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METABOLISM, STORAGE, AND EXCRETION
OF C¹⁴-ENDOSULFAN IN THE MOUSE.

The Ohio State University, Ph.D., 1965
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METABOLISM, STORAGE, AND EXCRETION OF C¹⁴-ENDOSULFAN IN THE MOUSE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of the Ohio State University

By

Prayoon Deema, B.Sc., M.S.

* * * * *

The Ohio State University

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Approved by

Adviser

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INTRODUCTION

The effectiveness of endosulfan, 6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin 3-oxide, for the control of wide range of insect pests of various crops is well recognized (U.S. Dept. Agric. 1964). The potential widespread use of this compound raises a problem concerning the manner in which an animal can detoxify and eliminate the ingested pesticide. The purpose of this investigation was to identify some of the metabolism products of endosulfan in the mouse organs, urine, and feces and to determine whether and where it is stored in animal tissue.

Endosulfan, which has the trade name of Thiodan, was synthesized by Farbwerke Hoechst A.G., Frankfurt, Germany, in 1956, and is protected by U.S. Patent 2,799,685 (Farbwerke Hoechst, 1955). It was developed in the United States by the Niagara Chemical Division, Food Machinery and Chemical Corporation. In the United Kingdom endosulfan is known chemically as 1,2,3,4,7,7-hexachloro-bicyclo (2,2,1)-2-hepten 5,6-bis (hydroxymethylen)-sulphite (Reimschneider, 1963). It has an empirical formula of $C_9H_6O_3Cl_6S$ and a molecular weight of 406.97. The chemical structure is shown in Figure 1. Technical endosulfan, which is a light-brown solid, contains up to 96% endosulfan and is made up of the low-melting isomer (melting at 106° - 108° C) and the high-melting isomer (melting at 208° - 210° C) in the approximate ratio of 2:1 with a melting range of 70° - 100° C. The remaining 4% is made up of related

ENDOSULFAN

ENDOSULFAN ALCOHOL

ENDOSULFAN ETHER

ENDOSULFAN SULFATE

Figure 1. The structures of endosulfan and related chemicals.

compounds and inert materials. Endosulfan is insoluble in water but moderately soluble in most organic solvents. It has a density of 1.745 at 20°C, and is sensitive to moisture, bases, and acids. It decomposes slowly by hydrolysis to endosulfan alcohol and SO₂ (Dawsey, 1964; Graham et al. 1964; Kenaga, 1963, and Martin 1961).

Lindquist and Dahm (1957) used column and paper chromatography, ultraviolet and infrared spectroscopy to separate the isomers of endosulfan and its alcohol. In tests against female house flies, from a non-resistant strain, the estimated LD₅₀ value was 6.7 ug/g. The acute oral LD₅₀ of purified technical grade endosulfan to male white rats was between 40 and 50 mg/kg of body weight.

Zweig and Archer (1960) used gas chromatography for the quantitative determination of endosulfan and its isomers in technical endosulfan and in emulsifiable concentrate formulations. Endosulfan extracts in benzene was eluted through a silicone chromosorb column, and its isomers was analyzed in quantities from 25 to 1000 ug with the Beckman GC-2 and the Wilkins Aerograph Model A-100. Terranova and Ware (1963) used gas chromatography and paper chromatography to determine endosulfan and related components in bean plants. Barnes and Ware (1964) applied the techniques of gas chromatography, paper chromatography, and autoradiograms in studying the penetration and metabolism products of endosulfan in the house fly, Musca domestica L.

An infrared spectrophotometric technique has been developed by Hooker Chemical Company for the assay of technical endosulfan. This method distinguishes between the isomers of endosulfan, can be used to determine the endosulfan alcohol and endosulfan ether impurities, and is precise to approximately 2-3% (Graham et al. 1964).

Cassil (1958) and Graham (1959) described various colorimetric methods for determining endosulfan residues. Their procedures have been used as a general method for the control and routine testing of endosulfan dusts, wettable powders, and emulsifiable concentrates. In this method, which is a sulfite-iodometric procedure, endosulfan reacts with alkali to form an inorganic sulfite. This salt is then titrated with iodine and a starch indicator. The sulfite technique does not differentiate between the two endosulfan isomers since both respond to this method in the same way. Endosulfan can be determined in the presence of endosulfan alcohol and endosulfan ether. Any compound that releases sulfur dioxide or other reducing substances upon treatment with alkali interferes. Maitlen, Walker and Westlake (1963) described an improved method wherein a pyridine-methanolic sodium hydroxide reagent was mixed with endosulfan extracts to develop a color, the density of which was proportional to the quantity of endosulfan and followed Beer's law.

MATERIALS AND METHODS

Eighty laboratory mice of the BALB/cJaxGnMc strain, sibling mated for more than 90 generations, were used in this study. Fifty males and thirty females were randomly selected from the healthy parents; the ages of these mice averaged about 90 days at the start of the experiments. Three to six mice of the same sex were usually housed in each cage. The cages were stainless steel 12 x 5 $\frac{1}{4}$ x 5 inch. (Figure 2). Water and food were given ad libitum. The cages were cleaned and cedar shavings litter changed weekly and the water changed twice a week. The food used throughout the experiment was Purina Laboratory Mouse Chow.¹ The animals were housed in an air-conditioned rearing room temperatures ranging from 21°-23°C.

Description of test compounds

The Niagara Chemical Division, Food Machinery and Chemical Corporation,² furnished all of the endosulfan and related materials as well as the C¹⁴-endosulfan. The unlabeled materials were purified endosulfan, technical endosulfan, endosulfan ether, endosulfan alcohol, low-melting isomer (melting at 106°-108°C), high-melting isomer (melting at 208°-210°C), and endosulfan sulfate.

¹Manufactured by the Ralston Purina Co., Checkerboard Square, St. Louis, Missouri.

²Middleport, New York.

Figure 2. The stainless steel rearing cage

The C¹⁴-endosulfan had a specific activity of 0.577 uc/mg and the C¹⁴-label was located in the hexachlorocycloidiene ring at carbons 5 and 6 (Figure 1, see asterisks).

Standard solutions

The standard solutions of endosulfan isomers used in laboratory analyses were prepared by dissolving 10 mg of the compound in 10 ml of glass-distilled acetone. One milliliter of the original mixture was then mixed with 9 ml of glass-distilled hexane. Dilutions were made until the final standard contained 0.1 nanogram (10^{-10} gram) in one microliter of the solution (0.1 ng/ul). These standard solutions were used in determining residues in the animal organs, by paper, thin-layer and gas chromatography.

Fifty milligrams of C¹⁴-endosulfan was dissolved in 10 ml of glass-distilled acetone. One microliter of this solution then contained 5 ug of C¹⁴-endosulfan.

Metabolism studies of unlabeled endosulfan

All mice used in the metabolism studies were fasted 12-18 hours and weighed prior to feeding the treated and untreated food. These mice were then weighed again at the end of each study or prior to sacrifice. Organs or tissues such as liver, kidney, visceral fat, stomach, small intestine, muscle, brain, blood, heart, and spleen were weighed and placed in separate screw-cap vials which were stored in the freezer at 0°C until extracted.

Five separate studies are involved. With normal or unlabeled endosulfan, mice were sacrificed 12 and 24 hours after a single dose and at weekly intervals during the feeding of 10 ppm dietary endosulfan. Using C¹⁴-endosulfan mice were sacrificed 24 hours after receiving a single dose, and after six daily doses.

