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Received for review April 9, 1973. Accepted July 9, 1973. Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Metabolism of Lindane-^{14}C in the Rabbit: Ether-Soluble Urinary Metabolites

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Uniformly labeled lindane-^{14}C (2.04 g) in gelatin capsules was fed to five rabbits over a period of 26 weeks. By the end of the feeding period, 54% of the administered radioactivity had been excreted in the urine and 13% in the feces. About 56% of the urinary metabolites were soluble in ether. From the ether-soluble urinary metabolites 2,3,5-, 2,4,5- and 2,4,6-trichlorophenol and 2,3,4,6-tetrachlorophenol were identified by comparing infrared spectra of the isolated chlorophenols and/or their anisole derivatives with those of the reference compounds. Three other metabolites, 2,3- and 2,4-dichlorophenol and 2,3,4,5-tetrachlorophenol, were identified by the gas chromatographic retention times and their mass spectra. Seven more chlorophenols and six chlorobenzenes were *tentatively* identified by their gas chromatographic retention times. These metabolites were: 2,5-, 2,6-, and 3,4-dichlorophenol, 2,3,4-, 2,3,6-, and 3,4,5-tetrachlorophenol, pentachlorophenol, 1,2-dichlorobenzene, 1,2,4-trichlorobenzene, 1,2,3,4-, 1,2,4,5-, and/or 1,2,3,5-tetrachlorobenzene, and pentachlorobenzene.

Over the past two decades, there have been several reports on the degradation of lindane (γ-1,2,3,4,5,6-hexachlorocyclohexane) in soil, plants, insects, and animals. These studies have been reviewed by Menzie (1969). Lindane is slowly degraded by soil and 2,3,4,5,6-pentachlorocyclohex-1-ene (γ-PCCH) has been the only product identified (Yule *et al.*, 1967). In plants, several metabolites of lindane have been indicated (Itokawa *et al.*, 1970) but only γ-PCCH has been identified (Bogdarina, 1957; San Antonio, 1959). Much work has been done on the metabolism of lindane in the housefly. While a significant portion of the metabolites still remains to be characterized, the following compounds have been identified: all the six isomers of dichlorothiophenol, 1,2,3- and 1,2,4-trichlorobenzene, 1,2,3,4- and 1,2,4,5-tetrachlorobenzene, pentachlorobenzene, γ-PCCH, and an isomer of PCCH (Menzie, 1969; Reed and Forgash, 1969, 1970).

Metabolism of lindane in mammals has been briefly studied with rats, rabbits, and dogs (van Asperen and Oppenoorth, 1954; Grover and Sims, 1965; Jondorf *et al.*, 1955; Koransky *et al.*, 1964; San Antonio, 1959). Although 1,2,4-trichlorobenzene, 2,4-dichlorophenylmercapturic acid, and 2,3,5- and 2,4,5-trichlorophenol have been identified, the characterization of all the metabolites is far from complete, as the identified products represented only a fraction of the total metabolites. After the present investigation was completed (Karapally *et al.*, 1971), Chadwick and Freal (1972) reported on the identification of six chlorophenols as urinary metabolites of lindane in the rat.

The object of the investigation reported here was to study the metabolism of orally administered lindane-^{14}C by rabbits. The results of the identification of ether-soluble urinary metabolites are reported here.

APPARATUS AND REAGENTS

A Nuclear Chicago Model Mark 1 liquid scintillation spectrometer was used to measure radioactivity. Infrared spectra were recorded on a Beckman 1R10 instrument equipped with beam attenuator and a beam condenser. The spectra were recorded in micro-KBr disks. Mass spectra were recorded with an AEI Model MS12 mass spectrometer.

Electron capture gas chromatography (ecgc) was done on an Aerograph Hi-Fy Model 600-D instrument. For thermal conductivity gas chromatography (tcgc), an Aerograph Model A90-P-3 gas chromatograph was used. The solid support for gas–liquid chromatography was 80–100-mesh acid-washed DMCS-treated Chromosorb W. All ecgc analyses were carried out with detector and injector temperatures of 210 and 190°, respectively, and a nitrogen flow rate of 30 ml/min. The following columns were used for gas chromatographic analyses: column a, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% SE-30 Chromosorb W; column b, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% QF-1 on Chromosorb W; column c, 5 ft × ⅛ in. i.d. aluminum tube packed with 5% OV-17 on Chromosorb W; column d, 10 ft × ⅛ in. i.d. aluminum tube packed with a 5% QF-1 on Chromosorb W; and column e, 5 ft × 3/16 in. i.d. copper tube packed with 15% OV-17 on Chromosorb W. This column was used only for tcgc analysis with a detector temperature of 230° and He flow rate of 40 ml/min. All column temperatures are reported later at appropriate places.

All solvents were reagent grade and further purified by distillation. The following reference compounds were obtained from commercial sources and purified by distillation or recrystallization followed by tcgc: 1,2-, 1,3-, and 1,4-dichlorobenzene; 1,2,3-, 1,2,4-, and 1,3,5-trichlorobenzene; 1,2,3,4- and 1,2,4,5-tetrachlorobenzene; pentachlorobenzene; 2,4-, 2,5-, 2,3-, 2,6-, and 3,4-dichlorophenol; 2,4,5- and 2,4,6-trichlorophenol; 2,3,4,6-tetrachlorophenol, and pentachlorophenol (all from Canadian Laboratory Supplies Ltd., Winnipeg, Man.), and 2,3,6-trichlorophenol (Aldrich Chemical Co., Milwaukee, Wis.). All melting points are uncorrected.

1,2,3,5-Tetrachlorobenzene was prepared from 2,4,6-trichloroaniline by diazotization in dilute hydrochloric acid

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followed by a Sandmeyer reaction. The product was purified by crystallization from ethanol and had a mp of 48–49° (Holleman, 1920).

2,3,4,5,6-Pentachlorocyclohex-1-ene (γ-PCCH) was prepared by alkaline monodehydrochlorination of lindane according to Nakajima *et al.* (1949) with 50% aqueous acetone as the solvent instead of methanol. The reaction mixture was extracted with hexane, and the hexane extract was washed with water, dried over anhydrous Na_2SO_4, and distilled under reduced pressure to remove the solvent. The product was purified by tcgc on column a at 120°. The main peak was collected from the gas chromatograph and characterized by mass spectrometry.

2,3,5-Trichlorophenol was prepared, starting from 2,4-dichloro-6-nitroaniline (Aldrich Chemical Co.) according to the method of Hodgson and Kershaw (1929). As the phenol was difficult to crystallize, a portion of the crude product was methylated with dimethyl sulfate and aqueous sodium hydroxide and the 2,3,5-trichloroanisole was crystallized from ethanol; mp 81–83°, lit. 84° (Hodgson and Kershaw, 1929). However, a small quantity of the phenol was obtained by collection from column c at 140° by tcgc and used as the reference compound along with the anisole derivative.

