Metabolism of Endosulfan in Milk Sheep

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A single oral dose of 0.3 mg. per kg. of endosulfan-^{14}C to two lactating sheep was almost completely eliminated in 22 days. About 50% of the carbon-14 was excreted in the feces, 41% in the urine, and 1% in the milk. During this period the concentration in milk gradually fell to 2 p.p.b. on the 22nd day. The concentrations did not exceed the 0.03-p.p.m. level in any organ and tissue in one animal sacrificed after 40 days. The compound was not completely metabolized; unchanged insecticide was present in the feces but absent from the urine. Two main metabolic products in the urine were: 1,4,5,6,7,7-hexachloro-2,3-bis-(hydroxymethyl)-bicyclo-2,2,1-heptene-5 (endosulfan alcohol) and 1-hydroxy-4,5,6,7,8,8-hexachloro-1,3,3*a*,4,7,7*a*-hexahydro-4,7-methanoisobenzofuran (1-α-hydroxy endosulfan ether).

A comprehensive review of the properties and path of metabolism of the insecticide endosulfan in insect and warm-blooded organisms has been published by Maier-Bode (1968). He indicated that the metabolism varies from species to species and that endosulfan sulfate does not accumulate in the adipose tissue of animals, but did not state whether endosulfan alcohol is present in the urine of sheep and whether metabolites other than endosulfan sulfate occur in the milk of sheep. Quantitative studies of absorption, distribution, and elimination have long been lacking. The object of the present paper is to provide the relevant data.

The presence or absence of the metabolites shown in Figure 1 was examined.

MATERIALS AND METHODS

The studies were carried out with endosulfan labeled with carbon-14 in the methylene group and with a specific activity of 95 mc. per gram (product of Radiochemisches Laboratorium, Farbwerke Hoechst AG). Radiochemical purity was established by thin-layer chromatography, which also showed that the preparation consisted of a mixture of 65% α- and 35% β-endosulfan, as in the commercial product.

Metabolites I, II, III, and IV were synthesized as nonradioactive products by Farbwerke Hoechst AG, Germany, and the purity was established by thin-layer and gas chromatography. Three East Friesian milk sheep were used in this study, one of which served as a control. The weights and ages were: sheep 1, 50 kg., age 3 years; sheep 2, 52 kg., age 3 years; and control, 49 kg., age 1.5 years. They were kept in metabolism cages under the conditions described by Herok and Götte (1963). After administration of the drug they received mixed feed of hay, oats, and turnip slices. The endosulfan-^{14}C was spread on cabbage leaves after it had been dissolved in acetone and leaves were fed to the sheep. The dose was approximately 0.30 and 0.26 mg. per kg. of body weight. The dosages of 14.8 and 13.5 mg., respectively, contained 1.4 and 1.3 mc. of carbon-14. The radioactivity in the blood was determined 2, 4, 6, 8, 12, 24, and 48 hours after

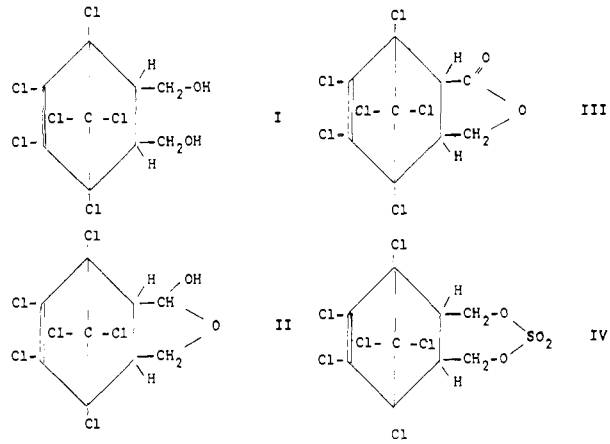

Figure 1. Structures

I. Endosulfan alcohol (Rahn, 1963)
II. α-Hydroxyendosulfan ether (Ballschmiter and Tölg, 1966)
III. Endosulfan lactone (Ballschmiter and Tölg, 1966)
IV. Endosulfan sulfate (Cassil and Drummond, 1965)

administration, then daily to the 21st day after withdrawal from a vein of the neck or leg with heparinized disposable syringes.