Twelve-hour studies

Three adult males were used in this study. Two mice were given the endosulfan-treated mouse food at the concentration of 0.30 mg/300 mg of food. A control mouse was given 300 mg of food treated with 60 ul of acetone. To prepare the standard solution, 50 mg of the purified endosulfan was dissolved in 10 ml of glass-distilled acetone, producing a concentration of 5 ug/ul. Sixty microliters of the standard mixture were dropped on 300 mg of food using a foot-activated microapplicator, ISCO Model M³ (Figure 3), fitted with a # 26 gauge needle. The microapplicator had been adjusted to deliver one microliter of liquid per signal.

After a mouse had eaten all the treated food, 15-30 minutes, it was placed in the Roth metabolism cage (Roth et al. 1948)(Figure 4). Untreated food was offered at intervals to these mice during the experimental period. Urine and fecal pellets were collected throughout the 12-hour period, and activities of each mouse were recorded until sacrificed.

Figure 3. The ISCO Model M microapplicator

Figure 4. The Roth metabolism cage, modified to accomodate the laboratory mouse.

Twenty four-hour studies

Purified endosulfan.— Ten adult males were used in this study. The microapplicator was used in preparing purified endosulfan-treated food at 0.30 mg/300 mg of food for each mouse. Nine mice were fed the test diet and one the control diet.

After each mouse had consumed all the food, it was placed in the Roth metabolism cage. Untreated food was fed to them during the experimental period. Urine and feces were collected throughout the 24-hour period, and activities of each mouse were recorded during the studies.

Low-melting isomer.— Three adult males were used in this study, two fed 0.30 mg of low-melting isomer and one the control diet. The treated and control foods were prepared as in the purified endosulfan experiment except for the use of low-melting isomer. Treatment and handling were the same as just described.

High-melting isomer.— Three adults males were used in this study, two for the 0.30 mg of high-melting isomer and one for the control diet. The treated and control foods were prepared as in the low-melting isomer experiment. Treatment and handling were also the same.

³Manufactured by Instrumentation Specialties Co., 5624 Seward, Lincoln, Nebraska.

Metabolism studies of purified endosulfan
longer than 24 hours

Twenty mice, 10 males and 10 females, were used in this study. They were held in the stainless steel cages, one mouse in each cage. Eight of each sex were fed the food containing 10 ppm of the purified endosulfan and two of each sex were fed with the control food treated with the control food treated with acetone.

To prepare the 10 ppm purified endosulfan diet, 6 kilograms of Purina Laboratory Mouse Chow were ground in a burr mill. The pulverized food was placed in an electric rotary mixer and 75 ml of purified endosulfan solution was evenly and gradually distributed over it. The purified endosulfan solution was made from 60 mg of the purified endosulfan in 75 ml of acetone. After the addition of 700 ml of tap water, the mixture was stirred 45 minutes. The treated chow was then pelleted into 3/8 inch diameter cylindrical pellets with a laboratory pellet mill⁴(Figure 5).

Thirty grams of 10 ppm purified endosulfan treated food were given to each mouse at the start of the experiment. The treated and untreated foods were added whenever necessary throughout the experiment. The amount of food consumed by each mouse per day was recorded until sacrificed. At the end of 7, 14, 21, 28, 35, 42 and 49 days a male and a female on the treated diet were sacrificed. A mouse fed with acetone treated food was sacrificed at the end of 14, 28, 42 and 49 days.

⁴Manufactured by the California Pellet Mill Co, Crawfordsville, Indiana.

The mice were weighed at the beginning of the experiment and also before sacrifice. Organs of the sacrificed mouse were dissected, weighed, placed in the screw-cap vials and kept in the freezer at 0°C until extracted.

Figure 5. The laboratory pellet mill.

Extraction procedures of
unlabeled endosulfan

The organs as well as urine and feces, were removed from the freezer and allowed to reach room temperature prior to extraction. Each organ was diced with scissors and macerated with mortar and pestle in 5x its weight of anhydrous sodium sulfate until powdered. The macerated organ was then extracted three times with a total of 25 ml glass-distilled acetone and additional maceration (Taylor, Rea and Kirby, 1964). The extracts were filtered through a Whatman # 1 filter paper⁵ into a 40 ml separatory funnel. Five milliliters of distilled water and ten milliliters of glass-distilled hexane were added to the filtrate, shaken well and allowed to stand for four minutes. The acetone portion was transferred into a second separatory funnel, and washed with ten milliliters of glass-distilled hexane, shaken well and allowed to stand for five minutes. The bottom portion was discarded and the hexane rinses combined in the screw-cap vial and dried in the hood. Five milliliters of glass-distilled hexane was added to the sample extract to dissolve the dried extracts prior to injection into the gas chromatograph. For the determination of endosulfan residues and the metabolism products of endosulfan in the animal organs and tissues by paper or thin-layer chromatography, only 0.5 ml of glass-distilled hexane was used for each sample spot. These extracts were again evaporated

⁵Manufactured by Messrs H. Reeve Angle & Co., Ltd., 9 Bridenvell Place, Clifton, New Jersey.

to dryness prior to returning to the freezer. At a later date in this investigation, benzene, rather than hexane and acetone, was used in the fecal extractions. Feces and 5x its weight of anhydrous sodium sulfate were macerated in a centrifuge tube. The feces-sodium sulfate was then extracted with 25 ml of benzene for approximately one hour, and the extract evaporated to five ml in the hood. One microliter of the extract was injected into the gas chromatograph. The gas chromatographic results showed that in the benzene-extracted feces, oxidized endosulfan was detected. The results indicate that benzene is a superior solvent for extracting feces of mice.

Metabolism studies of C¹⁴-endosulfan

The Roth metabolism cage was designed to collect urine, feces and respired air of an animal while it is metabolizing a radioactive material, particularly one tagged with C¹⁴. If these metabolic products are collected at regular intervals, rates of detoxication, metabolic oxidation and other metabolic processes can be measured.

The apparatus which was used is shown in Figure 6. A mouse was placed into the cage on a galvanized steel hardware cloth floor. Fresh air, dried and freed of CO₂ by passage through Drierite⁶ (6 mesh) and Ascarite⁷ (8-20 mesh), was drawn into the

⁶Manufactured by Drierite Co., Xenia, Ohio.

⁷Manufactured by Arthur H. Thomas Co., Philadelphia, Pa.

system through the opening tube at the top of the cage at a rate of approximately 40 ml per minute as measured by a flow meter.⁸ The exhaled CO₂ was collected in a train of five gas scrubbers containing 25 ml each of 0.5 N NaOH. Urine was collected in a vial and feces were collected from the cage in a glass tube beneath the cage. Water was supplied through the top of the cage by a water bottle. A single stick of mouse food 3/8 x 1 inch was provided through the feeding tube, which allowed the animal to eat without gnawing off any large particles and taking them into the cage. In this study the air was drawn through the animal chamber by a vacuum pump which caused a slight negative pressure on the outlet side. In this way labeled carbon dioxide would not be lost should a small leak develop in the cage.

Six adult mice were used in this study, two males and four females. Based on radioactivity measured by liquid scintillation of a standard collected at the time of food treatment, two were fed 0.30 mg, two fed 0.25 mg, and two fed 0.20 mg of C¹⁴-endo-sulfan in 300 mg of food. The microapplicator was used to apply the material as described above. A male was used as the control mouse and fed with the regular mouse food to which was added 40 µl of acetone. One mouse was studied each time in the Roth metabolism cage during the 24 hours. Urine and feces were collected and removed at each excretion.