2,3,4-Trichlorophenol was prepared from 1-nitro-2,3,4-trichlorobenzene (Canadian Laboratory Supplies Ltd.) according to the method of Tiessens (1931); mp 80–82°, lit. 83.5°.

3,4,5-Trichlorophenol was prepared from 2,6-dichloro-4-nitroaniline as the starting material. 1-Nitro-3,4,5-trichlorobenzene was prepared from 2,6-dichloro-4-nitroaniline (Canadian Laboratory Supplies Ltd.) by diazotization and Sandmeyer reaction. The 1-nitro-3,4,5-trichlorobenzene was then reduced to the corresponding amine by reduction with iron and hydrochloric acid. 3,4,5-Trichlorophenol was obtained by diazotization of the amine salt and decomposition of the diazonium salt in the usual manner; mp 100–101°, lit. 101° (Weast, 1968).

2,3,4,5-Tetrachlorophenol was prepared, starting from 1-nitro-2,3,4,5-tetrachlorobenzene (Aldrich Chemical Co.) according to Tiessens (1931); mp 115–16°, lit. 116–17°.

2,3,5,6-Tetrachlorophenol was obtained from 1-nitro-2,3,5,6-tetrachlorobenzene (Aldrich Chemical Co.) according to Tiessens (1931); mp 114–15°, lit. 115°. The anisole derivatives of all the chlorophenols were prepared by reacting the pure phenols with diazomethane.

Uniformly labeled lindane-^{14}C (sp act. 37.52 mCi/mmol) was obtained from Amersham/Searle Co., Toronto. The material was examined by thin-layer and electron capture gas chromatography and was more than 99% pure. The labeled lindane was diluted with the pure nonlabeled compound to give sp act. of 37.19 μCi/mmol and then fed to the rabbits. The 2,4,6-trichlorophenol-^{14}C (sp act. 37.19 μCi/mmol) used for determining glc collection efficiency was obtained from the rabbit urine.

Determination of Radioactivity. All radioactivity measurements were carried out in a scintillation counter. The radioactivity content of urine was determined by counting 0.5-ml aliquots in 10 ml of a 0.5% (w/v) solution of butyl-PBD in toluene–Triton X-100 (2:1 v/v) after acidification with a few drops of concentrated HCl. Nonaqueous samples (0.5 to 2 ml) were counted in 10 ml of 0.5% (w/v) butyl-PBD in toluene. Feces samples (*ca.* 100 mg) were suspended in toluene containing 6 g of PPO and 40 g of CaB-O-Sil per liter and counted in a liquid scintillation counter. The radioactivity contents of feces samples, as determined by the above method, were checked at random by dry combustion of 25-mg samples according to the method described by Saha (1971) and determining $^{14}CO_2$. The results obtained by these two methods agreed within ±10%.

Samples of rabbit tissues (*ca.* 200 mg) were solubilized in 2–5 ml of Soluene (Packard Instrument Co.) and counted in 10 ml of 0.5% butyl-PBD in toluene. Radioactivity contents of feces and tissue samples were determined by the internal standard method. For others, the channels ratio method was used and corrections were made for quenching. All counts were corrected for the background radioactivity.

PROCEDURE

Feeding of Lindane-^{14}C to Rabbits and Collection of Urine and Feces. A corn oil solution of uniformly labeled lindane-^{14}C (2.04 g, sp act. 37.19 μCi/mmol) in gelatin capsules was fed to five 6-month-old male New Zealand white rabbits (2–2.5 kg body weight) over a period of 26 weeks. Equal doses of lindane-^{14}C were given to the rabbits twice a week. Single dose per rabbit was about 3 mg for weeks 1 to 4, 6 mg for weeks 5 to 15, and 12 mg for weeks 16 to 26. The rabbits were kept in metabolic cages and were given food (rabbit pellets from commercial source) and water as required. They were all apparently healthy and gained weight similar to a sixth rabbit, which was not given any lindane.

Urine and feces samples from the five rabbits that were fed lindane-^{14}C were collected each day from the first day of feeding. Daily samples of urine were stored at room temperature in stoppered bottles, pooled into weekly batches, and extracted with ether. Daily collections of feces samples were dried at ambient temperature, pooled into weekly batches, ground in a Wiley mill, mixed thoroughly, and stored at 0° until their radioactivity contents were determined. Collection of excreta was continued for a total of 32 weeks, at which time the rabbits were sacrificed. The fat, muscle, liver, kidney, and brain tissues were separated from each rabbit. Tissue samples were stored at −20° until their radioactivity contents were measured by the method described above and the results were expressed as parts per million of lindane.

Isolation of Ether-Soluble Metabolites from Urine. Weekly urine samples were continuously extracted with diethyl ether for 48 hr. The ether extracts were dried over anhydrous Na_2SO_4, concentrated, and made up to 100 ml and radioactivity content was determined by counting aliquots in the scintillation counter.

Silica Gel Column Chromatography. The ether extracts of urine from weeks 17 to 26 were combined and concentrated by distillation using a fractionating column packed with Raschig rings to minimize loss of metabolites (less than 0.1% loss). The concentrate was chromatographed on a silica gel (Davison Chem., Baltimore, Md., Grade 12, 28–200 mesh and used as received) column (18 in. × 2 in. i.d.) and eluted successively with hexane (2.5 l.), 20% benzene in hexane (0.5 l.), and 40% benzene in hexane (4 l.). Column eluates were collected in 15-ml portions using an automatic fraction collector and radioactivity content of each fraction was determined by counting an aliquot in the scintillation counter. A plot of the concentration of radioactivity in each of the 470 tubes against the tube numbers showed two peaks with a shoulder on the second. The column eluates were combined into three fractions, as shown in Figure 1.

Finally, the silica gel column was washed with diethyl ether (2 l.) to obtain fraction IV.

