EXHIBIT E

EXPERIMENTS WITH 2.5.4'-TRICHLOROBIPHENYL

R. Felt

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INTRODUCTION

Since 1929 when the production of polychlorinated biphenyl compounds was begun in the United States, PCB's have steadily accumulated and become widely dispersed throughout the environment. The persistance and toxicity of these compounds has been fully recognized since 1972 when voluntary restrictions were placed on their use. However, the total amount of PCB's in the environment appears to be increasing substantially each year. The world production of PCB's has been estimated at 3.6×10^7 kg per year of which 25% or more is lost to the environment through vaporization, leaks, spills, landfills, paper recycling, and the burning of waste which contains PCB's.

The study of 2,5,4'-trichlorobiphenyl (2,5,4'-TCB) has been undertaken to examine in detail the effects of this compound in animals from rats to Rhesus monkeys. 2,5,4'-TCB is a particularly apt choice in that it is one of the major constituents of the lower chlorinated biphenyl formulations.

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SINGLE ORAL DOSE OF 2,5,4'-TRICHLOROBIPHENYL IN LONG-EVANS RATS

Materials and Methods

A sample of ^{14}C-labelled 2,5,4'-trichlorobiphenyl (2,5,4'-TCB) of 0.254 mCi/mmole specific activity and 99.9+% purity as determined by gas chromatography was dissolved in absolute ethanol to give a 0.032 M solution.

Five female and five male Long-Evans rats (obtained from Simson Laboratories, Ca.) were placed in individual plastic metabolism cages with free access to water and Purina rat chow. The female rats ranged in weight from 212 to 294 g and the males ranged in weight from 218 to 290g. After allowing the rats to acclimatize for three days an oral dose of 5 ppm by body weight was administered to each rat. The volume of dosing solution varied according to the weight of the animal from 128ul to 178 ul. After dosing the animals were placed in individual metabolism cages whivh were designed to allow seperate collection of urine and feces.

Elimination of 2,5,4'-TCB

Every 24 hours the urine from each rat was collected and its volume measured. An aliquot was withdrawn and the radioactivity determined in a Packard 2425 Tri-Carb liquid scintillation spectrometer. All the remaining female urine was combined as was the remaining male urine and acidified to pH 1 with concentrated sulfuric acid. The acidified urine was then extracted for 24 hours with diethyl ether in a continuous liquid extractor. Ether was removed from the urine extracts on a rotary evaporator and the concentrated urine extracts were plated on silica gel thin layer plates and developed in bezene/ethanol/formic acid (90/9/1). Radioscans of the TLC

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plates with a Berthold LB 2723 thin layer scanner revealed a single major peak, R_f 0.2.

Feces from each rat were collected daily, homogenized and weighed samples prepared. Three fecal samples per rat per day were burned in a Packard 306 sample oxidizer and the liberated $^{14}CO_2$ counted in a Packard 2425 Tri-Carb liquid scintillation spectrometer. All male fecal samples for a day were then combined and thoroughly mixed with anhydrous magnesium sulfate and then extracted in a Soxholet extractor for 24 hours with diethyl ether. The same procedure was applied to the female fecal samples. Removal of diethyl ether by rotary evaporation was followed by plating of the extracted material on silica gel thin layer plates. Development of the thin layer plates was carried out in benzene/ethanol/formic acid (90/9/1). Radio-scans of the developed plates revealed three major zones of activity of R_f value 0.02, 0.43 - 0.59, and 0.94 in the ratio of 5.6 : 16.6 : 1 respectively.