⁸ Manufactured by Fisher and Porter Co., Distributed by the Emil Greiner Co., 20-26 N. Moor Street, New York 13, N.Y.

Figure 6. Apparatus used in the studies of C^{14} -endosulfan metabolism in the mouse.

Extraction procedures
of C¹⁴-endosulfan

Urine samples were collected, measured, and diluted with 25 ml glass-distilled acetone. The urine-acetone solution was then extracted with 20 ml glass-distilled hexane after the addition of 5 ml of distilled water. The extract was air-dried in the hood. Ten ml of a liquid scintillator (5 g of 2,5-diphenyloxazole, PPO; 100 mg of 2,2' paraphenylene bis 5-phenyloxazole, POPOP; and 1000 ml of toluene)(Hayes, 1963) was added and the vial shaken thoroughly to dissolve the residue. The activity was read with the liquid scintillation spectrometer (Figure 7). This instrument was a Packard liquid scintillation spectrometer,⁹ Model 3314, which had a 200 sample capacity, a belt-type sample changer, three channels, and an automatic data lister. The data were collected from the red channel, the most appropriate for C¹⁴ and the gain set at 8.5%. The window was set at 50-1000 for the optimum reading. The operating temperature was -6°C. Twenty ml glass screw-cap counting vials⁹ were used. The reading times were 1, 2, and 10 minutes for each sample. Since the background was between 16-18 counts per minute, all counting was standardized for 17 counts per minute background.

⁹Manufactured by the Packard Instrument Co., Inc., Box 428
La Grange, Ill.

Figure 7. The tri-carb liquid scintillation spectrometer model 3314.

Feces were ground with 5x their weight of anhydrous sodium sulfate in a small mortar 2 3/4 inches in diameter. The ground material was extracted three times totaling 25 ml of glass-distilled acetone. The extracts were filtered through an 11 cm Whatman # 1 filter paper into a 40 ml separatory funnel. Five ml of distilled water and 10 ml of glass-distilled hexane were added to the filtrates which were shaken well and allowed to stand for four minutes. The acetone portion was transferred to a second separatory funnel and extracted with 10 ml of glass-distilled hexane, shaken and allowed to stand for five minutes. The hexane extracts were combined in 20 ml screw-cap vials and dried in the hood as also were the acetone-water extracts and feces-sodium sulfate mixture.

Respired carbon dioxide was collected in 0.5 N NaOH and converted to BaCO_3 by adding 10% BaCl_2 in excess. The BaCO_3 was precipitated, filtered through a Millipor filter (which retained particles 0.45 μ diameter or larger) and dried in the oven at the temperature of 100°C for 5 hours. The activities of 0.25 gm, 0.5 gm and 1.0 gm samples were measured in the liquid scintillation spectrometer.

Two female mice were sacrificed after the 24-hour observation period. Liver, kidney, brain, visceral fat, blood and small intestine were extracted with acetone and hexane, by the same procedure given for feces. The hexane extracts, acetone-water

extracts and the tissue-sodium sulfate portion were kept in the screw-cap vials and air dried. Ten ml of the liquid scintillator described above was added to each vial, shaken well and the activities were read with the liquid scintillation spectrometer.

The carcass of the sacrificed mouse, less organs, was weighed before and after the skin was removed. The skin was air-dried in the hood for 24 hours. The carcass was then diced with scissors and macerated with 5 times its weight of anhydrous sodium sulfate in a 7 inches mortar until powdered and then weighed again. Three one gm samples of the carcass-sodium sulfate were used in reading the activities with the liquid scintillation spectrometer.

The dried skin was also cut into small pieces with scissors and then mixed with the remaining carcass-sodium sulfate powder. The skin and carcass mixture was then divided into two parts and the CO_2 extracted from each by means of the Van Slyke procedure (Van Slyke and Folch, 1940; Van Slyke, Plazin and Weisiger 1951). The reagents used were based on the amounts of carbon in the animal tissues, which was estimated to be 60 percent of the dry weight of the mouse. Seventy grams of dry reagents ($\text{KIO}_3 + \text{K}_2\text{Cr}_2\text{O}_7$ 10:1 W/W) and 150 ml of an acid mixture (1000 ml H_3PO_4 , 1000 ml H_2SO_4 and 30 gm KIO_3) were used per gram of carbon in the dried tissue. The apparatus used for C^{14}O_2 production by Van Slyke procedure is shown in Figure 8.

Figure 8. Apparatus for $C^{14}O_2$ production by Van Slyke procedure.

The sample was heated with an open flame and the CO_2 produced by the reaction was passed through a stannous chloride tube to remove free iodine and was collected in a one liter glass tower filled with 900 ml 2 N NaOH. A number of marbles were placed in the bottom of the tower and a glass wool plug at the top to increase absorption of CO_2 by the NaOH. The combustion stopped after one hour of reaction and the system was flushed with air for 5 minutes. The NaOH solution in the tower was then transferred to a 2000 ml beaker, 400 ml of 10% BaCl_2 were added and the mixture was stirred. The BaCO_3 precipitated and was filtered through the Whatman # 1 filter paper. The BaCO_3 and the filter paper were dried in an oven at 100°C for 5 hours before the weight of BaCO_3 was determined with an analytical balance. The dried BaCO_3 was powdered with a 7 inches mortar and pestle and 0.25 gm, 0.5 gm and 1.0 gm samples were placed in 20 ml screw-cap vials. Ten ml of the liquid scintillator were added to the sample and the activities were read in the liquid scintillation spectrometer.

The activity of the C^{14}O_2 produced by digesting a pair of four grams carcass-sodium sulfate samples with the Van Slyke reagents (Van Slyke et al. 1951) was read on a model 6000 Dynacon electrometer.¹⁰ A four gm sample was placed in the sample vial and 14 grams of dry reagent was added. The vial was lubricated with H_3PO_4 and then attached to the system. The ionization chamber

¹⁰ Manufactured by Nuclear-Chicago Corporation, 333 East Howard Ave. Des Plaines. Ill.

was evacuated through a by-pass and filled with CO_2 through the by-pass two or three times and then connected to the Van Slyke system. The by-pass then was filled up with CO_2 . The liquid Van Slyke reagent was added to the sample vial (about $\frac{1}{2}$ inch above the lower surface of the entry tube) and the sample and the reagents were heated until the reaction stopped. Normal CO_2 was then bubbled through the sample vial and all CO_2 obtained from the reaction was flushed into the ionization chamber with the carrier gas (filled to atmospheric pressure). All stopcocks were closed and the ionization chamber was removed and installed into the model 6000 Dynacon electrometer system on which the activity of the sample was read in amperes. This value was compared with the value of a CO_2 standard or with the standardization chamber.

Metabolism studies of C^{14} -endosulfan for Autoradiograms

Three mice, two males and one female, were used in this study. A male and a female were each given 0.30 mg C^{14} -endosulfan on 300 mg of food. A male control mouse was fed control food treated with 60 ml glass-distilled acetone on 300 mg of food. These mice were placed in the metabolism cages. Urine and feces were collected and activities of the mice were recorded throughout the 24 hours. At the end of the study these mice were sacrificed and their organs extracted using the acetone-hexane method.

The extractions were dried in the hood and were kept in the freezer until paper chromatograms could be developed from which autoradiograms were made.

Metabolism studies of C¹⁴-endosulfan
longer than 24 hours

Ten mice, eight males and two females, were used in the studies. One male and a female were used for the control animals. The treated mice were fed with 0.1 mg C¹⁴-endosulfan twice daily for several days. These mice were sacrificed at intervals and the activities and location of radioactive materials were determined by using the autoradiogram technique.

