Metabolic Fate of Dieldrin in the Rat

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Dieldrin, a potent residual insecticide, is metabolized in the rat. Studies in vivo and in vitro indicate that this insecticide is attacked by three different enzyme systems, the most important degradation route being a glucuronic conjugation system upon one of the oxidation products of dieldrin in the liver. The major urinary metabolite (also known as Klein's metabolite) appears to be a by-product of this conjugation system.

Dieldrin has been used extensively as an insecticide for almost 20 years. Because of its physicochemical characteristics in resisting biological and physical degradation, it has become a common contaminant of the environment. Little is known of the mode of action or metabolic fate of this insecticide, despite the need of such information in relation to problems of public health importance.

It is apparent that in mammals dieldrin is either excreted or metabolized and excreted, because it does not accumulate above certain levels, even when mammals are fed relatively high daily doses (Korte, 1965). The fact that low concentrations of unchanged dieldrin are usually found in the feces and urine of animals on diets containing relatively high concentrations of dieldrin further indicates that the insecticide is degraded in the mammalian body or by intestinal microbes (Porter, 1967). Recently Korte and his associates demonstrated in vivo that dieldrin metabolism does occur in rats and rabbits (Korte, 1965; Korte *et al.*, 1963; Ludwig *et al.*, 1964; Ludwig and Korte, 1965). The actual sites of metabolism, however, have not been specified, nor have the reaction mechanisms through which dieldrin is degraded been explored.

Efforts to demonstrate significant dieldrin metabolism in vitro have not, to date, been fruitful. It has been possible, however, to demonstrate the conversion of aldrin to dieldrin in microsomal preparations from pig, rat, and rabbit liver (Brooks *et al.*, 1963; Nakatsugawa *et al.*, 1965; Wong and Terriere, 1965), or microsomal preparations from the housefly (Ray, 1967), cockroach fat bodies (Nakatsugawa *et al.*, 1965), and the midgut of the southern army worm (Krieger and Wilkinson, 1967). In every case, the reaction requires reduced nicotinamide adenosine dinucleotide phosphate (NADPH) and atmospheric oxygen. Pyrethrum synergists such as sesamex, piperonyl butoxide, and SKF 525A not only inhibit the epoxidation reaction of aldrin but increase the toxicity of dieldrin itself when the latter is directly given to the test insects (Sun and Johnson, 1960). None of the investigations of aldrin epoxidation in vitro have resulted in reports of further metabolism of dieldrin.

The liver is an important site of degradation of xenobiotics. Accordingly, the present work has been primarily designed to study the metabolism of dieldrin by the rat liver. An effort has been made to correlate the metabolism observed in an in vitro system with that observed by other workers in vivo (Damico *et al.*, 1968; Heath and Vanderkar, 1964; Richardson *et al.*, 1968). Partly because of the low solubility of dieldrin in water, it has not been possible to obtain enough of the in vitro metabolites for chemical characterization, but enough in vivo metabolites have been obtained for positive identification. These in vivo metabolites have been compared chromatographically with those formed in vitro.

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EXPERIMENTAL

Reduced nicotinamide adenine dinucleotide phosphate (NADPH), adenosine triphosphate (ATP), glucose-6-phosphate (G-6-P), and β-glucuronidase were obtained from the Nutritional Biochemical Corp. Other cofactors and materials used in this research were uridine diphosphate glucuronic acid (UDPGA), Sigma Chemical Co.; nicotinamide, Eastman Organic Chemicals; Sephadex, Pharmacia Fine Chemicals, Inc.; silica gel G, Brinkmann Instruments, Inc.; and Florisil, Fisher Scientific Co. Young adult male, albino rats, 170 to 190 grams, were obtained from Rolfsmeyer Farms, Madison, Wis. The film used in the detection of radioactive spots on chromatograms was Supreme x-ray film from the General Aniline and Film Corp. The ^{14}C-universally labeled dieldrin used in the work had a specific activity of 70.4 mCi. per millimole. It was obtained from the Radiochemical Centre, Amersham, England, and used after purification on thin-layer chromatography. Sesamex was obtained from Shulton Fine Chemicals, Shulton, Inc. The column used for gas chromatography was a prepacked SE-30 column (Catalog No. 71803) obtained from Beckman Instruments, Inc.

Collection, Extraction, and Cleanup of Fecal Metabolites. Four albino male rats were fed a diet of ground dog food containing 20 p.p.m. of dieldrin by weight for one month, after which the dieldrin in the diet was increased to 100 p.p.m. Feces and urine were collected separately. Feces were collected for 18 days, during which period the four rats consumed 1 kg. of ground dog food containing dieldrin (100 p.p.m.). The feces were desiccated under a stream of dry air at room temperature and ground into a fine powder. Ground feces were extracted in succession with 1 liter each of heptane, methylene chloride, and acetone, by homogenizing the feces with the solvent in a 2-quart Mason jar attached to a Lourdes homogenizer. The solid residues were removed through paper filtration and the filtrate was concentrated to 10 ml. A thick, oily residue was thus obtained. Each residue was partitioned three times with an equal volume of acetonitrile, which was pooled and concentrated to an oily residue. The residue was then taken up in *n*-pentane and cleaned up on a 2.5- × 16-cm. Florisil column (Florisil activated at 140° C. for 4 hours). The sample was transferred into the column with *n*-pentane, and eluted with 100 ml. each of (I) *n*-pentane (95)–diethyl ether (U.S.P.) (5), (II) *n*-pentane (80)–ether (20), (III) *n*-pentane (50)–ether (50), (IV) *n*-pentane (10)–ether (90), and (V) acetone.