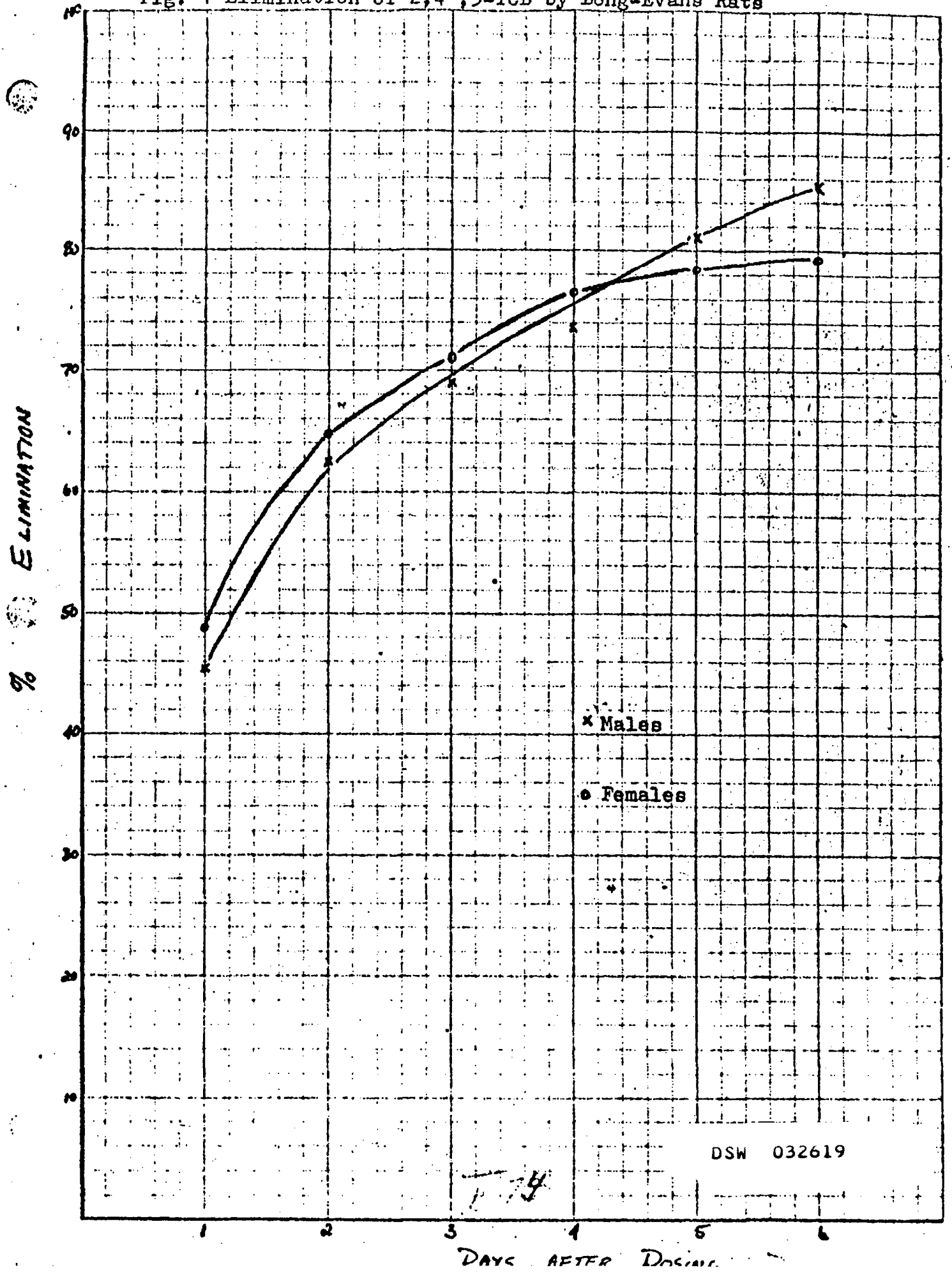
After six days the females had excreted 79.4% of the applied dose. Of this excreted activity 8.2% resided in the urine and 91.8% in the feces. The males after six days had excreted 84.6% of the applied dose, 4.2% of the activity was found to be in the urine and 95.8% in the feces. Figure 1 shows the total cumulative daily elimination for the female and male rats. The biological half-life of 2,5,4'-TCB was slightly more than 24 hours for these rats.

Body Distribution of 2,5,4'-TCB

Six days after the rats were dosed they were sacrificed and necropsies performed. The organs and tissues listed in Table 1 were removed from the rats, rinsed in saline solution where appropriate, placed in Whirl-Pack plastic bags and stored at -18°. Two carefully weighed portions of each tissue

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Fig. 1 Elimination of 2,4',5-TCB by Long-Evans Rats



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sample from each rat were burnt in a Packard 306 sample oxidizer and counted in a Packard 2425 Tri-Carb liquid scintillation spectrometer. Sample size for the sciatic nerve, adrenal, ovaries, hair and fat was approximately 100mg; approximately 200mg portions of the remaining tissue samples were oxidized. Table 1 lists the mean concentration of labelled compound (reported as 2,5,4'-TCB) found in the examined tissues of male and female Long-Evans rats.