Paper chromatography

Ascending, reversed phase paper chromatography was used to identify the residues of endosulfan and its metabolism products in mouse organ and tissue extracts. The methods used were based on those of Mitchell (1958) and Terranova and Ware (1963).

A sheet of Whatman # 1, filter paper,⁵ 18 $\frac{1}{4}$ x 22 $\frac{1}{2}$ inches, was cut to make four sheets 8 x 10 inches. The papers were cut in a manner that when developed the mobile solvent would move in the same direction as the grain of paper. A line $\frac{3}{4}$ inch from the bottom of the paper was then drawn the width of the sheet with a graphite pencil. This line was marked at nine points

3/4 inch apart ending one inch from either side of the paper. The scraps were used to make strips 1 x 8 inches which were folded lengthwise, placed over the top of the 8 x 10 inches sheet and stapled so that a 1/8 inch glass rod could be inserted in the fold to support the paper. Each sheet was washed 12 times with distilled water and glass-distilled acetone alternately and hung to dry at least 24 hours. The dried paper was then sprayed on both sides with a total of 50 ml of diethyl ether in which was dissolved 4% USP medium grade mineral oil (V/V) and dried for at least 24 hours prior to spotting with extracts. A 10 ul Hamilton micro-syringe¹¹ was modified for the spottings by cutting the pointed tip of the needle off and then bending the needle to a 90° angle. The standard solution and the extracts were dried with warm air from a hair dryer after each spotting to keep the spot small prior to the next spotting. Each spot was identified with a pencil mark. Up to six chromatograms were then put in the chromatographic developing tank, 9 x 12 x 13 inches hard rubber, which had been lined with filter papers. The chromatographic tank was then closed and sealed with masking tape.

Fifty ml of the mobile solvent, 65% glass-distilled methylcellulose and 35% distilled water, were placed in the trough through a side opening and allowed to equilibrate for 2 hours before adding more mobile solvent to raise the level $\frac{1}{2}$ inch above

¹¹ Manufactured by Hamilton Company Inc., Whittier, Calif.

The lower edge of the suspended chromatograms. The chromatograms were developed in the tank for at least six hours or until the mobile front reached a point one inch from the top. The chromatograms were removed from the tank, the front marked with a graphite pencil and hung to dry at room temperature. They were then sprayed with a chromogenic agent or exposed to Kodak No-screen Medical (8 x 10 inches) X-ray film.¹² The chromogenic agent used was based on the method of Mitchell (1958), which contained 0.85 gm AgNO_3 dissolved in 2ml of distilled water and 10 ml 2-phenoxy ethanol diluted to 200 ml with glass-distilled acetone. Both sides of the chromatogram were sprayed with 40 ml of the chromogenic agent, 20 ml per side. The sprayed chromatogram was air-dried prior to placing under three, 30 watt, ultraviolet lamps¹³ mounted in a specially built case six inches above the chromatograms, for 20 minutes or until the spots appeared. The spots were circled with a graphite pencil and then the chromatograms were washed twice with distilled water and air-dried.

R_f values were determined for the standard isomers of endosulfan and for technical endosulfan. These values were used as the basis for determining the results of the residues and metabolism studies.

¹²Manufactured by Eastman Kodak Co., Rochester 4, New York.

¹³Manufactured by the General Electric Co., Cleveland, Ohio

Gas chromatography

A Barber Colman Model 15 gas chromatograph¹⁴ was used for analysis of all endosulfan extracts. The chromatograph was equipped with an electron capture detector which utilized either radium 226 or tritium. The recorder, a single point series 8000 with 1/3 inch per minute chart speed, was used for recording the results from all extracts. The column used in this study was a 4-foot, 1/8 inch inside diameter, U-shaped, Pyrex glass column. The column was packed with 1.5% SE 52 silicone gum rubber coated on 60/80 mesh Gaschrom¹⁵ solid support. The anode voltages used in this study varied between 35-37.5 volts except where indicated. Throughout the experiment the temperatures of operation were the column at 205°C, the detector at 220°C, and the injection block at 250°C. The sensitivity of the machine was set either at 10 or 30, with an attenuation of one. A molecular sieve column connected the gas chromatograph and the tank of Hy-pure Nitrogen gas.¹⁶ The nitrogen flow measured on the effluent side of the detector was 100 ml/min with no scavenger flow.

A 10 µl Hamilton micro-syringe was used to inject the extracts and the standard mixtures. The amount of the sample

¹⁴Manufactured by the Barber-Colman Co., Rockford, Illinois.

¹⁵Manufactured by Applied Science Laboratory, 140 N. Barnard Street, State College, Pennsylvania.

¹⁶Manufactured by Liquid Carbonics Division of General Dynamics.

injected each time was carefully measured and controlled as described by Barnes and Ware (1964, in press). The injections were made twice for each extraction with 2 μ l or more for each injection. To identify an unknown peak from the animal extracts, the standard mixture and the unknown sample were combined and injected together. The standard mixtures were injected alternately with the animal extracts to check the sensitivity of the detector throughout the period of injection.

Thin-layer chromatography

Thin-layer chromatography on silica gel¹⁷ was used for the determination of endosulfan residues and endosulfan metabolites in the mouse organs, urine and feces. Walker and Beroza (1963) discussed the use of the thin-layer chromatography as a method for the cleanup of insecticides and 19 testing dyes. Kovacs (1963) also used thin-layer chromatography to detect chlorinated pesticide residues in various food products. He stated that thin-layer chromatography techniques could detect chlorinated pesticide residues as low as 1 part per billion. Morley and Chiba (1964) demonstrated the use of thin-layer chromatography for detecting chlorinated pesticide residues without cleanup.

The equipment used was a fixed thickness applicator,¹⁸ the chromatographic tank, plate holder, drying rack and

¹⁷Manufactured by E. Merck, Darmstadt, West Germany.

¹⁸Manufactured by the Research Specialties Co., 200 South Garrard Blve., Richmond, Calif.

8 x 8 inches glass plates. To coat 5 plates, 30 g of silica gel and 55 ml distilled water were mixed together. The plates were dried in position on the mounting board for 20 - 25 minutes and then dried in the oven for 30 minutes at 75°C. The plates were prewashed twice with distilled water (Kovacs, 1963) prior to spotting with the extracts. To apply the extracts on the plate, a pencil mark was made $1\frac{1}{2}$ inches from the bottom edge of the plate on both sides. An imaginary line between the two points indicated the sample spotting or origin line. A line was drawn, which removed the coating, completely across the plate $5\frac{1}{2}$ inches from the bottom edge, to represent the solvent front after development. On the lower edge of the adsorbent, starting $\frac{3}{4}$ inch from the left edge of the plate, 9 marks were made with a pencil at $\frac{3}{4}$ inch intervals, to serve as horizontal guides for sample application. The identities of the samples were etched above the solvent front. The sample was repeatedly transferred with the microsyringe to one of the origin points on the plate, dried after each application to restrict spot size. Standard solutions of endosulfan were transferred to other spots on the same plate with the microsyringe. The chromatographic tank was cleaned and dried before using. The mobile solvent used was 4% redistilled acetone in n-heptane¹⁹(V/V). When the solvent front just reached the pencil line 10 cm above the spotting line, the plates were removed and allowed to air-dry in the hood for

¹⁹Manufactured by Phillips Petroleum Co., Bartlesville, Okla.

five minutes. The plates were then sprayed with the chromogenic agent, prepared with 1.7 gm AgNO_3 dissolved in 2 ml of distilled water and 10 ml 2-phenoxy ethanol diluted to 200 ml with glass-distilled acetone, using lateral motions of the spray bottle parallel to the direction of solvent flow. After spraying, the plates were dried in the hood for five minutes, then in a forced draft oven at 75°C for 15 minutes. The plates were removed from the oven and cooled then exposed to the ultraviolet lamps until the spot for the standard of the lowest concentration appeared.