The eluates were concentrated to 5 ml. each, and assayed for

dieldrin metabolites by thin-layer chromatography with silver nitrate as a chromogenic agent. The stationary phase was a layer of silica gel G (0.25-mm. thickness) and the mobile phase was a mixture of hexane-acetone (8 to 2). Silver nitrate reagent was prepared by dissolving 1 gram of silver nitrate in 5 ml. of deionized water, adding 10 ml. of 2-phenoxyethanol, and making up the volume to 200 ml. with acetone. A small drop of 40% hydrogen peroxide was added to the silver nitrate reagent as a preservative. Chlorinated hydrocarbons were detected as black spots when the thin-layer plates were sprayed with the silver reagent and exposed to a strong ultraviolet light source.

Fractions containing dieldrin metabolites were further cleaned up by chromatography on 20- × 20-cm. thin-layer plates coated with a 1-mm. thick layer of silica gel G.

The major fecal metabolite was crystallized by dissolving the cleaned-up residue in 0.5 ml. of acetone in a 3-ml. vial. One milliliter of hexane was added to this solution, and the mixture was evaporated under a gentle stream of dry air. Evaporation of the solvents resulted in crystallization. The major fecal metabolite was recrystallized in this manner three times, each time from fresh solvents.

Collection, Extraction, and Cleanup of Urinary Metabolites. The urine sample collected in the previously described feeding studies was allowed to dry in the container used for its collection. Urinary metabolites were collected by repeated washings of the collecting apparatus with 2 liters of acetone. The acetone wash was concentrated by evaporation to 20 ml. of an oily residue which was extracted three times, each time with 20 ml. of acetonitrile. Acetonitrile from the extraction was pooled and concentrated by evaporation to an oily residue, which was taken up in 10 ml. of pentane. Cleanup of the major urinary metabolite by activated Florisil, thin-layer chromatography, and recrystallization from hexane was accomplished by the procedure previously described for the major fecal metabolite.

Spectroscopic Analysis of Metabolites. Infrared spectra of the purified fecal and urinary metabolites were taken with a Beckman IR-5A, by using a 0.1-mm. microcell. Metabolites were dissolved in carbon tetrachloride to make approximately 10% (weight per volume) solutions. In some cases the concentrations used were less than 10% because of the low solubilities of some of the metabolites in carbon tetrachloride.

Mass spectra of the fecal and urinary metabolites were obtained at 70 e.v. of energy at 200° C. under a vacuum of 2×10^{-7} torr in a Hitachi-Perkin-Elmer mass spectrometer.

Gas Chromatography. Gas chromatography was carried out on a Beckman GC-4 gas chromatograph using a Beckman prepacked $^1/_8$-inch × 6-foot stainless steel column containing silicone SE-30, 0.75%, on Chromosorb-W 60/80. The column temperature was 175° C. The sample was eluted at the rate of 62 ml. per minute of helium and detected by the dual hydrogen flame detectors.

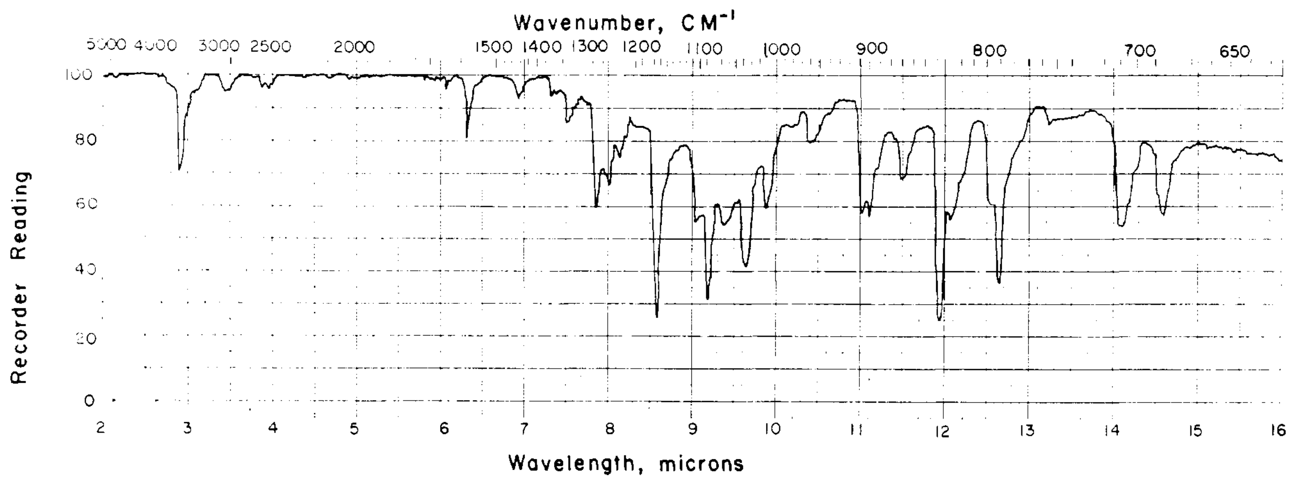
Preparation of Microsomal Fraction. Rats were stunned by a cerebral concussion and the liver was sampled immediately. The liver samples were first rinsed in ice-cold distilled water, and then in ice-cold 0.2*M* potassium phosphate buffer at pH 7.4. The samples were blotted of excess liquid on filter paper, weighed, and homogenized at 30% (weight per volume) in fresh, ice-cold 0.2*M* potassium phosphate buffer. An attempt was made to standardize the method of homogenization: The mortar portion of a Potter-Elvehjem type homogenizer with the liver sample in buffer was attached to the fitting Teflon pestle (mortar-pestle clearance 0.05 inch at 20° C.). The pestle was turned by a Lourdes homogenizer with the rheostat set at 22% (approximately 1000 r.p.m.); the mortar portion was then moved up and down for eight complete successive trips in approximately 45 seconds.