Tissue levels are highest in abdominal and subcutaneous fat. Adrenals and the sciatic nerve also bear substantial concentrations of 2,5,4'-TCB. For the most part tissue levels are comparable for males and females except for a level of 1.30 ± 0.32 ppm found in the ovaries compared to 0.16 ± 0.04 ppm found in the testes. Also noteworthy are the significant levels found in the skin and hair.

Metabolism

Purification of the material isolated from fecal extracts of R_f value 0.94 (TLC on silica gel, benzene/ethanol/formic acid) was accomplished by repeated TLC using developing solutions of methylene chloride/ethyl acetate (95/5) and hexane. Gas chromatography-mass spectrometry revealed this material to be unmetabolized 2,5,4'-TCB. Urine extracts afforded a substance which upon TLC (silica gel; benzene/ethanol/formic acid) gave a radioactive peak of R_f 0.2. Elution of this peak followed by acid hydrolysis with 9N sulfuric acid yielded a peak of R_f 0.45 under the same chromatographic conditions as above. Further purification of this metabolite was accomplished by silica gel TLC with methylene chloride/ethyl acetate (95/5) developer, R_f 0.60. Gas chromatography-mass spectrometric examination of this material showed it to be a trichloro-biphenylol.

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Table 1 Mean Concentrations (ppm) of 2,5,4'-TCB in Tissues of Long-Evans Rats after Single Oral Dose

Tissue	ppm 2,5,4'-TCB Males	Females
Heart	0.19	0.25
Lungs	1.15	2.58
Duodenum	0.37	0.33
Liver	0.56	0.53
Abdominal Fat	4.84	4.31
Subcataneous Fat	2.66	3.67
Kidney	0.41	0.52
Testes	0.16	
Ovaries		1.30
Uterus		0.62
Adrenal	0.93	1.25
Sciatic Nerve	1.04	2.05
Blood Plasma	0.30	0.29
Muscle	0.15	0.17
Hair	0.39	0.23
Skin	0.47	1.06
Thymus	0.24	0.32

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SINGLE ORAL DOSE OF 2,5,4'-TRICHLOROBIPHENYL IN THE RHESUS MONKEY

Materials and Methods

A sample of ^{14}C-labelled 2,5,4'-TCB of specific activity 0.252 mCi/mmole and 99.9+% purity as determined by gas ohromatography was dissolved in acetone to give a 0.240N dosing solution."

A 12.0kg male and a 4.5kg female Rhesus monkey were placed in individual metabolism cages. Each monkey was allowed one liter of water a day (usually not entirely consumed); the male was given 175g of Purina monkey chow biscuits and the female 110g a day. This basic diet was supplemented with one half an apple or banana daily for each monkey. After allowing three days for acclimitization to the metabolism cages the male was given a piece of apple spiked with 727ul of the above dosing solution and the female received a piece of apple spiked with 273ul of the above dosing solution. This dose corresponds to 3.74mg 2,5,4'-TCB per kilogram body weight.

Elimination

Every 24 hours urine from each monkey was collected and the volume measured. An aliquot was withdrawn and the radioactivity determined in a Packard 2425 Tri-Carb liquid scintillation spectrometer. The urine was acidified to pH 1 with concentrated sulfuric acid and extracted for 24 hours with diethyl ether in a continuous liquid extractor. Ether was removed from the urine extracts by rotary evaporation and the concentrated urine extracts were plated on silica gel thin layer plates which were developed in benzene/ethanol/formic acid (90/9/1). Radioscans of the TLC plates with a Berthold LB 2723 thin

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layer scanner revealed three zones of activity. Most of the activity (> 85%) resided in the starting zone. The bulk of the remaining activity was in a peak of R_f 0.35. The remaining radioactive peak of high R_f disappeared after the second day.