Autoradiography

Autoradiography is concerned principally with the location of the radioactive atoms. Basically, the autoradiographic technique consists of placing a radioactive specimen in contact with a photographic emulsion. The radiation, passing through the film, sensitizes the silver grains which upon development will show the location of radioactive atoms in the specimen.

The paper chromatograms which were spotted with extracts from organs, urine and feces of the mice fed C^{14} -endosulfan were developed in the usual manner as nonlabeled endosulfan. After 6-7 hours of developing in the chromatographic tank, they were removed, air-dried and then placed under the Kodak No-screen Medical X-ray film (8 x 10 inches) in Kodak cassettes in the darkroom. They were left in the cassette for at least 25 days

before developing in DK-50 developer²⁰ for five minutes, fixed for 15 minutes and washed in 20°-21°C running tap water for two hours. The developed X-ray film was then air-dried and the spots were identified by comparing with the chromogenically produced spots of the paper chromatogram.

²⁰Manufactured by Eastman Kodak Co., Rochester 4, New York.

RESULTS AND DISCUSSION

Paper chromatographic analyses of both the purified endosulfan and its isomers are shown in Figure 9. The purified endosulfan produced two spots whose R_f values corresponded to those of the low- and high-melting isomers. The R_f values of the isomers of endosulfan are summarized in Table 1. A gas chromatographic analysis of all components and the endosulfan sulfate standards are presented in Figure 10.

Figure 9. Paper chromatograph trace of 5 ug quantities of endosulfan standards. Six-hour development at 24-25°C. Mobile phase, 65% methyl cellosolve in water. Stationary phase, 4% mineral oil in diethyl ether.

Table 1. - R_f values of technical endosulfan and related derivatives using reverse phase ascending paper chromatography.

Standard	R_f^*
Endosulfan Low-Melting Point isomer (LMP)	0.11, 0.06, 0.06
Endosulfan High-Melting Point isomer (HMP)	0.33, 0.26, 0.29
Endosulfan Ether (EE)	0.23, 0.26, 0.29
Endosulfan Sulfate (ES)	0.67, 0.75, 0.76
Endosulfan Alcohol (EA)	0.84, 0.83

* Mobile phase, 65% methyl cellosolve in water.
Stationary phase, 4% mineral oil in diethyl ether.

Figure 10. Gas chromatogram of endosulfan isomers and related products.

Detector : Tritium
 Injection temperature : 225°C
 Column temperature : 210°C
 Detector temperature : 222°C

Results of the metabolic studies by using C^{14} -endosulfan in mice are summarized in Tables 2 and 3. Two adult mice were sacrificed at the end of each experiment. The radioactivity of organs, urine, feces, and the expired gas of one of the mice were read with the liquid scintillation spectrometer and the Model 6000 Dynacon electrometer which is shown in Table 2. Only the liquid scintillation spectrometer was used in reading the radioactivity of the other test mouse (shown in Table 3). By using the Model 6000 Dynacon electrometer and liquid scintillation spectrometer, the percent recovery was greater (65.7%) than when the liquid scintillation spectrometer was used alone (21%). This may be due to the settling of $BaCO_3$ to the bottom of the vial or a mechanical quenching by the $BaCO_3$. The author found that by shaking the vial vigorously prior to reading in the liquid scintillation spectrometer, the radioactivity was highest and then decreased rapidly with time. At the end of ten minutes the activity decreased about 33% compared with the first reading (Figure 11). Color in organ, urine and fecal extracts may have caused some quenching in the system which in turn reduced the radioactivity read by the spectrometer. In using the Model 6000 Dynacon electrometer, the problem of quenching and settling effect were erased because the system produced $C^{14}O_2$ was readily read with the electrometer. If the Model 6000 Dynacon had been used in reading the radioactivity from the second mouse along

with the liquid scintillation spectrometer, the percentage of recovery should have been identical to the first test mouse.

Based on two mice, the amounts of radioactivity found from these experiments were in the following sequence: Feces> small intestine and contents>urine>visceral fat>liver> kidney> respiratory CO_2 > blood. From 0.1 to 0.2 percent of the radioactivity was found in the exhaled gas indicating that the animal can metabolize the cyclo diene ring and ultimately convert the products to CO_2 .

Based on the specific activity (activity/ weight) of the organs and excreta the following sequence was obtained: Feces 98452 cpm)> visceral fat (7053.5 cpm)> urine (3746 cpm)> liver (2883 cpm)> small intestine and contents (2080 cpm)> kidney (1390 cpm)> Brain (424.6 cpm)> respiratory CO_2 (302 cpm)> blood (92.3 cpm).

Table 2.- Radioactivity found in organ extracts, respired CO_2 , urine and feces 24 hours following oral dose of C^{14} -endosulfan in a mouse. The radioactivity were measured with a Tri-Carb liquid scintillation model 3314 and a model 6000 Dynacon electrometer.

Source	Extract fraction	CPM less background	Total CPM	% of recovered radioactivity
Liver	Hexane	1538.0	3447.0	2.12
	Acetone: H_2O	1439.0		
	Na_2SO_4 :tissue	470.0		
Kidney	Hexane	120.5	431.4	0.27
	Acetone: H_2O	39.3		
	Na_2SO_4 :tissue	271.6		
Small Intestine	Hexane	434.2	916.1	0.58
	Acetone: H_2O	205.9		
	Na_2SO_4 :tissue	276.0		
Brain	Hexane	68.0	165.2	0.104
	Acetone: H_2O	25.2		
	Na_2SO_4 :tissue	72.0		
Visceral fat	Hexane	1825.0	2014.0	1.27
	Acetone: H_2O	0.0		
	Na_2SO_4 :tissue	189.0		
Blood	Hexane	30.0	92.3	0.06
	Acetone: H_2O	34.3		
	Na_2SO_4 :tissue	28.0		
Urine # 1	Hexane	237.6	623.6	0.40
	Acetone: H_2O	386.0		
Urine # 2	Hexane	76.7	429.0	0.27
	Acetone: H_2O	352.3		
Urine # 3	Hexane	210.0	1057.0	0.67
	Acetone: H_2O	847.0		
Feces # 1	Hexane	69.8	124.1	0.078
	Acetone: H_2O	5.3		
	Na_2SO_4 :feces	49.0		

Table 2. (Contd.)

Source	Extract fraction	CPM less background	Total CPM	% of recovered radioactivity
Feces # 2	Hexane	153.0	1317.0	0.83
	Acetone:H ₂ O	61.0		
	Na ₂ SO ₄ :feces	1103.0		
Feces # 3	Hexane	729.0	3682.0	2.33
	Acetone:H ₂ O	464.0		
	Na ₂ SO ₄ :feces	2489.0		
Feces # 4	Hexane	722.0	5242.0	3.32
	Acetone:H ₂ O	243.0		
	Na ₂ SO ₄ :feces	4277.0		
Contents in Gastrointestinal tract	Hexane	105.0	2364.0	1.50
	Acetone:H ₂ O	2205.0		
	Na ₂ SO ₄ :contents	54.0		
Respiratory CO ₂	BaCO ₃	207.0	207.0	0.13
Carcass	BaCO ₃	130 795 .0*	130795 .0	82.93
Equipment		4800.0	4800.0	3.10
Total			158 706.7	
C ¹⁴ -endosulfan standard			240 000.0	

Percent recovered = 65.7 %

* Equivalent CPM from C¹⁴O₂ activity measured with the Dynacon 6000 electrometer.