Centrifugation of the homogenate at 0° to 2° C. for 20 minutes at 10,000 × gravity resulted in sedimentation of undisrupted cells, nuclei, mitochondria, cell walls, and other tissue debris. The resulting supernatant contained the microsomal plus soluble portions of the cells—i.e., the microsomal fraction.

Incubation of Dieldrin-^{14}C with Microsomal Fraction. In a typical experiment, 1 ml. of microsomal fraction was incubated for 2 hours with 0.01 μmole of dieldrin-^{14}C (0.704 μc.) plus various combinations of cofactors in a 25-ml. flask. The cofactors such as NADPH, nicotinamide, ATP, or G-6-P were added at the desired concentrations in 0.2*M*, pH 7.4 potassium phosphate buffer. Each incubation mixture was finally made up to 5 ml. with 0.2*M*, pH 7.4 potassium phosphate buffer. Flasks were shaken at approximately 60 cycles per minute open to the atmosphere at 37° C. for 2 hours in a metabolic shaker. The reactions were stopped by immediate extraction with ether. Each 5-ml. incubation mixture was extracted three times with 10 ml. each of diethyl ether by vigorous agitation in a 2.5- × 30-cm. test tube on a Vortex Jr. vibrator. The three ether extracts were combined, and the solvent was removed under a gentle stream of dry air. The residue was taken up in 5 ml. of ether and a 0.5-ml. portion was added to 10 ml. of liquid scintillation counting solution. Counting solution was made by dissolving 16.5 grams of 2,5-diphenyloxazole (PPO) and 0.9 gram of 1,4-bis-[2-(4-methyl-5-phenoxazolyl]-benzene (POPO) in 1.5 liters of methyl Cellosolve and 1.5 liters of toluene.

The amounts of ether-inextractable metabolites of dieldrin formed during the incubation period were assessed by first removing the precipitated proteins from the water phase by brief centrifugation, and sampling a 0.5-ml. portion of this phase and transferring it to a second 10 ml. of counting solution. Radioactivity in each phase was determined by counting the sample in a 20-ml. glass counting vial in a Packard Tri-Carb Model 314-E liquid scintillation spectrometer. Corrections for radioactive quenching were made by observing the quenching of a known count of dieldrin-^{14}C by 0.5-ml. aliquots of each phase from a blank liver incubation mixture. Under the conditions tested, the 0.5-ml. aliquot of ether phase quenched the count by 2.8%; the 0.5-ml. aliquot of water phase quenched the count by 21.6%. Radioactivity found in each phase was totaled, and the total recovery rates were checked (consistently greater than 95%). The per cent of the total which was inextractable from water with ether was initially used as the index for the degree of dieldrin metabolism.

Water-Soluble Metabolite. Dieldrin-^{14}C (0.01 μmole) was added to eight 5-ml. incubation mixtures which contained microsomal fraction (1 ml.), NADPH (2.4 μmoles), ATP (3.6 μmoles), and G-6-P (6.5 μmoles); then the systems were shaken at 37° C. for 2 hours. Each mixture was then extracted with a double volume of ether three times, and precipitated proteins were removed by brief centrifugation. The clear water phases were pooled and concentrated to 4 ml. by lyophilization. Sephadex G-25 was used to purify the radioactivity which remained in the water phase after extraction with ether. Sephadex G-25 had been preswollen overnight. A 1-ml. portion of the concentrated sample was transferred to the 1.4- × 40-cm. Sephadex G-25 column and the system was eluted with distilled water at the rate of 20 ml. per hour. Two-milliliter fractions were collected and assayed for ultraviolet absorption at 280 mμ. Radioactivity in each of the