Identification of Metabolites. Fraction I from silica gel column chromatography (Figure 1) was concentrated and made up to 100 ml with ether. Extraction of an aliquot of this fraction with 10% sodium hydroxide solution did not remove any radioactivity, indicating that the metabolites in this fraction were not acidic. Analysis by ecgc on columns a and b at 120° and on column c at 150° indicated the presence of 1,2,4-trichlorobenzene, 1,2,3,4-tetrachlorobenzene, 1,2,3,5- and/or 1,2,4,5-tetrachlorobenzene, and pentachlorobenzene (Figure 2), as shown by comparison of

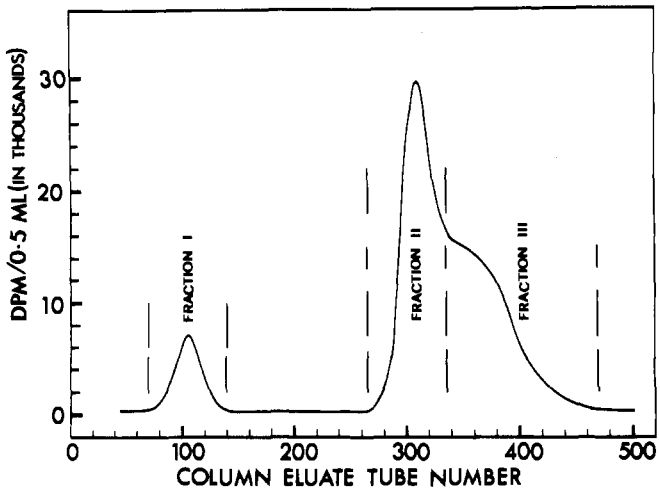

Figure 1. Silica gel column chromatogram of the ether-soluble urinary metabolites of lindane-^{14}C.

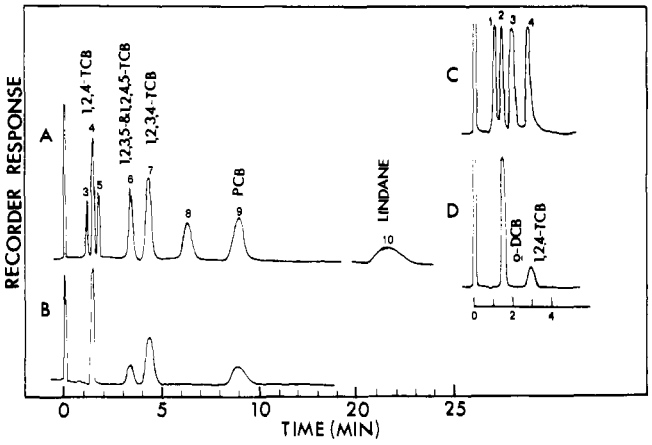

Figure 2. Gas chromatogram (ecgc) of silica gel column fraction no. 1 and reference compounds on 5% SE-30 column. A. Reference compounds at 120°. B. Lindane metabolites, silica gel column fraction no. 1 at 120°. C. Reference compounds at 95°. D. Lindane metabolites, silica gel column fraction no. 1 at 95° (partial chromatogram). (1) *m*- and *p*-dichlorobenzene; (2) *o*-dichlorobenzene; (3) 1,3,5-trichlorobenzene; (4) 1,2,4-trichlorobenzene; (5) 1,2,3-trichlorobenzene; (6) 1,2,3,5- and 1,2,4,5-tetrachlorobenzene; (7) 1,2,3,4-tetrachlorobenzene; (8) γ-PCCH; (9) pentachlorobenzene; and (10) lindane.

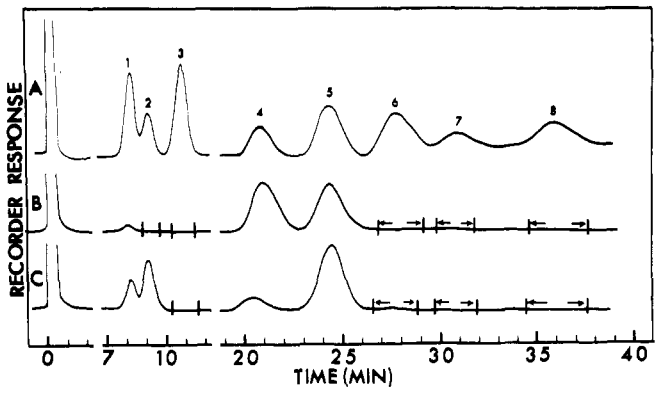

Figure 3. Gas chromatogram (tcgc) of silica gel column fractions II and III and reference compounds on 15% OV-17 at 140°. A. Reference compounds. B. Fraction II. C. Fraction III. (1) 2,4- and 2,5-dichlorophenol; (2) 2,3-dichlorophenol; (3) 2,6-dichlorophenol; (4) 2,3,5-trichlorophenol; (5) 2,4,5- and 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol; (6) 2,3,4-trichlorophenol and 3,5-dichlorophenol; (7) 2,3,6-trichlorophenol; and (8) 3,4-dichlorophenol.

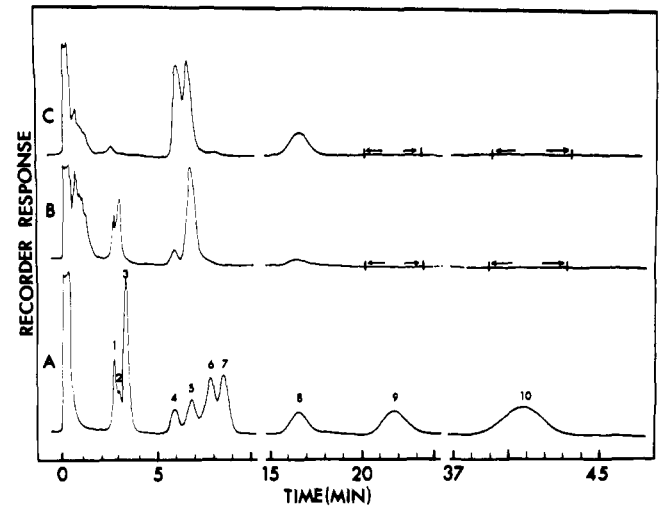

Figure 4. Gas chromatogram (tcgc) of reference compounds and silica gel column fractions II and III on OV-17 column at 180°. A. Reference compounds. B. Fraction III. C. Fraction II. (1) 2,4- and 2,5-dichlorophenol; (2) 2,3-dichlorophenol; (3) 2,6-dichlorophenol; (4) 2,3,5-trichlorophenol; (5) 3,5-dichlorophenol, 2,4,5- and 2,4,6-trichlorophenol and 2,3,5,6-tetrachlorophenol; (6) 2,3,4- and 2,3,6-trichlorophenol; (7) 3,4-dichlorophenol; (8) 2,3,4,6- and 2,3,4,5-tetrachlorophenol; (9) 3,4,5-trichlorophenol; and (10) pentachlorophenol.

their retention times with those of reference samples. Analysis by ecgc on columns a and b at 95° and column c at 110° showed the presence of 1,2-dichlorobenzene. This fraction was further analyzed by tcgc on column a at 120° and at 95° and compounds representing each peak were collected and the radioactivity content was determined by liquid scintillation counting. The concentration of metabolites was determined by ecgc on column a at 120° and at 95° by the internal standard method, using 1,3,5-trichlorobenzene as the internal standard for the estimation of 1,2-dichlorobenzene and pentachloroanisole for the other components.