The sheep were milked in the morning and evening and the portions were collected daily.

Urine and feces were collected separately throughout the experiment and the excretions over a 24-hour period were examined.

Forty days after administration one animal was sacrificed, and various organs and tissues were removed for the determination of radioactivity. The levels in blood and in organs and tissues were calculated as micrograms of endosulfan per gram of whole blood from the percentage of radioactivity, regardless of the metabolization of the compound. The levels in milk, urine, and feces are percentages of the radioactivity administered.

PREPARATION OF SAMPLES FOR MEASUREMENT OF RADIOACTIVITY

Three parallel 0.1-ml. samples of whole blood were pipetted onto cotton and, after drying, burned in an

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atmosphere of oxygen, as described by Kalberer and Rutschmann (1961). After absorption of the CO_2 in 10 ml. of methanol-butylamine (4 to 1) and addition of an aliquot portion to 10 ml. of toluene scintillator, the radioactivity was measured in a liquid scintillation counter.

For direct determination of radioactive substance in the milk 0.1 ml. of freshly homogenized milk was dissolved in 1 ml. of 1*N* Hyamine hydroxide 10/X solution, 0.5 ml. of 0.1*N* sodium hydroxide, and 10 ml. of dioxane scintillator.

The radioactivity in the urine was directly determined after mixing an aliquot portion of 0.1 to 2.0 ml. with 15 ml. of dioxane scintillator.

The feces, organs, and tissues were carefully homogenized, and four to eight samples were weighed on filter paper. The subsequent working up, including the determination, was as for the blood. The dioxane scintillator consisted of 5 grams of 2,5-diphenyloxazole (PPO), 100 mg. of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP), and 100 grams of naphthalene per liter of dioxane. The toluene scintillator consisted of almost the same combination, with the exception of POPOP, 150 mg. per liter of which was present. These reagents were purchased from Merck AG, Darmstadt. The over-all error of individual values for blood, milk, urine, and feces was up to 10%, including errors in administration, sampling, pipetting, preparation, and measurement of samples.

All samples were counted in a Packard Tri-Carb liquid scintillation spectrometer, Series 3000. All quenching corrections were made with the aid of automatic external standardization.

EXTRACTION AND PURIFICATION OF RADIOACTIVE MATERIAL FROM TREATED ANIMALS

Feces. Two hundred grams of feces were mixed for a few minutes with acetone in a homogenizer. After a brief interval for sedimentation, the acetone was decanted into a round-bottomed flask through a filter. After two further extractions with the same quantities of solvent, the acetone was evaporated in a rotary evaporator. The aqueous residue was diluted with water to 150 to 200 ml. and extracted first with 100 ml. then with seven 50-ml. portions of benzene. The combined extracts were dried with anhydrous sodium sulfate and concentrated to 100 ml. in a rotary evaporator. Aliquots of the water and benzene phase were examined for metabolites by gas chromatography, thin-layer chromatography (TLC), and determination of radioactivity.

Measurement of the activity in the solid residue of the feces after extraction with acetone gave an estimation of the concentration of radioactive substances.

Urine. Fifty milliliters of urine were shaken with 50 ml. of benzene. Both phases had to be centrifuged (13,000 r.p.m.) for better separation before the extraction could be repeated with 50 ml. and then 25 ml. of benzene. The benzene phase was dried with anhydrous sodium sulfate and concentrated in a rotary evaporator, and the remaining solution was made up to 10 ml. with benzene. The aqueous solution separated was made up to 100 ml. with water, and an aliquot of this solution was adjusted to a 5% H_2SO_4 content and refluxed for 1 hour. After cooling, the solution was shaken with three 25-ml. portions of ether. The combined ether phases were then concentrated, and the volume was adjusted to 25 ml.