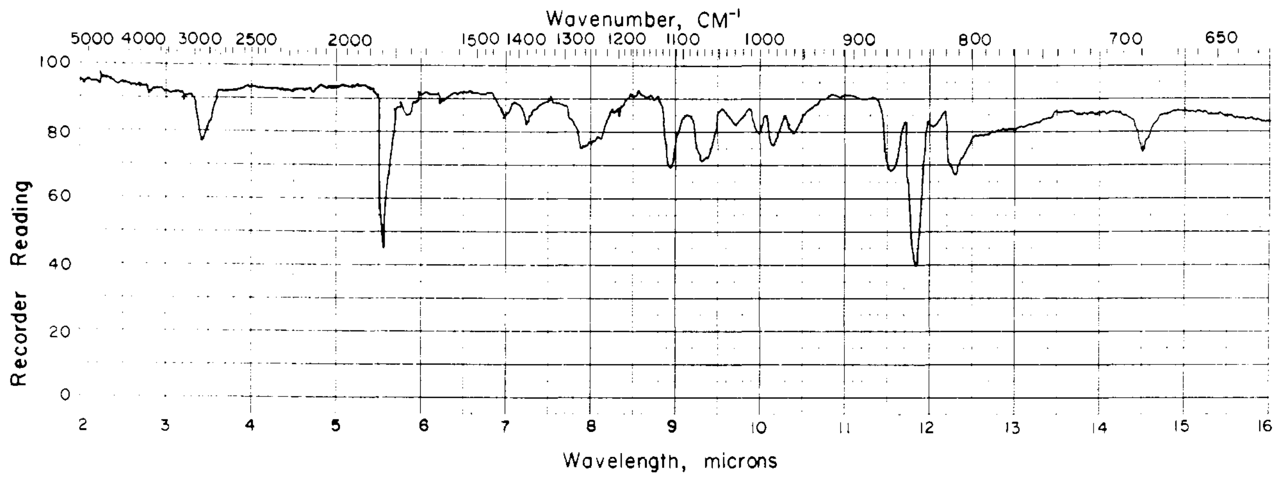


Figure 1. Infrared spectra for two major dieldrin metabolites in the rat in vivo

Upper. F-1
Lower. U-1

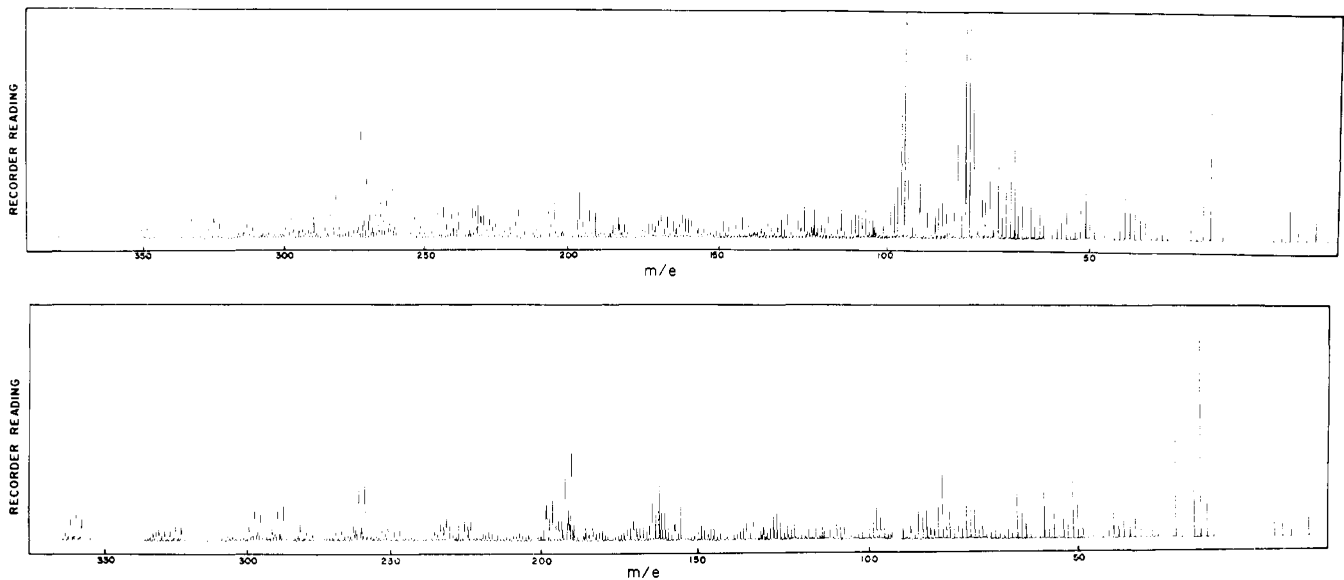


Figure 2. Mass spectra for two major dieldrin metabolites in the rat in vivo

Upper. F-1
Lower. U-1

fractions was determined by counting a 0.1-ml. aliquot in a liquid scintillation counter.

Two-milliliter fractions from the Sephadex column containing radioactivity were incubated with various amounts of β-glucuronidase, 70,000 to 100,000 units per gram. The incubation conditions were varied with respect to time, temperature, and pH to determine the optimum conditions for hydrolysis of the glucuronide conjugate. Degrees of hydrolysis were determined by extracting the β-glucuronidase digest three times with a double volume of ether. The rate in per cent of hydrolysis was determined by dividing the amount of radioactivity transferred to the ether extract by the total radioactivity in the original aqueous sample before extraction.

RESULTS

In Vivo Studies. Two metabolites of dieldrin were isolated from the feces and two were isolated from the urine of four male rats which were fed dieldrin. Twelve milligrams of the major fecal metabolite, F-1, were isolated in pure crystal form from the feces collected during the consumption of 2 kg. of dog food containing dieldrin at 100 p.p.m. Approximately 1 mg. of the major urinary metabolite, U-1, was isolated in pure form from the urine collected during the same period. The minor fecal metabolite, F-2, was also present in greater quantities than the minor urinary metabolite, U-2; neither of the minor metabolites was isolated in pure form.

The white needle-shaped crystals of F-1, obtained after three recrystallizations from hexane, were pure according to thin-layer and gas-chromatographic analysis. The crystals of F-1 consistently melted at 154–56° C. Both infrared (Figure 1, *a*) and mass spectral analysis (Figure 2, *a*) indicated that it was probably the same compound as the one isolated by Richardson *et al.* (1968). The addition of an —OH group to dieldrin was indicated by both spectroscopic analyses. Furthermore, the sharpness of the —OH absorption in the infrared spectrum at 2.9 microns indicated that it is an angular —OH, free movement of which is possibly restricted.