Fractions II and III from silica gel column chromatography (Figure 1) were concentrated separately and made up to 100 ml with ether. Extraction of aliquots of these fractions with 10% sodium hydroxide solution removed more than 98% of the radioactivity from the organic layer, indicating the presence of acidic metabolites. Analysis of these two fractions by tcgc on column e at 140 and 180° indicated the presence of all the isomers of dichlorophenols except 3,5-dichlorophenol, all the isomers of trichlorophenol, 2,3,4,6- and 2,3,4,5-tetrachlorophenol, and pentachlorophenol. The dichlorophenols and the trichlorophenols, except 3,4,5-trichlorophenol, were eluted from the column at 140° (Figure 3) and the others were eluted at 180° (Figure 4). The retention time of each suspected compound was compared with that of the reference samples under identical conditions. The compounds representing these peaks were collected by tcgc whenever possible and their infrared and/or mass spectra were recorded and compared with those of the reference compounds. The phenolic metabolites collected by tcgc were also reacted with diazomethane and, if required, their mass and infrared spectra recorded. Peaks containing more than one metabolite were collected by tcgc from column e (at either 140 or 180°), converted to the corresponding anisole derivatives, and then analyzed by both ecgc and tcgc on columns b and d at 130°.

The chlorophenols could not be quantitated by ecgc, as the peaks were broad due to severe tailing and, more seriously, the detector responses could not be reproduced. The ec detector also lost its sensitivity very rapidly when

chlorophenols were injected. However, these chlorophenols could be easily estimated by tcgc on column e at 140 or 180°. For this purpose an aliquot of this fraction containing over 50,000 dpm was mixed with sufficient amounts of reference samples of the suspected compounds, if needed, so that a reasonable detector response would be obtained. The compounds representing the various peaks were collected into 5 ml of diethyl ether contained in a scintillation vial and the radioactivity content was determined by scintillation counting. The efficiency of the collection procedure was between 90 and 97%, as indicated from the injection and collection of known amounts of lindane-^{14}C and 2,4,6-trichlorophenol-^{14}C.

RESULTS AND DISCUSSION

Retention and Excretion of Lindane-^{14}C by the Rabbit. By the end of the 26-week feeding period, about 54% of the administered radioactivity was excreted in the urine and about 13% in the feces (see "Supplementary Material Available" paragraph at end of article). After 6 more weeks, an additional 3% was excreted in the urine and about 1% in the feces. The amount excreted in the feces increased steadily and nearly doubled by the end of the feeding period. On the other hand the amount excreted in the urine increased from 43 to 54% during the same period. These results were in agreement with those obtained by Koransky *et al.* (1964) and Chadwick *et al.* (1971). It appears that a rapid equilibrium was established between the intake of lindane and its excretion, a fact observed with other organochlorine insecticides in animals. It also appears that the rate of excretion of lindane from the animal body was rather slow after the intake had been stopped.

A similar study on the metabolism of dieldrin-^{14}C in rabbits by Korte and Arent (1965) showed a much slower rate of excretion of dieldrin from the animal body. Thus, by the end of the 21-week feeding period, about 28% of the orally administered dieldrin-^{14}C was excreted in the urine and about 12% in the feces. After a further 21-week period, the total amount excreted in the urine was only 43% and that in the feces was 13%.

Up to the time at which the animals were killed, 71% of the administered radioactivity had been excreted in urine or feces. Some radioactivity from the feces might have been lost by volatilization during the drying process. Some lindane-^{14}C was lost by regurgitation, as the rabbits occasionally spat out the gelatin capsules within a few minutes after administration. Examination of the different tissues of the rabbits showed that a maximum of 2.0 ppm of lindane and/or lindane-derived residues was present in the fat, 0.4 ppm in liver, 0.3 ppm in kidney, 0.4 ppm in muscle, and 0.1 ppm in the brain. Thus, more residue was found in adipose tissue than in the other types that were examined. Preferential concentration of lindane residues in the adipose tissues of animals has been observed by Davidow and Frawley (1951) and by Nakajima *et al.* (1949). Considering a weekly intake of 6–24 mg/rabbit or 2–8 mg/kg of body weight, the concentrations in the body tissues were low indeed, indicating that lindane was not stored in the animal body to a great extent.

Identification of Metabolites in Urine. Extraction of rabbit urine with diethyl ether removed about 56% of the total radioactivity in the urine or about 32% of the administered radioactivity. Lindane and its metabolites that were not conjugated would be expected to be present in the ether extract. The metabolites (44%) that could not be extracted with ether probably contained products that were conjugated as glucuronides, sulfates, or mercapturic acids. Such conjugates have been suggested by Jondorf *et al.* (1955) and Grover and Sims (1965).

Silica gel column chromatography effected a partial separation of the ether-soluble metabolites (Figure 1). Fraction I contained 6.4% of the radioactivity in the total ether extract, fraction II had 34.6%, and fraction III had 30.9%. The ether washings of the silica gel column (fraction IV) contained 21.0% of the ether-soluble urinary metabolites and the remaining 7.1% presumably could not be eluted from the column.

The gas chromatogram of silica gel column eluate fraction I on the SE-30 column at 120° showed four peaks having retention times of 1.5, 3.5, 4.5, and 9.0 min (Figure 2B). These retention times were identical with those of reference 1,2,4-trichlorobenzene, 1,2,3,5- and 1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene, and pentachlorobenzene, respectively (Figure 2A). This column could separate all the three isomers of trichlorobenzene, 1,2,3,4-tetrachlorobenzene, and pentachlorobenzene, but failed to resolve 1,2,3,5- and 1,2,4,5-tetrachlorobenzene. Thus, the peak with a retention time of 3.5 min could be due to either one or both of these compounds. Although the concentrations of these metabolites were sufficient for analysis by ecgc, they were too low for detection by tcgc. To prove that the compounds representing the peaks in Figure 2B were derived from lindane-^{14}C, an aliquot of fraction I was mixed with sufficient amounts of the five suspected metabolites so that reasonable recorder response would be obtained by tcgc. The mixture was then analyzed by tcgc on the same SE-30 column and under similar operating conditions as were used for ecgc analysis. The compounds representing these peaks were then collected and their radioactivity contents were measured by liquid scintillation counting. As only the radioactivity contents of the compounds representing these peaks were measured, the incorporation of nonlabeled reference compounds did not affect the conclusions derived from such measurements. Since the compounds representing all these peaks contained radioactivity, they must have been derived from lindane-^{14}C and thus were metabolites of lindane. However, the total amount of radioactivity contained in these four peaks (Figure 2B) accounted for only 5% of the radioactivity present in this fraction and the remainder of the radioactivity was present in the compounds representing the solvent peak. Ecgc of this fraction on the same column at a lower temperature (95°) showed an additional peak (Figure 2D) with a retention time of 1.7 min and the same as that of 1,2-dichlorobenzene (Figure 2C). Although 1,3- and 1,4-dichlorobenzene could not be resolved into two peaks, there was no indication of their presence by ecgc, even when the peak due to 1,2-dichlorobenzene gave more than 100% detector response. Examination of this fraction by tcgc showed that 1,2-dichlorobenzene accounted for about 94% of the radioactivity present in this fraction.