Table 3.- Radioactivity found in organ extracts, respired CO_2 , urine and feces 24 hours following oral dose of C^{14} -endosulfan in a mouse. The radioactivity were measured with a Tri-Carb liquid scintillation model 3314.

Source	Extract fraction	CPM less background	Total CPM	% of recovered radioactivity
Liver	Hexane	623.0	3790.22	1.33
	Acetone: H_2O	1164.0		
	Na_2SO_4 :tissue	2003.22		
Kidney	Hexane	70.0	361.00	0.98
	Acetone: H_2O	54.0		
	Na_2SO_4 :tissue	237.0		
Small Intestine	Hexane	205.0	2064.70	5.62
	Acetone: H_2O	376.0		
	Na_2SO_4 :tissue	1483.70		
Stomach	Hexane	209.0	529.58	1.41
	Acetone: H_2O	22.0		
	Na_2SO_4 :tissue	298.58		
Visceral fat	Hexane	843.0	1099.67	2.99
	Acetone: H_2O	111.0		
	Na_2SO_4 :tissue	145.67		
Blood	Hexane	4.0	14.0	0.04
	Acetone: H_2O	10.0		
Urine # 1		1058.0	1058.0	2.83
Urine # 2		2124.0	2124.0	5.78
Urine # 3		2197.0	2197.0	5.98
Feces # 1	Hexane	717.0	5537.30	15.10
	Acetone: H_2O	392.0		
	Na_2SO_4 :feces	4428.30		
Feces # 2	Hexane	337.0	1002.95	2.73
	Acetone: H_2O	81.0		
	Na_2SO_4 :feces	684.95		

Table 3. (Contd.)

Source	Extract fraction	CPM less background	Total CPM	% of recovered radioactivity
Contesnts in Hexane		148.0		
Gastrointes- Acetone:H ₂ O		178.0	3177.47	8.65
tinal tract Na ₂ SO ₄ :contents		2851.47		
Respiratory BaCO ₃		397.8	397.8	1.08
CO ₂				
Carcass BaCO ₃		12120.0	12120.0	30.3
Equipment		5602.0	5602.0	16.1
Total			36695.0	
C ¹⁴ -endosulfan standard			195000.0	

Percent recovered = 21 %

Figure 11. Decrease in radioactivity as shown by liquid scintillation spectrometer due to settling of the sample in the vial. (Na_2SO_4 :feces)

Results of gas chromatographic analysis of the 12-hour studies of mice fed with 0.30 mg purified endosulfan are summarized in Table 4. Oxidized endosulfan (the cyclic sulfate of endosulfan) was detected in the liver, kidney, small intestine, muscle, and visceral fat extracts. Large amounts of oxidized endosulfan were found in the liver, small intestine, and visceral fat extracts, while only a trace of it was found in the muscle and kidney extracts. It is possible that oxidized endosulfan was found in the muscle and kidney extracts due to fatty tissue surrounding these organs since in later extractions of these organs from other test animals no oxidized endosulfan was found. The low- and high-melting isomers of endosulfan were found in the stomach as well as in small intestine extracts. Some of the stomachs contained undigested food at the time of extraction and this may have resulted in the detection of both low- and high-melting isomers. Feces in the small intestine were included in the small intestine extracts, which contained large amounts of oxidized endosulfan detected with gas chromatography.

Neither of the isomers nor their metabolites were detected in the blood extracts.

Urine and feces of these mice were extracted using the hexane-acetone procedure. Neither isomer of endosulfan nor their metabolites were detected when the radium detector was used. When the tritium detector was used, gas chromatograms

Table 4. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed 12 hours following ingestion of 0.30 mg purified endosulfan.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	++	-	-
Kidney	-	-	-	*	-	-
Stomach	++	++	-	-	-	-
Small Intestine	++	++	-	++	-	-
Muscle	-	-	-	*	-	-
Visceral Fat	-	-	-	++	-	-
Blood	-	-	-	-	-	-
Urine	-	-	-	-	-	++
Feces	++	++	-	++	++	-

Symbols: ++ = readily detectable by gas chromatography.

* = trace-between 0.01-0.02 ng endosulfan sulfate

- = undetectable-less than 0.01 ng endosulfan sulfate; 0.02 ng purified endosulfan; 0.005 ng endosulfan ether; and 0.4 ng endosulfan alcohol.

LMP = Low-melting point

HMP = High-melting point

showed all isomers of endosulfan, endosulfan alcohol and oxidized endosulfan in the fecal extracts. In urine extracts, a metabolite was found with the tritium detector which has the same retention time as endosulfan alcohol. These results indicate that the tritium detector is considerably more sensitive than the radium detector. Operating temperatures of both systems were the same throughout the investigation : the detector at 220°C, the column at 210°C, and the injection block temperature at 225°C.

The results of paper chromatographic separation of these organs were not conclusive. Not all spots obtained from the organ extracts were readily detectable and they were whitish in color instead of the usual dark brown AgCl hue as obtained from the endosulfan isomer standards.

From the preliminary studies, it was determined that 15 mg of purified endosulfan per kilogram of body weight was the maximum tolerated dosage for this strain of white mice. At 20 mg/kg, most of the test mice did not survive the 24-hour period following ingestion of the treated food. From this study the LD₁₀ of purified endosulfan was estimated to be 15-20 mg/kg of body weight for the laboratory mouse.

In the 24-hour studies of mice fed 0.30 mg of purified endosulfan, the results of gas chromatographic analysis of organ extracts were identical to those of the 12-hour studies (Table 5). Urine and feces were extracted with the hexane-acetone method and

the residues were identified with gas chromatography, paper chromatography, and thin-layer chromatography. These three methods failed to show any isomers of endosulfan or its metabolic products in urine or feces extracts. This may be due to the fact that some of the metabolic products are insoluble in hexane and remained in the acetone-water portion. This was partially confirmed by the results from the metabolism of C^{14} -endosulfan in mice. The acetone-water portion of urine extracts from mice fed C^{14} -endosulfan showed the highest radioactivity (Table 2 and 3). With the tritium detector, a metabolic product which has same retention time as endosulfan alcohol was detected in the urine extracts. However, it is not believed that the urine metabolite is endosulfan alcohol for two reasons: (1) Its peak shape is not characteristic of the alcohol, and (2) theoretically the alcohol should not be urine- or water-soluble.

All isomers of endosulfan, endosulfan alcohol and oxidized endosulfan were detected in the fecal extracts. Most of the radioactivity in fecal extracts obtained from mice which were fed C^{14} -endosulfan was in the sodium sulfate-feces portion (Tables 2 and 3).

In the metabolism studies of mice fed with 0.30 mg of low-melting isomer (Table 6), only the low-melting isomer was detected in extracts of the stomach, small intestine, and feces. The oxidized endosulfan was detected by gas chromatography in the liver, small intestine, visceral fat, and fecal extracts.