After three recrystallizations from hexane, crystals of U-1 had the appearance of very short lengths of string which had been snipped off with scissors. The U-1 crystals melted at 165–66° C., and were pure as judged by thin-layer and gas chromatographic analyses. Infrared (Figure 1, *b*) and mass spectroscopic analysis (Figure 2, *b*) indicated that U-1 was identical to the compound isolated by Damico *et al.* (1968) and Richardson *et al.* (1968) (Figure 4).

The minor fecal and urinary metabolites of dieldrin were chromatographically identical and they behaved chromatographically in the same way as 6,7-*trans*-dihydroxydihydroaldrin previously isolated and described by Korte's group from rabbit urine (Korte and Arent, 1965; Ludwig and Korte, 1965). The rats used in the in vivo studies of dieldrin metabolism remained lively, alert, and in apparent good health throughout the experiment.

In Vitro Studies. WATER-SOLUBLE METABOLITES. Preliminary experiments indicated that in the presence of reduced nicotinamide adenine dinucleotide phosphate (NADPH) the liver microsomal fraction converted 7 to 10% of the dieldrin-^{14}C into ether-inextractable metabolites in a 2-hour incubation period. This observation appeared significant in view of the phenomenon that, in the absence of NADPH, only 1 to 2% of the radioactivity from the dieldrin-^{14}C became inextractable to ether from the water phase. Numerous organic solvents were tested for extraction of the ether-inextractable material; these included ether, benzene, chloroform, methylene chloride, and various combinations of solvents—e.g., benzene-methanol and chloroform-methanol. With all of these solvents, the per cent of the total radioactivity which remained in the water phase was relatively constant.

The effects of NADPH and other cofactors were studied both alone and in various combinations. Table I shows the results of this study and the concentrations of each cofactor used in attempts to optimize the metabolic conditions for a 5-ml. incubation mixture. Greater activity was observed when higher concentrations of microsomal fraction were used. The enzyme activity remained relatively constant over a wide pH range (pH 7 to 8.0).

Table I. Effect of Various Combinations of Cofactors of Dieldrin Metabolism in Vitro[a]

	Flask Number																	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Dieldrin (0.01 μmole)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Microsomal fraction (1 ml.)			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NADPH (2.4 μmoles)		+		+				+	+	+	+	+	+	+				
Nicotinamide (16.3 μmoles)		+			+			+			+	+		+	+	+		+
G-6-P (6.5 μmoles)		+				+			+		+		+	+	+		+	+
ATP (3.6 μmoles)		+					+			+		+	+	+		+	+	+
% total ^{14}C in H_2O phase	0.23	0.25	1.45	7.26	3.59	3.84	2.67	8.58	7.69	8.92	7.85	10.50	11.68	11.10	3.32	5.86	3.06	7.24

[a] Microsomal fraction prepared by centrifuging rat liver homogenate at 10,000 G for 20 minutes. All incubation mixtures made up to 5 ml. with 0.2*M* potassium phosphate buffer, pH 7.4. Incubation at 37° C. in a shaking incubator for 2 hours, followed by extraction of incubation mixtures with ether. Metabolism measured by the "% total ^{14}C in H_2O phase" after extraction with ether.

Table II. Formation of Ether-Soluble Metabolites of Dieldrin in Vitro
(data expressed in percentages of total dieldrin recovered in ether phase)

Treatment (Flask No.)[a]	^{14}C Ether-Extractable Metabolite[b] M-1	M-2	M-3	M-4	M-5[c] M-6	Dieldrin
Blank (2)	0.11	0	0	0	0	99.5
Control (3)	0.07	0.04	0.05	0.15	0.44	97.9
Control + NADPH (4)	0.25	0.15	0.15	0.84	2.34	89.0
Control + cofactors (13)	0.31	0.36	0.13	1.28	3.97	82.7
Control + cofactors − NADPH (18)	0.23	0.21	0.11	0.68	2.81	88.6

[a] Flask numbers correspond to flask contents (Table I).
[b] Analysis of ether-extractable metabolites by thin-layer chromatography of silica gel G with mobile phase of ether–hexane (1 to 1) and exposure to x-ray film to detect radioactive spots. Silica gel at exact position of each radioactive spot removed and counted in a liquid scintillation counter.
[c] Two metabolites (M-5 and M-6).

Table III. R_f Values[a] for Dieldrin and Its Metabolites

Mobile Phase	Metabolites and Their R_f Values: M-1	M-2[b] (=F-2) (=U-2)	M-3	M-4 (=U-1)	M-5 (=F-1)	M-6 (=C-1)	Dieldrin
Hexane–acetone (8:2)	0.0	0.10	0.12	0.35	0.40	0.38	0.80
Ether–hexane (1:1)	0.0	0.07	0.10	0.33	0.52	0.52	0.83
Benzene–ethyl acetate (3:1)	0.0	0.15	0.17	0.80	0.75	0.68	0.93
Methylene chloride	0.0	0.0	0.0	0.83	0.74	0.45	0.95
Ether–hexane (9:1)	0.0	0.52	0.62	0.94	0.95	0.95	1.0

[a] In all cases stationary phase was a thin-layer (0.25 mm.) of silica gel G.
[b] Identical to 6,7-*trans*-dihydroxydihydroaldrin.