Further indications of the identities of the compounds present in this fraction were obtained by ecgc on column b at 120° and on column c at 150°, when four peaks with retention times the same as those of reference 1,2,4-trichlorobenzene, 1,2,3,5- and/or 1,2,4,5-tetrachlorobenzene, 1,2,3,4-tetrachlorobenzene and pentachlorobenzene were obtained. Similarly, further indication for the presence of 1,2-dichlorobenzene was obtained by ecgc on these two columns at 95 and at 110°, respectively, where this additional peak cochromatographed with reference 1,2-dichlorobenzene.

The concentrations of these metabolites were insufficient for infrared or mass spectral analyses. However, the suspected metabolites had the same retention times as those of reference compounds and they cochromatographed with them on three different columns, and gas chromatographic data were considered adequate for their *tentative* identification.

The most abundant metabolite in silica gel column eluent fraction I was 1,2-dichlorobenzene (94%), followed by 5% of 1,2,4-trichlorobenzene (Table I). Only 0.2% of

Table I. Ether-Soluble Urinary Metabolites of Lindane-^{14}C in the Rabbits

Metabolite	Abundance, % of Fraction[a] I	II	III	Total ether-soluble metabolites
1. o-Dichlorobenzene	94.0			6.0
2. 1,2,4-Trichlorobenzene	5.0			0.3
3. 1,2,3,4-Tetrachlorobenzene	0.2			t
4. 1,2,3,5- and/or 1,2,4,5-tetrachlorobenzene	t			t
5. Pentachlorobenzene	t			t
6. 2,4-Dichlorophenol		1.7	12.5	4.4
7. 2,5-Dichlorophenol		t	t	t
8. 2,3-Dichlorophenol		0.3	15.3	4.8
9. 2,6-Dichlorophenol		0.4	0.9	0.4
10. 3,4-Dichlorophenol		0.4	0.5	0.3
11. 2,3,5-Trichlorophenol		35.7	6.6	14.4
12. 2,4,5-Trichlorophenol		t	49.6	15.3
13. 2,4,6-Trichlorophenol		37.0	t	12.8
14. 2,3,4-Trichlorophenol		0.8	3.5	1.3
15. 3,5,6-Trichlorophenol		1.3	0.8	0.7
16. 2,3,4-Trichlorophenol		1.8	0.5	0.7
17. 2,3,4,6-Tetrachlorophenol		t	2.4	0.7
18. 2,3,4,5-Tetrachlorophenol		16.0	1.6	6.0
19. Pentachlorophenol		1.0	0.3	0.4
				68.5

[a] Silica gel column chromatographic fraction (Figure 1). (t = ce amtarount less than 0.1%).

1,2,3,4-tetrachlorobenzene and trace amounts of 1,2,3,5- and/or 1,2,4,5-tetrachlorobenzene and pentachlorobenzene were present in this fraction. The peaks due to these compounds were appreciable (Figure 2B) due to the fact that the electron capture detector was much more sensitive to them than to trichlorobenzene. Although the columns used for the gas chromatographic analyses could separate 1,2,3- and 1,3,5-trichlorobenzene and γ-PCCH from the other reference compounds, there was no indication of their presence among the metabolites. Lindane also could not be detected.

Nearly all of the radioactivity (more than 98%) present in silica gel column fractions II and III could be extracted with aqueous alkali, indicating that only acidic metabolites were present in these fractions. Analysis of fraction II by tcgc on column e at 140° (Figure 3B) gave three peaks having retention times of 8.2, 20.7, and 24.5 min. Analysis of fraction III under the same conditions gave an additional peak with a retention time of 9.3 min (Figure 3C).

The first peak had the same retention time (8.2 min) as those of reference samples of 2,4- and 2,5-dichlorophenols. These two isomers could not be separated on this column, neither could they be separated on column d. But their anisole derivatives could easily be separated by tcgc on column d at 130°. The compound(s) representing the peak with a retention time of 8.2 min and suspected to contain these two phenols was collected by tcgc of fraction III on column e at 140° (Figure 3C) and reacted with diazomethane. The gas chromatogram of the anisole derivative on column d at 130° showed only one peak having the same retention time as that of 2,4-dichloroanisole. The mass spectrum of the compound representing this peak was similar to that of reference 2,4-dichloroanisole (see "Supplementary Material Available" paragraph at end of article), confirming that this peak was due to this compound. However, ecgc analysis on column d at 130° of the anisole derivative of the collected material showed one major peak due to 2,4-dichloroanisole and indicated a trace amount of 2,5-dichloroanisole. Similar ecgc analysis

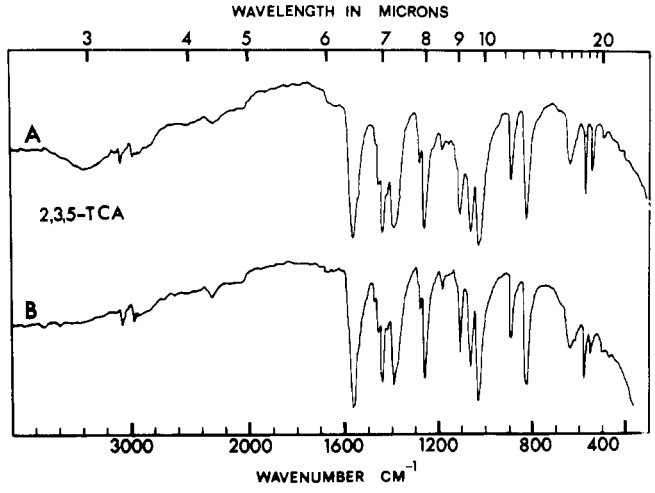

Figure 5. Infrared spectra of the anisole derivative of 2,3,5-trichlorophenol isolated from rabbit urine (A) and (B) reference 2,3,5-trichloroanisole (2,3,5-TCA).

of the anisole derivative of the compounds representing the peak with a retention time of 8.2 min (Figure 3B) in fraction II showed mainly 2,4-dichloroanisole and trace amounts of the 2,5 isomer. Thus, this peak in both silica gel column fractions II and III contained mainly 2,4-dichlorophenol and probably trace amounts of the 2,5 isomer.