Feces from each monkey were collected daily, homogenized, weighed and weighed samples prepared. Three fecal samples per monkey per day were burned in a Packard 306 sample oxidizer and the liberated $^{14}CO_2$ counted in a Packard 2425 Tri-Carb liquid scintillation counter. The fecal material was thoroughly mixed with either anhydrous sodium sulfate or anhydrous magnesium sulfate and extracted with diethyl ether for 24 hours in a Soxholet extractor. Removal of diethyl ether by rotary evaporation was followed by plating of the extracted material on silica gel thin layer plates. Development of the thin layer plates was carried out in benzene/ethanol/formic acid (90/9/1). Radioscans of the developed plates revealed that in the first 48 hours after dosing more than 97% of the activity excreted in the feces was unchanged 2,5,4'-TCB. This is probably a result of failure of the animal to absorb the dose. From the third day on no more unmetabolized 2,5,4'-TCB could be detected in the feces. There were two major zones of radioactivity of approximately equal intensity.

The elimination of 2,5,4'-TCB by Rhesus monkeys after a single oral dose is shown in Figure 2.* After 14 days the male monkey had eliminated about 40% of the applied dose and the female had eliminated 58%. The male monkey eliminated 72% of the excreted material in the urine and 28% in the feces, whereas the female eliminated 58% in the urine and 42% in the feces.

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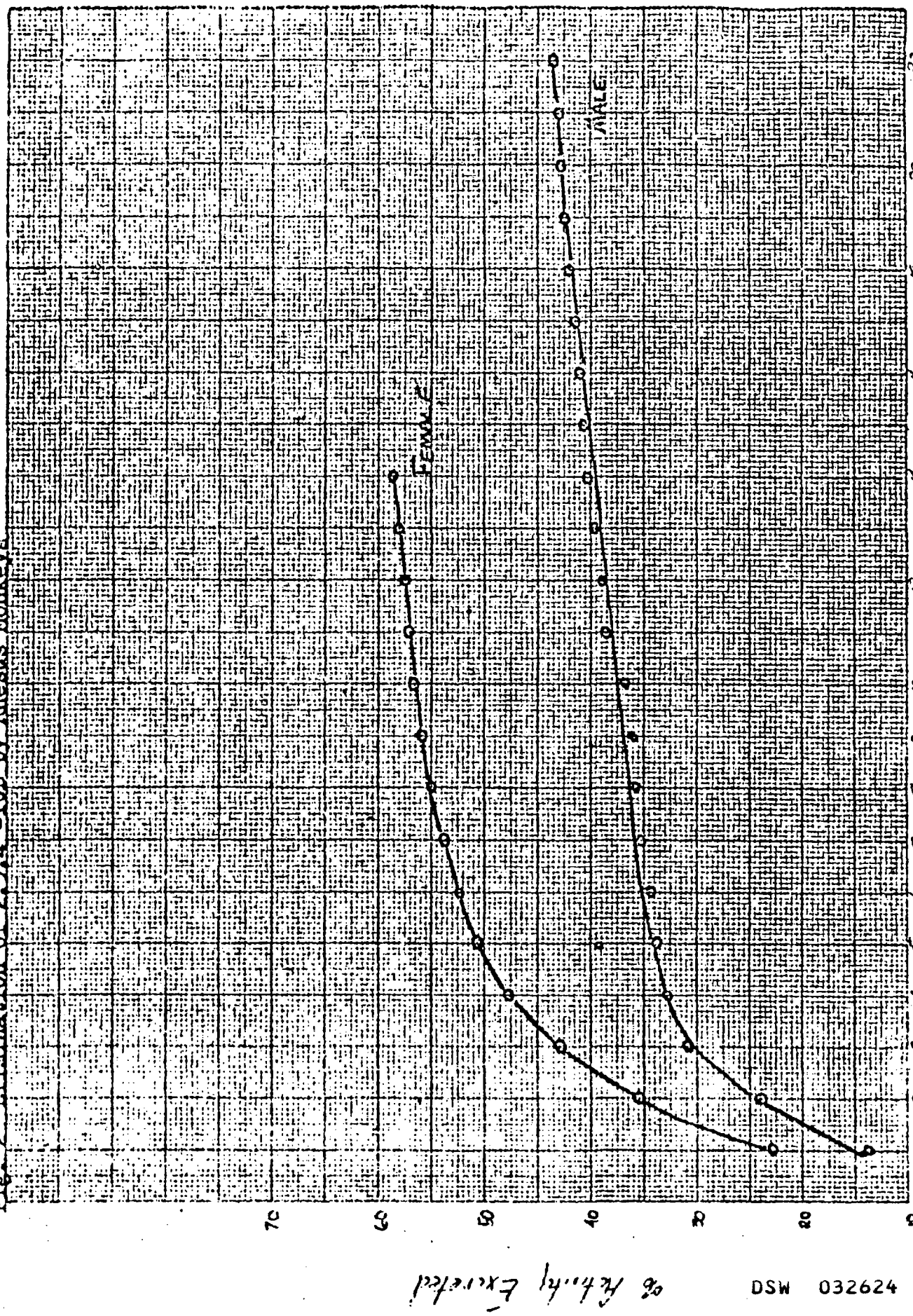