Milk. Fifty milliliters of milk were centrifuged at 13,000 r.p.m. and then well cooled in the centrifuge beaker before the fat phase could be removed with a spatula as solid platelets from the skim milk. The fat was transferred to a 100-ml. glass beaker and stirred with 5 grams of anhydrous sodium sulfate. The fat was then extracted in portions with 40 ml. of hexane with gentle warming, and the solution was transferred to a shaking funnel. After thorough shaking of the hexane phase with 75-, 50-, and 25-ml. portions of acetonitrile, extracts were concentrated in a rotary evaporator. The still moist evaporation residue was taken up with a total of 2 ml. of benzene. The benzene solution so obtained was then diluted to 20 ml. with methanol and cooled to $-20°$ C., a portion of the fat being frozen out. The clear supernatant methanol layer was decanted and then evaporated in a rotary evaporator. The still moist evaporation residue was dissolved and transferred to a graduated microcylinder with a total of 1 ml. of benzene. The solution so obtained was used for examination by gas and thin-layer chromatography.

THIN-LAYER CHROMATOGRAPHY

The technique described by Stahl (1967) was used. Spots were detected by radiometric scanning and by spraying with Rhodamine B as reagent (Ballschmiter and Tölg, 1966).

GAS CHROMATOGRAPHY

The gas chromatograph used had a built-in module for combustion of the eluted components (Gorbach, 1965). The microcoulometer of Dohrmann Instruments (Coulometer C 200, T 300 chlorine cell, T 300 P sulfur cell) was used as detector. The gas chromatographic parameters were as follows:

Column packing
- I. 10% SE 30 on Chromosorb W, 60- to 80-mesh (preparation according to Cassil, 1962)
- II. 10% QF 1 on Chromosorb W, 60- to 80-mesh (preparation according to Cassil, 1962)

Column material. Glass
Column dimensions. Length 1000 mm., i.d. 4 mm.
Column temperature. 200° C.
Temperature of injection port. 270° C.
Temperature of combustion oven. 830° C.
Carrier gas. Nitrogen, 100 to 200 ml. per minute
Oxygen. 100 ml. per minute
Recorder. 1 mv., chart speed 1140 mm. per hour

All V_{ik} values (relative gross retention volumes) are based on the retention volume of α-endosulfan as standard ($V_{ik} = \lambda$).

RESULTS AND DISCUSSION

Blood. The activity in the blood had reached a maximum in both sheep after 24 hours with levels of 4.3 and 4.5×10^{-4}% of activity administered per milliliter of whole blood, equivalent to 0.07 μg., of endosulfan per ml. After 21 days, the remaining concentration in the blood was only a tenth of the maximum—i.e., 0.007 μg. per gram (Table I).

Milk. The elimination of activity in the milk was approximately the same for both sheep in the first 2 days after administration, 0.4% of that administered. The

highest concentration measured was 0.15 μg. per gram (sheep 2, 24 hours) and was thus about three times that in the blood. After one week, the concentration had fallen below one tenth of the maximum. On day 17, only 0.002 μg. per gram could still be determined. Altogether, 0.87 and 1.82%, respectively, of the quantities administered were eliminated in 17 days (Table II). After the whole milk was separated into skim milk and cream by centrifugation, analysis showed that up to 88% of the radioactive materials remained in the cream.

Cream from the first-day sample of sheep 2 was cleaned up as described above. The radioactive material was shown to be endosulfan sulfate by gas and thin-layer chromatography. The identification was confirmed by eluting the endosulfan sulfate from the thin-layer chromatoplates and characterizing by gas chromatography on two columns of different polarity (Table III). The quantitative determination with the microcoulometric detector revealed that the activity contained in the milk fat was carried to about 100% by the endosulfan sulfate. The nature of the remaining radioactive material in the skim milk was not investigated.

Feces. Maximum excretion of activity in the feces was reached on day 2 with 20.8 and 18.6%, respectively, of the radioactivity administered. On day 22, the animals excreted 0.03% of the activity administered. Altogether 49 and 52%, respectively, were excreted in 22 days (Table IV).

The average radioactivity in the various feces extracts as described above is shown in Figure 2.

The main carriers of the radioactivity in the benzene phase (Figure 2, item 4) were α- and β-endosulfane, as shown by gas and thin-layer chromatography (Table V). The lactone, diol, and hydroxyether of the endosulfan were not detectable and if present ranged below 0.5 p.p.m.

There was no attempt to determine the nature of the substance remaining in feces after the acetone extraction (Figure 2, item 2).