The compound with the retention time of 9.3 min (Figure 3C) was suspected to be 2,3-dichlorophenol, as its retention time was identical to that of the reference compound. The compound representing this peak was collected by tcgc and was reacted with diazomethane. The anisole derivative also had a retention time identical to that of 2,3-dichloroanisole. Further proof of the identity of this compound was obtained from the mass spectrum of the anisole derivative of the collected material. The mass spectrum (see "Supplementary Material Available" paragraph at end of article) clearly showed that it was a dichloroanisole, and gas chromatographic data showed earlier that it was the 2,3 isomer. Thus, this peak was due to 2,3-dichlorophenol.

The retention time of the compound eluting at 20.7 min (Figure 3B and C) was the same as that of reference 2,3,5-trichlorophenol. The compound representing this peak was collected by tcgc and reacted with diazomethane. The infrared spectrum of the resulting anisole derivative was identical to that of 2.3.5-trichloroanisole (Figure 5), showing that this peak was due to 2,3,5-trichlorophenol.

The compound(s) representing the peak with a retention time of 24.5 min (Figure 3B) had a retention time the same as those of reference 2,4,5-trichloro-, 2,4,6-trichloro-, and 2,3,5,6-tetrachlorophenols. However, the infrared spectrum of the material collected by tcgc was identical with that of reference 2,4,6-trichlorophenol (Figure 6), indicating that this peak was due to 2,4,6-trichlorophenol and the other two suspected compounds, if present at all, could only be so in trace amounts. Further proof of the identity of the suspected compound was obtained from the infrared spectrum of the anisole derivative of the collected material. The anisole derivatives of the three suspected compounds could be resolved into two peaks on column b at 130°, one representing 2,4,6-trichloroanisole and the other representing 2,4,5-trichloro- and 2,3,5,6-tetrachloroanisole. The material representing this peak was collected by tcgc on column e at 140° (Figure 3B) and converted to the anisole derivative by reacting with diazomethane. Tcgc of the anisole derivative on column b at 130° gave only one peak having a retention time the same

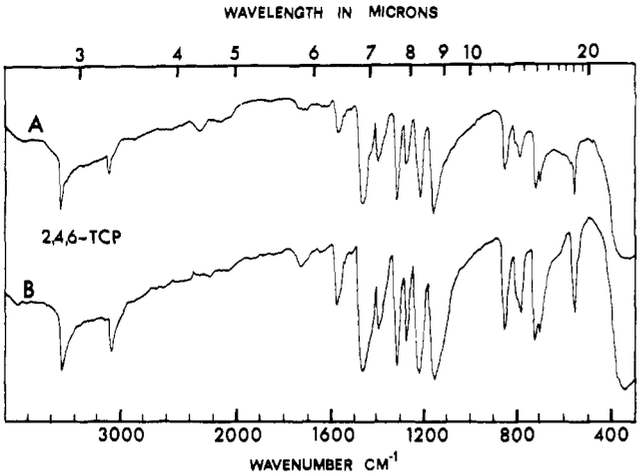

Figure 6. Infrared spectra of 2,4,6-trichlorophenol (2,4,6-TCP) isolated from rabbit urine (A) and reference compound (B).

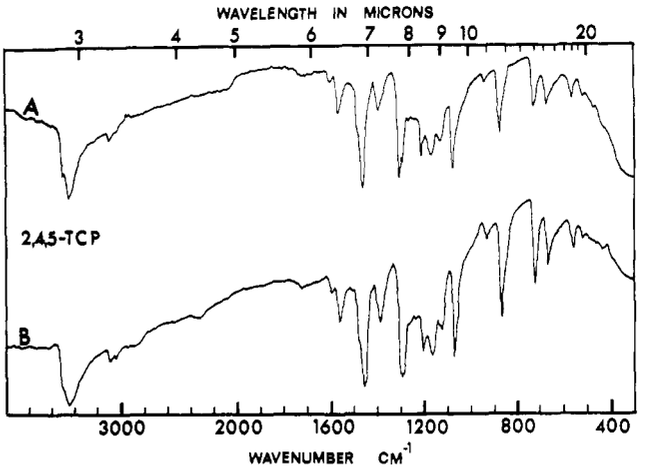

Figure 7. Infrared spectra of 2,4,5-trichlorophenol (2,4,5-TCP) isolated from the rabbit urine (A) and reference compound (B).

as that of 2,4,6-trichloroanisole. The infrared spectrum (see "Supplementary Material Available" paragraph at end of article) of the anisole derivative was the same as that of reference 2,4,6-trichloroanisole, indicating that the suspected metabolite was 2,4,6-trichlorophenol.

The gas chromatogram of the silica gel column chromatographic fraction III also gave a peak having a retention time of 24.5 min (Figure 3C), the same as that of reference 2,4,5-trichloro-, 2,4,6-trichloro-, and 2,3,5,6-tetrachlorophenols. But the infrared spectrum of the compound representing this peak was similar to that of 2,4,5-trichlorophenol (Figure 7), indicating that the other two suspected compounds could only be present, if at all, in trace amounts. Tcgc analysis of the anisole derivative of the collected material gave only one peak on column b at 130°. This peak had a retention time the same as those of 2,4,5-trichloro- and 2,3,5,6-tetrachloroanisole. But the infrared spectrum (see "Supplementary Material Available" paragraph at end of article) of the anisole derivative of the collected metabolite was the same as that of reference 2,4,5-trichloroanisole, indicating that the peak with a retention time of 24.5 min in fraction III (Figure 3C) was due to 2,4,5-trichlorophenol. Thus, it appears that this peak in silica gel column fraction II (Figure 3B) was due to 2,4,6-trichlorophenol, while the same peak in fraction III was due to the 2,4,5 isomer, indicating that column chromatography of the ether-soluble metabolites was very effective in separating these two compounds. Since the infrared spectra of the isolated metabolites were identical with those of reference 2,4,5- and 2,4,6-trichlorophenol, the presence of 2,3,5,6-tetrachlorophenol in either of these two fractions is extremely doubtful.

The compounds representing the peaks in Figures 3B and C accounted for 74 and 84% of the radioactivity present in fractions II and III, respectively, indicating that other metabolites in these fractions were present either in small quantities (and hence not detected by tcgc) or they were not being eluted from the gas chromatographic column. To test the first assumption, an aliquot of fraction II was mixed with sufficient amounts of reference compounds (so that a reasonable recorder response would be obtained in tcgc) represented by peaks 2, 3, 6, 7 and 8 (Figure 3A) and examined by tcgc on column e at 140°. The compounds representing these peaks were collected and measurement of radioactivity content of the collected materials indicated the presence of lindane-^{14}C metabolites in them, as all of them contained radioactivity. Radioactivity in the collected materials could only come from some lindane-^{14}C metabolites that cochromatographed with the admixed reference compounds. The presence of the suspected compounds was later indicated by ecgc analysis of the anisole derivatives. For this purpose an aliquot of fraction II (that was not mixed with any reference compound) was injected into the thermal conductivity gas chromatograph. The eluates emerging from the column between the times indicated by arrows in Figure 3B and possibly representing the phenols represented by peaks 2, 3, 6, 7, and 8 in Figure 3A were collected. The collected materials were reacted with diazomethane and examined by ecgc on column b at 130°. The electron capture gas chromatogram of the collected materials showed peaks having retention times the same as those of the anisole derivatives of reference 2,3-dichlorophenol, 2,6-dichlorophenol, 2,3,4-trichloro- and 2,3,6-trichlorophenol and 3,4-dichlorophenol, indicating that fraction II contained trace amounts of the corresponding phenols. Similar analysis of fraction III indicated trace amounts of 2,6-dichlorophenol, 2,3,4-trichloro- and 2,3,6-trichlorophenol, and 3,4-dichlorophenol.

