compound. Among these, differences in the degradation of the compound and variations in its interaction with the target enzyme are the most important aspects to be considered. Although it was suggested that methylenedioxy synergists act by inhibiting the detoxifying enzymes in the microsomes (Lewis *et al.*, 1967; Philleo *et al.*, 1965), the exact nature of the target enzymes is not known. However, the latter workers showed that 2,3-methylenedioxynaphthalene was 167-fold more active in the inhibition of the housefly microsomal epoxidation of aldrin than was the comparable enzyme for pig liver. Although in vitro studies do not always coincide with the over-all in vivo picture, this inhibition of epoxidation may serve as a model and explain in part the phenomenon of selectivity.

Equally important are the differences in rate and extent of degradation of the synergist in the two organisms. 2,3-Methylenedioxynaphthalene is extremely stable in houseflies, yet it is extensively metabolized in mice. The two organisms are only similar qualitatively in their degradation of the synergist. Cleavage of the methylenedioxynaphthyl ring occurs faster in the mouse, also hydroxylation is both more rapid and more versatile in mammals. The flies, on the other hand, were shown to be ineffective in carrying out these essential reactions.

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