Ether-Extractable Metabolites. In the standard incubation mixtures—e.g., experiment 13, Table I—generally over 11% of the total radioactivity remained in the water phase after extraction with ether. The ether extracts were also assayed for dieldrin metabolites by chromatography and autoradiography. Table II gives the percentages of the total ^{14}C found in each spot detected on the chromatograms by autoradiography and the R_f's at which these spots were detected. The in vitro metabolites have been referred to as M-1, M-2, M-3, M-4, and M-5 with respect to their increasing R_f's on silica gel G, when diethyl ether (U.S.P.)–hexane (1 to 1) was used as the mobile phase. This chromatographic system resolved all of the metabolites except two (M-5 and M-6) which had an R_f of 0.52. These two metabolites were resolved when methylene chloride was used as the mobile phase.

Chromatographic Comparison of in Vivo and in Vitro Metabolites. The ether-extractable metabolites formed in vitro were compared by cochromatography with those obtained in vivo. The metabolites observed in vitro were detected on the chromatograms by means of autoradiography. The metabolites observed in vivo were detected with silver nitrate as a chromogenic reagent. Chromatograms on which both in vitro and in vivo metabolites were developed were first exposed to x-ray film for the necessary period of time to detect the metabolites from dieldrin-^{14}C, and then the positions of the in vivo metabolites were detected by means of the silver nitrate spray reagent. The results of these chromatographic comparisons are seen in Table III.

Inhibition of Metabolism by Sesamex. The requirement of NADPH by the microsomal fraction for dieldrin metabolism is indicative of oxidative degradation by microsomal enzymes. Another observation which indicates microsomal oxidation is the drastic decrease in ether-inextractable radioactivity from the water phase in the presence of added sesamex in the incubation mixture (Table IV). Sesamex is a well known inhibitor of microsomal oxidative systems. The inverse correlation between the amounts of water-soluble metabolites and the concentrations of sesamex is indicative of competitive inhibition which has been proposed as the mode of action of sesamex (Philleo *et al.*, 1965).

Table IV. Sesamex Inhibition of Ether-Extractable Radioactivity as Shown by Chromatography and Autoradiography[a]

Sesamex	Water-Soluble Metabolite	Ether-Soluble Metabolites M-1	M-2	M-3	M-4	M-5	M-6	Remaining Dieldrin
0.0	9.38	0.20	0.17	0.10	2.41	0.92	0.34	84.0
0.01	6.53	0.32	0.23	0.12	1.92	1.55	0.21	87.8
0.02	4.95	0.33	0.17	0.16	1.42	1.40	0.15	90.5
0.05	1.54	0.10	0.13	0.15	0.43	1.60	0.05	95.5
0.10	0.55	0.03	0.15	0.09	0.15	1.30	0.01	97.2
0.15	0.28	0.05	0.10	0.05	0.10	0.88	0	98.4
0.20	0.21	0.05	0.07	0.04	0.07	0.74	0	98.7

[a] Radioactivity in each spot formed on x-ray film exposed to chromatogram of ether extracts from incubation mixtures containing 1 ml. of rat liver microsomal fraction, 2.4 mmoles of NADPH, 6.5 mmoles of G-6-P, and 3.6 mmoles of ATP plus various concentrations of Sesamex. Chromatography done on silica gel G using ether–hexane (1:1) as mobile phase.

At higher concentrations (above 0.06 μmole of sesamex per 5 ml.) radioactivity in the water phase became even lower than that produced by the microsomal fraction alone. This observation provides evidence of the presence of endogenous NADPH or an NADPH-generating system in the microsomal

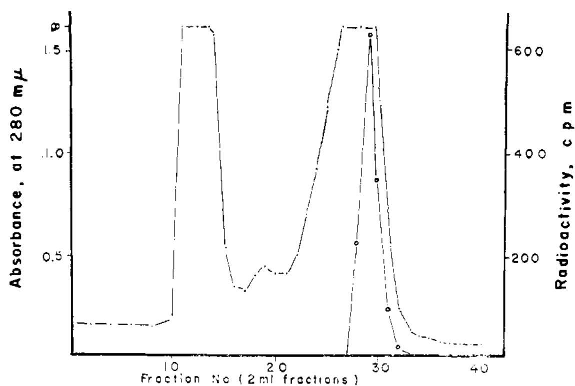


Figure 3. Sephadex elution, radioactivity *vs.* absorption at 280 mμ

Fractionation, on Sephadex G-25, of 1 ml. of concentrated, ether-extracted water phase from an active incubation mixture plus dieldrin-^{14}C

fraction which permits a small amount of dieldrin-^{14}C metabolism without added cofactors.

Table IV illustrates the effect of sesamex on the ether-extractable metabolites, in terms of autoradiographed thin-layer chromatograms. In this experiment M-4 was found to be more abundant than M-5 in the uninhibited incubation mixture. The production of the ether-extractable metabolites, M-4 and M-6, and of the water-soluble metabolite was almost completely inhibited, but that of M-5 was almost unaffected. The enzyme responsible for the production of metabolite M-2, which is identical to 6,7-dihydroxydihydroaldrin, was also relatively resistant to inhibition by sesamex.

Analysis of Water-Soluble Metabolite. The nature of the radioactivity, which remained in the water phase after extraction with ether, was studied by pooling the reaction products from the optimum incubation mixture (Table I, flask 13, eight tubes), removing ether-extractable materials, precipitating proteins, and concentrating the clear water phase to 4 ml. One milliliter of this concentrate was placed on a 1.4- × 40-cm. Sephadex G-25 column and eluted with distilled water at the rate of 20 ml. per hour. The eluate was collected in 2-ml. fractions which were assayed for absorption at 280 mμ and for radioactivity (Figure 3).