Tcgc of fractions II and III on column e at 180° showed one peak having a retention time of 16.5 min (Figure 4), in addition to the peaks observed at a lower temperature (140°) on the same column (Figure 3). Some of the early eluting compounds that could be resolved at the lower temperature could not, however, be resolved at the higher temperature. Since the early eluting compounds (retention time up to 9 min, Figures 4B and C) were identified before, no attempt was made to identify these peaks from the tcgc at 180°. The retention time of the additional peak at 16.5 min was the same as that of 2,3,4,6- and/or 2,3,4,5-tetrachlorophenol. The compound(s) representing the peak at 16.5 min (Figure 4C) from fraction II was collected by tcgc and converted to the anisole derivative by reacting with diazomethane. The infrared spectrum of the anisole derivative was the same as that of reference 2,3,4,6-tetrachloroanisole (Figure 8), confirming that this peak was due to 2,3,4,6-tetrachlorophenol.

Although 2,3,4,6- and 2,3,4,5-tetrachlorophenol could not be separated on column e at 180°, their anisole derivatives could easily be separated on the same column at the same temperature. The compound(s) representing the peak at 16.5 min in fraction III (Figure 4B) was collected by tcgc and converted to the anisole derivative by reacting with diazomethane. Tcgc analysis of the anisole derivative showed two peaks in the proportion 2:3 and having retention times the same as those of reference 2,3,4,6- and 2,3,4,5-tetrachloroanisole. The mass spectrum (see "Supplementary Material Available" paragraph at end of article) of the compound representing the larger peak was similar to that of reference 2,3,4,5-tetrachloroanisole. Thus, fraction III contained both 2,3,4,5- and 2,3,4,6-

tetrachlorophenol, while fraction II contained only the latter metabolite.

Tcgc of fractions II or III did not show any peak (Figures 4B and C) due to 3,4,5-trichlorophenol or pentachlorophenol, which had retention times of 21.5 and 41.0 min, respectively (Figure 4A). To test whether traces of these compounds were present in fractions II and III, effluents from tcgc of these fractions were collected between times indicated by arrows in Figures 4B and C and their radioactivity contents were measured. Since the collected material contained radioactivity, the presence of these two compounds was suspected. Although the collected materials did not give any measurable response in the thermal conductivity detector, they were enough for detection by ecgc. The presence of these two suspected compounds was then indicated by ecgc analysis of the collected material and the anisole derivatives of the collected materials as well (by comparing their retention times with those of the corresponding reference compounds).

Thus, in all, 14 chlorophenols were identified from the ether extract of the rabbit urine (Table I). Four of these metabolites were identified by infrared spectroscopy, three by mass spectrometry, while the remaining seven were *tentatively* identified by comparing gas chromatographic retention times of the chlorophenols and their anisole derivatives with those of reference compounds. The relative abundances of the various metabolites were determined by collecting the gas chromatographic effluents representing the various compounds by tcgc and measuring their radioactivity contents. As mentioned before, in some cases, incorporation of nonradioactive reference compounds was necessary for obtaining detector response in tcgc. Since quantitative measurements were based on radioactivity determination, incorporation on nonradioactive materials would not affect the validity of the data. The most abundant metabolite was 2,4,5-trichlorophenol, followed by 2,3,5-trichlorophenol, 2,4,6-trichlorophenol, 2,3,4,6-tetrachlorophenol, 2,3-dichlorophenol, 2,4-dichlorophenol, and 2,3,4-trichlorophenol in decreasing order (Table I). The other metabolites were present in quantities less than 1% or in trace amounts. Chadwick and Freal (1972) identified 3,4-dichlorophenol, 2,3,5-, 2,4,5-, and 2,4,6-trichlorophenol, and 2,3,4,6- and 2,3,4,5-tetrachlorophenol from the urine of rats that were fed lindane. In addition to these six chlorophenols identified by Chadwick and Freal (1972), the present investigation showed the presence of eight more chlorophenols as urinary metabolites from rabbit urine. Chadwick and Freal (1972) found pentachlorocyclohexenol (PCCOL) among the urinary metabolites of lindane in the rat. This compound was not found in the present study. Pentachlorocyclohexenol may have been present in the rabbit urine as a conjugate. Since the rabbit urine was not subjected to acid hydrolysis prior to extraction with ether, any conjugate of PCCOL, if present, was not extracted by ether. Chadwick and Freal (1972) acidified the rat urine prior to extraction with benzene and thus may have hydrolyzed conjugates of PCCOL and/or the phenolic metabolites.

The 14 chlorophenols accounted for 62% of the radioactivity in the ether extract of the rabbit urine (Table I). The six chlorobenzenes accounted for another 6.3% of the ether-soluble urinary metabolites. The smallest molecular weight compound so far identified among the metabolites of lindane in the animal system is 3,4-dichlorophenol. The smallest molecular weight metabolite found in the present study was 1,2-dichlorobenzene, and it was present in rather significant quantities. Obviously 1,2-dichlorobenzene could not have been formed by the dehydrochlorination reaction alone, although the trichlorobenzene was formed by this mechanism. Dehydrochlorination to trichlorobenzene, followed by dechlorination, appears to be the likely pathway for the formation of 1,2-dichlorobenzene. Pentachlorobenzene could be visualized as resulting from dehydrochlorination and dehydrogenation reactions. Similar mechanisms may be involved in the formation of the tetrachlorobenzenes.