Urine. The excretion of activity with the urine reached a maximum in the first 24 hours at 18.5% of the dose administered. The concentration then fell steadily, and on

Table I. Radioactivity Level in Blood Calculated as Micrograms[a] of Endosulfan per Gram of Whole Blood (P.P.M.)

Time after Administration	P.P.M. Endosulfan	
	Sheep 1	Sheep 2
2 hours	0.016	0.022
4	0.025	0.038
6	0.027	0.050
8	0.037	0.054
12	0.061	0.058
24	0.064	0.061
28	0.062	0.059
2 days	0.047	0.050
3	0.038	0.039
7	0.018	0.024
14	0.010	0.010
21	0.006	0.007

[a] Values in micrograms calculated from percentage content of radioactivity, disregarding metabolization of compound.

Table II. Radioactivity of Milk in Per Cent of That Administered

Time after Administration, Days	Sheep 1		Sheep 2	
	Sample vol., ml.	%	Sample vol., ml.	%
1	560	0.35	430	0.46
2	460	0.33	500	0.45
3	290	0.13	460	0.44
4–7	200	0.037	3 200	0.35
8–12	490	0.019	2 900	0.088
13–17	200	0.002	3 100	0.036
Total		0.868		1.824

Table III. R_f and V_{ik} (Relative Retention Volume) Values of the Metabolite in Milk Compared with Reference Substance

	Metabolite	Endosulfan Sulfate
	Thin-Layer Chromatography[a]	
R_f	0.6	0.6
	Gas Chromatography	
V_{ik} (QF 1)	3.6	3.7
V_{ik} (SE 30)	1.6	1.6

[a] Layer, Al_2O_3 Merck G, 0.5 mm. Solvent system, Acetone-hexane (1 + 1).

Table IV. Radioactivity of Urine and Feces (Per cent of that administered)

Time after Administration, Days	Urine, %		Feces, %	
	Sheep 1	Sheep 2	Sheep 1	Sheep 2
1	18.5	18.5	9.8	11.6
2	13.4	3.6	20.8	18.6
3	5.6	13.0	6.0	7.6
4	2.1	2.9	3.6	4.6
5	0.84	1.2	1.5	2.3
6–7	0.67	1.0	1.2	3.4
8–12	0.48	0.82	5.8	3.4
13–17	0.21	0.19	0.28	0.44
18–22	0.11	0.11	0.18	0.20
Total	41.91	41.32	49.16	52.14

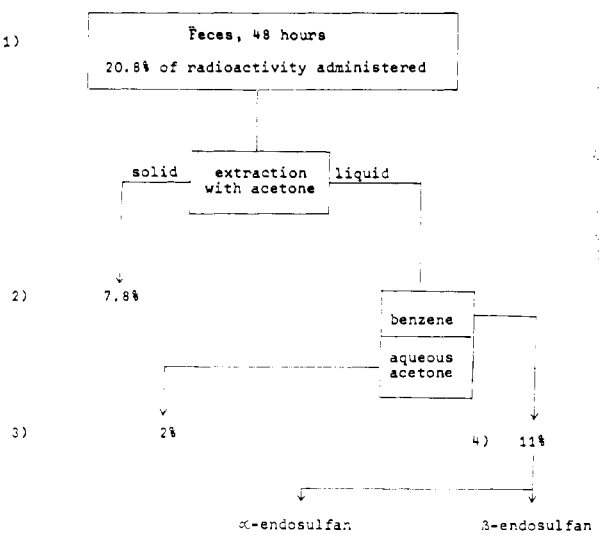

Figure 2. Schedule of activity distribution in feces of first sheep on second day after administration

day 22 only about 0.01% was still being excreted. Altogether 41.9 and 41.3%, respectively, of the activity administered were excreted via the urine in 22 days (Table IV).

The urine was not a homogeneous solution. After standing for a short time, a precipitate formed which contained only ~10% of the total activity in the urine. The experiments were therefore carried out with the clear supernatant urine above the sediment. Extraction and cleanup of urine were carried out as described above. A part of the endosulfan-^{14}C equivalent was transferred to the benzene phase, and could be separated into two parts by thin-layer chromatography (Figure 3, item 4).