Table 5. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed 24 hours following ingestion of 0.30 mg purified endosulfan.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	+ +	-	-
Kidney	-	-	-	*	-	-
Stomach	+ +	+ +	-	-	-	-
Small Intestine	+ +	+ +	-	+ +	-	-
Muscle	-	-	-	*	-	-
Fat	-	-	-	+ +	-	-
Blood	-	-	-	-	-	-
Urine	-	-	-	-	-	+ +
Feces	+ +	+ +	-	+ +	+ +	-

Symbols: Same as Table 4.

Table 6. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed 24 hours following ingestion of 0.30 mg low-melting point isomer.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	+	-	-
Kidney	-	-	-	*	-	-
Stomach	+	-	-	-	-	-
Small Intestine	+	-	-	+	-	-
Muscle	-	-	-	*	-	-
Visceral Fat	-	-	-	+	-	-
Blood	-	-	-	-	-	-
Urine	-	-	-	-	-	+
Feces	+	-	-	+	+	-

Symbols: Same as Table 4.

The paper chromatograms did not show a good resolution. The spots obtained from organ extracts of test animals and control animals appeared whitish in color, as opposed to the spots produced by isomer standards, which were dark-brown in color.

In the 24-hour studies of mice fed 0.30mg of high-melting isomer, gas chromatographic analysis showed oxidized endosulfan in the liver, kidney, small intestine, muscle, and visceral fat extracts (Table 7). Large amounts of oxidized endosulfan were detected in the liver, small intestine, and visceral fat extracts. The oxidized endosulfan found in kidney and muscle extracts may have been due to attached fat tissue. Later in this investigation when the methods of obtaining samples improved, oxidized endosulfan was not detected in the kidney and muscle extracts.

In urine extracts, the high-melting isomer was detected by gas chromatography. This may have been due to contamination by the mouse in the metabolism cage. When a mouse holds insecticide-treated food between his front feet, his feet become contaminated. Then, in walking around the metabolism cage, the mouse contaminates the cage, which in turn might contaminate any urine which comes in contact with the floor of the cage. In later experiments, the animals' feet were rinsed with acetone prior to housing in the metabolism cage. As a result, none of the isomers of endosulfan were detected in the urine extracts. A metabolic product which has same retention time as endosulfan alcohol was detected by gas chromatograph equipped with the tritium detector but not when the radium detector was used. In the fecal extracts large amounts

of high-melting isomer, endosulfan alcohol and oxidized endosulfan were detected.

Table 7. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed 24 hours following ingestion of 0.30 mg high-melting point isomer.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	+ +	-	-
Kidney	-	-	-	*	-	-
Stomach	-	-	-	-	-	-
Small Intestine	-	+ +	-	+ +	-	-
Muscle	-	-	-	*	-	-
Visceral Fat	-	-	-	+ +	-	-
Blood	-	-	-	-	-	-
Urine	-	-	-	-	-	+ +
Feces	-	+ +	-	+ +	+ +	-

Symbols: Same as Table 4.

Table 8. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 7 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	++	-	-
Kidney	-	-	-	-	-	-
Stomach	++	++	-	-	-	-
Small Intestine	++	++	-	++	-	-
Muscle	-	-	-	-	-	-
Visceral Fat	-	-	-	++	-	-
Urine	-	-	-	-	-	++
Feces	++	++	-	++	++	-

Symbols: Same as Table 4.

Table 9. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 14 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	++	-	-
Kidney	-	-	-	-	-	-
Stomach	++	++	-	-	-	-
Small Intestine	++	++	-	++	-	-
Muscle	-	-	-	-	-	-
Visceral Fat	-	-	-	++	-	-
Urine	-	-	-	-	-	++
Feces	++	++	-	++	++	-

Symbols : Same as Table 4.

Table 10. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 21 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	+	-	-
Kidney	-	-	-	-	-	-
Stomach	+	+	-	-	-	-
Small Intestine	+	+	-	+	-	-
Muscle	-	-	-	-	-	-
Visceral Fat	-	-	-	+	-	-

Symbols : Same as Table 4.

Table 11. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 28 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	++	-	-
Kidney	-	-	-	-	-	-
Stomach	++	++	-	-	-	-
Small Intestine	++	++	-	++	-	-
Muscle	-	-	-	-	-	-
Visceral Fat	-	-	-	++	-	-
Urine	-	-	-	-	-	++
Feces	++	++	-	++	++	-

Symbols : Same as Table 4.

Table 12. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 35 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol	Metabolite
Liver	-	-	-	++	-	-
Kidney	-	-	-	-	-	-
Stomach	-	-	-	-	-	-
Small Intestine	++	++	-	++	-	-
Muscle	-	-	-	-	-	-
Visceral Fat	-	-	-	++	-	-
Brain	-	-	-	-	-	-

Symbols : Same as Table 4.

Table 13. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 42 days on 10 ppm endosulfan treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol
Liver	-	-	-	++	-
Kidney	-	-	-	-	-
Stomach	-	-	-	-	-
Small Intestine	++	++	-	++	-
Muscle	-	-	-	-	-
Visceral Fat	-	-	-	++	-
Brain	-	-	-	-	-

Symbols : Same as Table 4.

Table 14. - Electron capture gas chromatographic analysis of organ extracts from mice sacrificed after 49 days on 10 ppm treated food.

Organs	LMP isomer	HMP isomer	Endosulfan Ether	Endosulfan Sulfate	Endosulfan Alcohol
Liver	-	-	-	++	-
Kidney	-	-	-	-	-
Stomach	-	-	-	-	-
Small Intestine	++	++	-	++	-
Muscle	-	-	-	-	-
Visceral Fat	-	-	-	++	-
Brain	-	-	-	-	-

Symbols : Same as Table 4.

Figure 12. A chromatogram trace of 2 μ l of fecal extracts from a female mouse after ingesting 10 ppm endosulfan treated food for 28 days.

Detector : Tritium
Temperature injection : 225°C
Column temperature : 210°C
Detector temperature : 222°C

Results of gas chromatographic analysis of organ extracts from mice fed 10 ppm of purified endosulfan for 7, 14, 21, 28, 35, 42 and 49 days are summarized in Tables 8, 9, 10, 11, 12, 13 and 14. The oxidized endosulfan was detected in the liver and visceral fat extracts of all ten mice. Less oxidized endosulfan was found in these organs than was found in the liver and visceral fat of mice fed with 0.30 mg purified endosulfan for 12 and 24-hour studies. The oxidized endosulfan found in organs of test mice fed 10 ppm decreased as the investigation progressed. This can not be explained on the basis of what is presently known of cyclo-diene insecticide metabolism and storage in mammalian tissue.

The low- and high-melting isomers of endosulfan were detected in the stomach extracts of mice fed 10 ppm endosulfan for 7, 14, 21, and 28 days. It is possible that prior to the test the mice examined at 35, 42, and 49 days did not eat the treated food immediately prior to dissection; otherwise the results should have been the same as in mice examined at 7, 14, 21, and 28 days. Neither of the endosulfan isomers nor the metabolic products of endosulfan were detected in the brain extracts. Apparently the same metabolic product which was described as found in the 24 hour tests having the endosulfan alcohol retention time was detected in urine extracts of the mice fed 10 ppm. Both isomers and all the metabolites including oxidized endosulfan in large amounts, were found in benzene extracts of feces from mice fed 10 ppm for 7, 14 and 28 days (Figure 12).