The major peak of absorption at 280 mμ occurred in the 11th through the 15th fractions. This was approximately the retention volume of the column and the expected position for proteinaceous material. The absence of any detectable radioactivity in the 11th through the 15th fractions indicates that dieldrin is not bound to proteinaceous material to any significant degree. Smaller peaks of absorption at 280 mμ occur between the 22nd and 32nd fractions. These peaks are probably due to the elution of nucleotides, nucleosides, polypeptides, water-soluble lipids, and amino acids. The comparatively sharp radioactive peak occurs between the 28th and 31st fractions and accounts for all of the radioactivity which was added to the column.

β-Glucuronidase is an enzyme which hydrolyzes glucuronide conjugates to yield the free aglycone and glucuronic acid. Twenty milligrams (1400 to 2000 units) of a β-glucuronidase (Nutritional Biochemical Corp., Cleveland) were incubated overnight at 37° C., pH 4.8, with several of the radioactive fractions from the Sephadex column. This incubation increased the ether-extractable radioactivity from approximately 5% to approximately 51% of the total radioactivity in each fraction. The metabolite freed by β-glucuronidase, hereafter referred to as C-1, behaved chromatographically identical to ether-extractable metabolite M-6 rather than any of the metabolites isolated in vivo.

Uridine diphosphate glucuronyltransferase forms conjugates with glucuronic acid and appropriate aglycones only when glucuronic acid is present in the form of uridine diphosphate glucuronic acid (UDPGA). Optimum incubation mixtures were supplemented with UDPGA to determine if it was rate-limiting for the conjugation reaction, as judged by an increase in inextractable radioactivity in the water phase. The results were variable (Table V), possibly because of the variation in the amounts of endogenous UDPGA in different liver preparations. In some cases the addition of UDPGA resulted in an increase of 2 to 4% of the total radioactivity in the water phase; in other cases it had no effect. The addition of glutathione or sulfate ions to the system never increased the water-soluble radioactivity.

Incubation mixtures in which additional UDPGA had the most marked effect were those in which dieldrin metabolism was the highest. Thus it appears that the amounts of endogenous UDPGA present in the microsomal fractions are marginal, and it becomes the limiting factor only when the per cent of dieldrin metabolism is relatively high—that is, only when metabolism is naturally high, or is increased by larger amounts of NADPH, does the addition of UDPGA to the incubation mixture result in increased radioactivity in the water phase. Since the concentration of endogenous UDPGA, as well as the rate of dieldrin-^{14}C metabolism, varies from animal to animal through normal biological variation, there is no precise point at which it can be predicted that UDPGA will become the rate-limiting factor.

Conjugation of *trans*-6,7-Dihydroxydihydroaldrin. Korte and his coworkers (1963) have shown *trans*-6,7-dihydroxydihydroaldrin (*trans*-aldrindiol) to be one of the metabolites of dieldrin produced by rabbits in vivo. In view of the importance of conjugation systems in the metabolism of dieldrin in the rat, this compound was also tested for the possibility of conjugation in the rabbit liver systems.

trans-Aldrindiol was synthesized according to the method described by Korte and Arent (1965). Radioactive dieldrin was used in the synthesis to facilitate the work in vitro. The experimental design with UDPGA was similar to those described for dieldrin metabolism with microsomal fractions from rat liver. Young adult male rabbits were used as the source of liver, and the microsomal fraction was prepared in the same manner as above. Results of the incubation of rabbit liver microsomal fractions with *trans*-aldrindiol are shown in Table VI.

Rat liver microsomal fraction was also found to form a conjugate of *trans*-aldrindiol and glucuronic acid. The activity of this system is similar to that described for rabbit liver microsomal fraction—i.e., it also incorporates 40 to 50% of the total radioactivity into the water phase.

DISCUSSION

The major metabolite observed in the in vivo studies, F-1, was isolated in approximately 10 times greater amounts than U-1, which was the only other metabolite isolated in large enough quantities for chemical identification. Because of the differences in the material from which they were extracted and the fact that the extraction procedures may have favored one compound over the other, the actual amount of each metabolite isolated in pure form is only a rough estimate of the relative amounts produced. The minor metabolites were not purified, but by chromatographic observation it was possible to estimate

Table V. Effect of UDPGA on Rates of Production of Metabolites in Vitro

Treatments	Expt. No.	Water-Soluble Metabolite	Ether-Extractables M-1	M-2	M-3	M-4	M-5,6	Dieldrin Remaining
Control[a]	1	1.40	0.07	0.05	0.05	0.15	0.24	97.7
Control + UDPGA[b]	2	1.31	0.11	0.05	0.03	0.14	0.23	98
Control + NADPH[b]	3	13.92	0.72	0.25	0.18	3.24	1.91	78.8
(3) + UDPGA	4	15.50	0.72	0.17	0.13	3.23	0.73	79.5
(4) + sesamex[c]	5	1.08	0.08	0.07	0.04	0.09	0.33	97.2

[a] Control. 1 ml. of microsomal fraction plus 0.01 μmole of ^{14}C dieldrin. 2 hours shaking at 37° C.
[b] Added UDPGA, 0.7 μmole and NADPH, 3.6 μmoles.
[c] Sesamex added 1.5 μmoles.

Table VI. Conjugation of *trans*-Aldrindiol with Glucuronic Acid by Rabbit Liver Microsomal Fraction

	Treatments[a] Control	Control + NADPH	Control + UDPGA	Control + NADPH + UDPGA
Water-soluble metabolites	8.91	14.17	40.40	43.43

[a] Conditions as in Table IV.

that the quantity of the compound present in the feces was severalfold higher than that in the urine.