Fig. 2 Elimination of 2,5,4'-TCB by Rhesus Monkeys

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Metabolism

Thin layer chromatography of urine extracts gives three radioactive zones. It has been possible to purify these zones and to elucidate the structures of the compounds which make up these zones. The entire scheme is outlined in Figure 3.

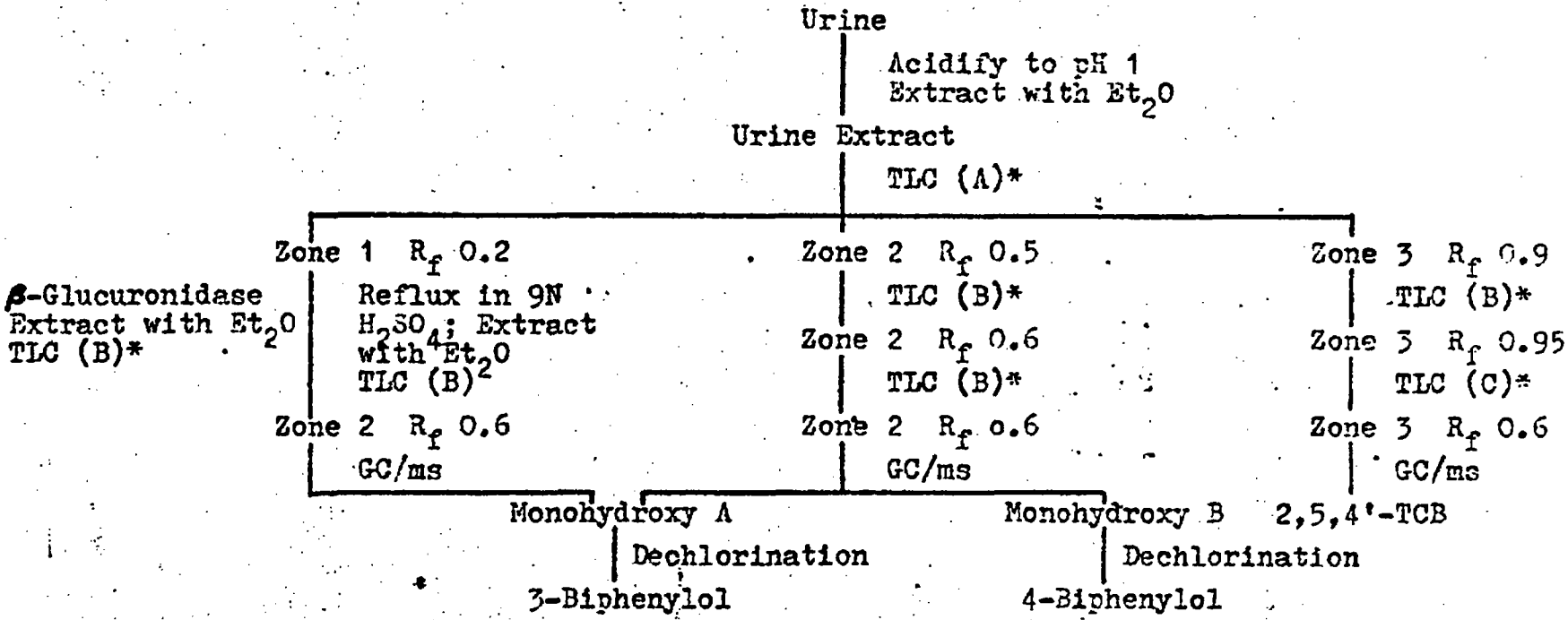
The zone of highest R_f value from the first plating of the urine extracts was further purified by two successive platings on silica gel. Development with methylene chloride/ethyl acetate (95/5) was followed by TLC on silica gel with hexane development. Verification of the identity of this compound was carried out with a Finnigan 3000D gas chromatograph-mass spectrometer. The compound isolated by the above procedure had identical retention time on a 1m x 2mm 3% OV-1 column as authentic 2,5,4'-TCB.