One of the R_f values obtained was identical with that of endosulfan alcohol and the other with that of α-hydroxyendosulfan ether. To confirm the identification, the corresponding portions of the thin-layer chromatogram were eluted with methanol, and the two eluates were characterized by gas chromatography via their retention times in two columns of different polarity (Table VI). According to the gas chromatograph, the components were in the ratio of 70% endosulfan alcohol to 30% α-hydroxyendosulfan ether. A radiogram of the thin-layer chromatograph gave a ratio of 80 to 20.

The activity in the aqueous phase (Figure 3, item 5) could be separated into two main fractions on silica gel GF_{254} with 1-propanol and ammonia (1 to 1), one of which remained near the start line and the other attained an R_f of approximately 0.4. These metabolites are not yet identified.

After saponification of the aqueous phase in 5% sulfuric acid (1 hour of reflux) a substance was further extractable, which behaved in gas chromatography and TLC like endosulfan alcohol.

Organs. With the exception of the liver, large intestine, and fat, the organs and tissues of sheep sacrificed after 40 days revealed concentrations of less than 0.02 μg. per gram; in the liver, which had the highest concentration, 0.03 μg. per gram was detected.

Table V. R_f and V_{ik} (Relative Retention Volume) Values of Chief Substances Extracted from Feces, Compared with Reference Substances

	Radioactive Spot 1	α-Endosulfan	Radioactive Spot 2	β-Endosulfan
	Thin-Layer Chromatography[a]			
R_f	0.70	0.68	0.84	0.86
	Gas Chromatography			
V_{ik} (QF 1)	1	1	1.7	1.7
V_{ik} (DC 200)	1	1	1.3	1.3

[a] Layer. Silica gel, Merck GF_{254}. Solvent system. Benzene + 5% ethyl acetate. Spray reagent. Rhodamine B.

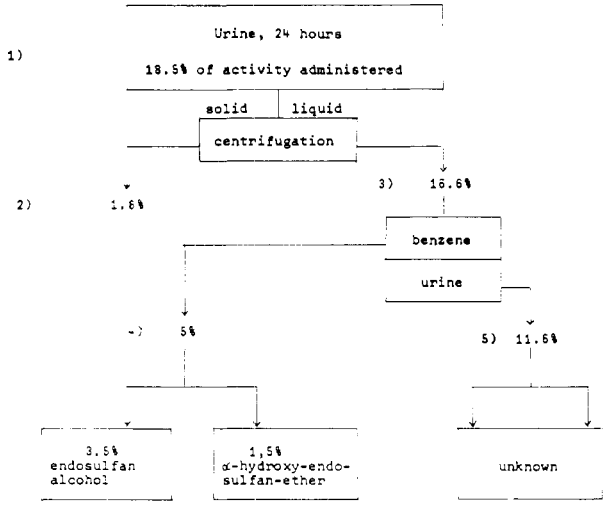

Figure 3. Schedule of activity distribution in 24-hour urine sample of second sheep

Table VI. R_f and V_{ik} (Relative Retention Volume) Values of Metabolites in Benzene Extract of Urine, Compared with Reference Substances

	Metabolite A	Endosulfan Alcohol	Metabolite B	Hydroxy-endosulfan Ether
	Thin-Layer Chromatography[a]			
R_f	0.2	0.21	0.46	0.45
	Gas Chromatography			
V_{ik} (SE 30)	1.35	1.37	0.74	0.73
V_{ik} (QF 1)	1.58	1.54	0.60	0.62

[a] Aluminum oxide Merck GF_{254}, 0.25 mm. Solvent system. Acetone-hexane (1 to 1). Spray reagent. Rhodamine B (Ballschmiter and Tölg, 1966).

CONCLUSIONS

The total radioactivity recovered amounted to nearly 95% of that administered. About one half of the activity was excreted by the feces and the other half by the urine. A certain amount of active substances remained unidentified in the feces (Figure 2, item 2) and urine (Figure 3, item 5).

The investigations are being continued by another working group.

Very important for the application of the compound as an insecticide is the fact that no fat-soluble metabolite other than endosulfan sulfate was detected in milk, and that no major metabolite is retained in fat or in one of the organs for a longer time.

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