Metabolites F-1 and U-1 were identified by their infrared and mass spectra to be identical to the compounds isolated by Richardson *et al.* (1968) from rat feces and urine, respectively (Figure 4, F-1 and U-1). The identity of the urinary metabolite has been independently reported by Damico *et al.* (1968) as having the same molecular structure as that reported by Richardson *et al.* (1968). The fact that only M-6, and not F-1 (= M-5), forms conjugates with glucuronic acid supports the view that the —OH group is readily available for M-6 but not for F-1.

The major in vitro metabolites extracted from the incubation mixture by ether were M-4 and M-5. They were present at levels varying from 3.3 to 5.2% of the total dieldrin added to the optimum in vitro system. Metabolite M-6, which cochromatographed with M-5, when ether-hexane (1 to 1) was used as the mobile phase, accounted for 0.34% of the radioactivity (Table II). Thus, M-6 was the third most abundant ether-extractable metabolite. By far the highest amount of dieldrin metabolite produced in vitro was, however, found in the water phase (9 to 11% of total dieldrin added). The balance of evidence indicates that the major degradation route is through the glucuronic conjugation system (Figure 4).

The production of the water-soluble metabolites, M-4 and M-6 of the ether-extractable metabolites, appears to be competitively inhibited by the addition of sesamex to the incubation mixture (Table IV). Furthermore, their formation is inhibited by similar percentages by the addition of sesamex.

The enzyme or enzyme system responsible for the formation of metabolite M-5 is apparently not the enzyme responsible for the formation of the other major dieldrin metabolites. This is obvious because of its insensitivity to sesamex. An enzyme which requires NADPH as a cofactor for the oxidation of a xenobiotic, and is at the same time insensitive to sesamex, is, to our knowledge, unique among microsomal enzymes.

The presence of a third (minor) system is indicated by the fact that the production of M-2—i.e., *trans*-aldrindiol—appeared to be only slightly influenced by either sesamex or NADPH (Tables II and IV).

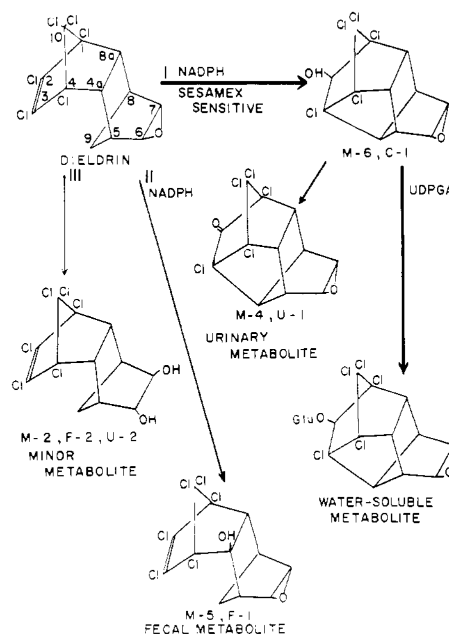


Figure 4. Degradation pathways of dieldrin in the rat

Pathways I, II, and III depend on three independent enzyme systems. Metabolite M-4 is a by-product of the conjugation system involving M-6—i.e., M-4 is spontaneously formed from unspent M-6 whenever the glucuronic conjugation system becomes a limiting factor.

The major metabolite of dieldrin produced in vitro has not been previously reported, probably because it stays with the aqueous phase as a glucuronide conjugate. This metabolite has not been identified, but its conjugation with glucuronic acid indicates that it must have a hydroxyl group at an easily accessible position, probably at one end of the molecule. The conjugated metabolite is much less polar than *trans*-aldrindiol and it is therefore thought to have only one hydroxyl group. The inhibitions of the conjugated metabolite and metabolite M-4 by sesamex are very closely correlated, indicating that these compounds may be formed from a similar intermediate. In fact, it was possible to show that M-6 could spontaneously convert to M-4 and other polar compounds upon storage at 4° C. Based on these observations, it is proposed that the conjugated metabolite is the product of dieldrin dechlorination and hydroxylation at the number 2 carbon. It has also been speculated that the molecule undergoes a rearrangement to form a hydroxylated and "caged" structure such as C-1 in Figure 4. This rearrangement may or may not

occur until the hydroxylated compound is further oxidized to form metabolite M-4.

trans-Aldrindiol has been shown to be actively conjugated with glucuronic acid by a microsomal fraction prepared from either rabbit or rat liver. Forty to 50% of the total *trans*-aldrindiol is incorporated into the water phase when the microsomal fractions are supplemented with UDPGA. When Korte and Arent (1965) fed *trans*-aldrindiol to rats, they found 16% of the dose to be excreted as an unidentified polar metabolite. This unidentified polar metabolite could have been a glucuronide conjugate of *trans*-aldrindiol. *trans*-Aldrindiol is also conjugated by the rat liver in vitro system, but the enzyme system responsible for the production of "*trans*-diol" does not appear to be as active in the rat because in the experiments with the rat liver, this compound was never liberated from the water phase by β-glucuronidase in detectable quantities.

CONCLUSIONS

In the rat, dieldrin is metabolized through the three different degradation routes drawn in Figure 4. Judging from the in vitro studies alone, the major metabolite of dieldrin produced in the liver is conjugated with glucuronic acid. This metabolite is probably excreted in both the urine and feces. Metabolite U-1 (M-4) is probably a by-product of the conjugated metabolite, C-1.

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