Thin-layer chromatographic analysis of both the purified endosulfan and its isomers are summarized in the Table 15. The R_f values obtained from the thin-layer chromatogram are different from those obtained from the paper chromatograms. The endosulfan alcohol which has the R_f value highest in the paper chromatograms is the lowest in the thin-layer chromatograms. The thin-layer chromatograms produced more distinctive spots of the organ extracts than those shown on the paper-chromatograms. The spots which were apparent on the thin-layer chromatograms were scraped from the plate with a razor blade and were separately dropped into screw-cap vials which contained 5 ml glass-distilled hexane. Each vial was shaken and allowed to settle for five minutes and then 5 μ l of the hexane portion was injected in the gas chromatograph. The peaks obtained by the thin-layer chromatographic cleanup method corresponded with those obtained from the organ extracts before cleanup.

Table 15. - R_f values of technical endosulfan and related derivatives separated by thin-layer chromatography.

Standard	R_f *
Endosulfan Low-Melting Point isomer	0.16, 0.15
Endosulfan High-Melting Point isomer	0.6 , 0.67
Endosulfan Sulfate	0.08, 0.08
Endosulfan Alcohol	0.03, 0.03

* Mobile phase, 96% heptane in acetone.
Stationary phase, Silica Gel.

Faint, but readily detectable spots were produced on an autoradiogram made from a paper chromatogram which had been spotted with 20 μ l each of the hexane-acetone extracts of various organs from the mice each fed with 0.30 mg C^{14} -endosulfan and sacrificed at the end of 24 hours. Only the stomach extracts showed four definite spots which corresponded to the four components of C^{14} -endosulfan standard. Neither of the isomers of endosulfan nor their metabolic products were found in other organ extracts on the autoradiogram. This may be because insufficient material from each sample was spotted on paper chromatograms or not enough residue was in the hexane portion of extractions which were used in spotting the paper chromatograms.

An autoradiogram obtained from liver and kidney extracts of a mouse which died after eating 0.6 mg C^{14} -endosulfan over a period of six days produced four spots corresponding to the low and high-melting isomers, endosulfan ether and oxidized endosulfan. The spots obtained from kidney extracts separated nicely unlike the spots produced from liver extracts. The liver extracts produced tailing, but separate spots. It is possible that too much liver extract had been placed on the paper chromatogram. The autoradiogram was placed above the original paper chromatogram and the spots were carefully marked on the paper chromatogram. These spots were then cut from the chromatogram with scissors and extracted with 5 ml hexane. A 5 μ l portion of each spot was injected into the gas chromatograph. The extracted spots corresponded with those obtained from endosulfan

isomers and their metabolic products. This indicated that in a mouse which died after ingesting endosulfan, liver and kidney contained isomers of endosulfan and their metabolic products including a very small amount of oxidized endosulfan.

In another phase of this study, mice were fed either 0.30 mg purified endosulfan and sacrificed after 12 hours, 0.30 mg high-melting point isomer (sacrificed after 24 hours), or 0.30 mg low-melting point isomer (sacrificed after 24 hours). When the extracted organs from these mice were analyzed by gas chromatography, the results differed from the autoradiographic results. The only metabolite found was oxidized endosulfan, which was present in large quantities in liver extracts but only in trace amounts (0.02 ng) in kidney extracts. An animal which detoxifies endosulfan at the same rate in which it is ingested survives.

It appears that when endosulfan is oxidized to the sulfate it is detoxified, and is stored and excreted per se. Further studies are needed with all endosulfan components to learn their fate and toxicity in the mouse.

Daily food consumption of all mice, both test and control, was recorded throughout the investigation. The average daily food consumption of mice eating 10 ppm endosulfan-treated food for 49 days was 4.52 g for male and 4.41 g for female. Average daily food consumption of control mice was 4.52 g (Table 16).

Table 16. - Average daily food consumption of mice fed 10 ppm endosulfan-treated food and the control food.

Sex	Length of experiment Days	Initial Wt. (g)	Wt. prior sacrificed (g)	Average daily food consumption (g)
Male	7	17.00	20.00	4.20
	14	16.80	21.20	4.10
	21	30.50	30.70	4.30
	28	16.60	21.60	4.26
	35	28.30	31.20	4.87
	42	28.70	32.80	5.10
	49	27.60	30.20	4.80
	28	23.80	-	4.00
Control	28	26.10	-	4.86
Female	7	24.80	24.50	4.27
	14	22.80	23.00	4.14
	21	21.80	23.50	4.20
	28	25.30	24.80	4.30
	35	22.80	23.00	4.66
	42	20.80	22.80	4.00
	49	23.80	24.50	5.30
	28	23.00	-	4.66
Control	28	26.30	-	4.58

CONCLUSIONS

From this investigation the following conclusions are drawn:

1. Endosulfan fed continuously at 10 ppm in the total dry diet is a sublethal dose to the BALB/cJaxGnMc strain of laboratory mouse.
2. The maximum tolerated dosage of endosulfan for this strain of mouse was 15 mg/kg, which is the estimated LD₁₀.
3. Oxidized endosulfan (the cyclic sulfate) is the only material stored in any tissue of mice fed endosulfan one dose or continuously for 49 days.
4. An unidentified metabolite is found in the urine of mice fed endosulfan which has the same retention time as endosulfan alcohol by gas chromatography. However, its peak characteristics are not the same and it is not believed to be the alcohol.
5. The two isomers of endosulfan are not completely absorbed from the gastrointestinal tract when administered with the food at a sublethal dosage, but are excreted in the feces with the two metabolites, endosulfan sulfate and alcohol.
6. Neither the known metabolites nor isomers of endosulfan are found in blood or brain extracts. A trace of oxidized endosulfan is detected in kidney and muscle extracts but this may be due to small quantities of fat contained in these organs.

7. Approximately 65% of the radioactivity was recovered from a mouse fed with C^{14} -endosulfan during 24-hour period. Recovery from a second mouse whose radioactivity was measured somewhat differently, was only 21%. Based on the specific activity (activity/weight) of the organs and excreta the following sequence was obtained: Feces > visceral fat > urine > liver > small intestine and contents > kidney > brain > respiratory CO_2 > blood.

8. In the sacrificed mice, a large amount of oxidized endosulfan was detected in the liver and a trace in the kidney. In a mouse that died after eating endosulfan, both isomers of endosulfan, endosulfan alcohol, endosulfan ether and oxidized endosulfan were found in the liver and kidney, indicating that both of these organs enter into the metabolism and excretion of endosulfan.

9. The principal metabolic products of endosulfan in the mouse are the sulfate and alcohol.

AUTOBIOGRAPHY

I, Prayoon Deema, was born July 28, 1933, in Utaradit Province, Thailand, which is about 350 miles north of Bangkok, near the border of Thailand and Laos. In 1940 my parents sent me to Bangkok to attend private schools. I graduated from Sitabutrabumroong High School in 1952, and then attended the Kasetsart University for my undergraduate study. The B.Sc. degree in Agriculture, was received in 1957. Since then I have been employed in the Plant Pest Control Section, Plant Industry Division, Department of Agriculture, Ministry of Agriculture, in Bangkok.

In January, 1960, I received a two-year fellowship from the Agency for International Development to study for a higher degree in the United States. I attended the University of Arkansas from 1960 until March 1962, and obtained the M.S. degree in Entomology. Since then I have been working toward the Ph.D. degree in Entomology, specializing in insect toxicology and pesticide residue analysis. During this time the Ohio Agricultural Experiment Station has employed me as a Research Assistant in the Pesticide Residue Laboratory at the Ohio State University.

I was married in June 1962 to Nualchantra Homgrailars, a Thai student here at the Ohio State University.

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