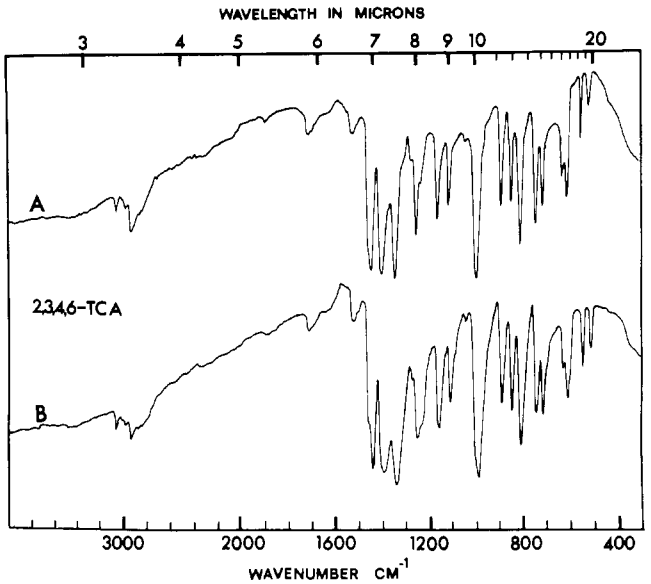

Figure 8. Infrared spectra of the anisole derivative of the 2,3,4,6-tetrachlorophenol isolated from the rabbit urine (A) and (B) reference 2,3,4,6-tetrachloroanisole (2,3,4,6-TCA).

No lindane was found in the ether extract of urine, nor was there any indication of the presence of γ-PCCH (γ-pentachlorocyclohexene), which has been identified as a metabolite of lindane in the housefly (Reed and Forgash, 1969, 1970; Sternburg and Kearns, 1956). This has also been suggested as an intermediate in the metabolism of lindane in rats (Grover and Sims, 1965), although γ-PCCH has never been identified as a metabolite from rats. Even if γ-PCCH was formed as an intermediate, it is likely that it was degraded to other compounds rapidly. In fact it has been shown that γ-PCCH is rapidly metabolized by rats to 2,3,5- and 2,4,5-trichlorophenol and 2,4-dichlorophenylmercapturic acid. These two trichlorophenols were also in the major metabolites found in the present study.

The results of the present study indicate that the metabolism of lindane by mammals is more complex than previously realized. Although it is tempting to suggest a metabolic pathway for the formation of the observed metabolites, it is perhaps better not to do so in the absence of adequate evidence. However, it is apparent that the formation of chlorobenzene and chlorophenols involves reactions such as dehydrochlorination, dehalogenation, dehydrogenation, and hydroxylation.

ACKNOWLEDGMENT

The authors are thankful to the National Research Council of Canada for the award of a Postdoctoral Fellowship to J. C. Karapally.

Supplementary Material Available. Additional data and mass spectra will appear following these pages in the microfilm edition of this volume of the journal. Photocopies of the supplementary material from this paper only or microfiche (105 × 148 mm, 20× reduction, negatives) containing all of the supplementary material for the papers in this issue may be obtained from the Journals Department, American Chemical Society, 1155 16th St., N.W., Washington, D. C. 20036. Remit check or money order for $3.00 for photocopy or $2.00 for microfiche, referring to code number JAFC-73-811.

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Received for review October 10, 1972. Accepted May 23, 1973. Contribution No. 493, Canada Agriculture Research Station, University Campus, Saskatoon, Saskatchewan, Canada. Presented at Division of Pesticide Chemistry, 162nd National Meeting of the American Chemical Society, Washington, D. C., September 1971.

Tissue Residue Studies in Swine Treated with Virginiamycin

C. John DiCuollo,* James A. Miller, and Cecil R. Miller

Virginiamycin was administered as a feed supplement to swine at a level of 170.5 ppm (155 g/ton) for a period of 18 weeks. Residues of the antibiotic greater than 0.1 ppm could not be detected in muscle, liver, kidney, fat, and skin, even in animals not subjected to a withdrawal period.

Virginiamycin is an antibiotic produced by a mutant of *Streptomyces virginiae,* exhibiting activity *in vitro* and *in vivo* primarily against gram-positive organisms (Van Dijck, 1969). Its biological and chemical properties resemble those of other complex antibiotics, such as mikamycin, ostreogrycin, PA 114 factor, pristinamycin, streptogramin, and vernamycin. These antibiotics have in common the presence of two factors which exhibit separate biological activities against several microorganisms but synergistic activity against a single bacterium (Vazquez, 1966).

Factor M, present in the highest concentration, is a macrocyclic lactone containing an oxazole ring (Figure 1). It strongly resembles ostreogrycin A (Delpierre *et al.,* 1966; Vanderhaeghe *et al.,* 1957). Factor S, the minor component, consists of a cyclopeptide lactone ring (Vanderhaeghe and Parmentier, 1960) (Figure 2) which is similar to the B factor of the ostreogrycin complex (Eastwood *et al.,* 1960) and the I_A component of pristinamycin (Jolles *et al.,* 1965).

Both factors M and S exhibit bacteriostatic activity separately but in combination, while exhibiting their synergistic effect, are bactericidal. Apparently, the primary target for both factors of virginiamycin is the process of translation in which inhibition of peptide chain formation occurs, thereby accounting for their synergistic action (Cocito, 1969).

To date, the primary application of virginiamycin has been its use as a feed additive for the growth promotion of swine and for the prophylaxis and treatment of swine dysentery. In this respect, it is an ideal agent, since the gram-positive nature of this antibiotic avoids the problems of resistance transfer through R factor.

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Its growth promotant effects have been reported on by a number of investigators. In one study, virginiamycin at levels of 44 ppm (20 mg/lb) in feed resulted in significant increases in average daily gains in swine (Jones and Pond, 1963). Griffin *et al.* (1961) reported on the use of virginiamycin in feeds at levels of 22–44 ppm (20–40 g/ton). Again, the results demonstrated significant increases in weight gains of swine. Numerous other investigations confirm the activity of virginiamycin as a growth promotant (Barnhart *et al.,* 1960; Griffin and Lidvall, 1962; Miller and Barnhart, 1961).

Reports from abroad first disclosed the activity of virginiamycin in young pigs against blood scours following artificial challenge with swine dysentery in controlled studies (Scholtz and Philip, 1970). Most recently, virginiamycin was shown to protect young pigs effectively against an artificial challenge of the virulent strain of swine dysentery (Miller *et al.,* 1972). In that study, virginiamycin and tylosin were administered to pigs at levels of 28, 55, and 110 ppm (25, 50, and 100 g/ton) in feed. Virginiamycin was found to be more effective than tylosin at all dose levels in decreasing the incidence of diarrhea and death loss, while weight gains and feed efficiency were all improved.

Recently, we performed studies with virginiamycin in swine to determine its potential for the production of tissue residues under practical conditions. These studies are presented in detail in the following report.

MATERIALS AND METHODS

Chemicals and Supplies. Standard plastic petri dishes having an inside diameter of 100 mm and height of 15 mm with plane bottoms were employed. Stainless steel cylinders with internal diameters of 6.35 mm and heights of 10.0 mm were purchased from J. L. Behmer, Inc., Philadelphia, Pa. Trypticase-soy agar, BBL No. 11043, and trypticase-soy broth, BBL No. 11768, were employed in the assay procedure. All reagents employed were Fisher,