Zone 1, R_f of 0.2 in the first plating, turned out to be a glucuronide of a monohydroxy-2,5,4'-TCB. This was demonstrated in two ways. The material from zone 1 was refluxed in 9N sulfuric acid, extracted, and plated on a silica gel thin layer plate. Development of the plate with methylene chloride/ethyl acetate (95/5) gave a radioactive peak of R_f 0.6. Also when the material from zone 1 was incubated with a commercial β-glucuronidase (Ketodase from General Diagnostics) and then extracted and plated on silica gel, a radioactive peak of R_f 0.6 was obtained upon development with methylene chloride/ethyl acetate (95/5). This material, whether obtained by β-glucuronidase or acid hydrolysis, upon examination by gas chromatography-mass spectrometry gave the same monohydroxy-2,5,4'-TCB.

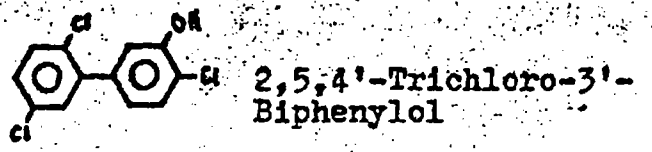
The material in zone 2 was purified by TLC on silica gel using methylene chloride/ethyl acetate (95/5) as the developing solution. Gas chromatography-mass spectrometry of this material revealed two monohydroxy-2,5,4'-TCB's in an approximate ratio of

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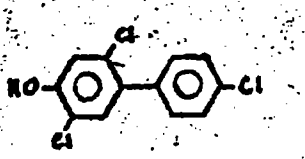
Fig. 3 Isolation, Purification and Identification of 2,5,4'-Trichlorobiphenyl in Rhesus Monkey Urine



*TLC Developing Solutions : A Benzene/Ethanol/Formic Acid (90/9/1)
B Methylene Chloride/Ethyl Acetate (95/5)
C Hexanes



2,5,4'-Trichloro-3'-Biphenylol
Monohydroxy A



2,5,4'-Trichloro-4-Biphenylol
Monohydroxy B

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20 : 1. The most abundant monhydroxy compound was the same as the one observed in the work up of zone 1. Both monhydroxy compounds could possibly have been present in zone 1, but the minor one might not have been present in sufficient quantity to observe.

Dechlorination of the purified material from zone 2 was carried out by refluxing the material in toluene with sodium dihydroxy-bis(2-methoxyethoxy) aluminate. Analysis of the dechlorination products by high pressure liquid chromatography on a u Porasil column revealed two peaks identical in retention volume to 3- and 4-biphenylol. These two peaks were collected as they eluted from the column and were counted in the liquid scintillation counter and shown to contain the labelled material. The major peak corresponded to 3-biphenylol and the minor peak corresponded to 4-biphenylol. Thus the major component of zone 2 is 2,5,4'-trichloro-3'-biphenylol, which as the glucuronide is the major component of zone 1. And therefore the minor component of zone 2 is 2,5,4'-trichloro-4-biphenylol.

The same scheme which has successfully elucidated the structure of the major urine metabolites is being applied to the fecal metabolites. Greater difficulty is encountered with the fecal extracts because of the greater difficulty in purifying them.

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LONG TERM FEEDING STUDY OF 2,5,4'-TRICHLOROBIPHENYL IN THE RHESUS MONKEY

Materials and Methods

A sample of ^{14}C-labelled 2,5,4'-TCB of 0.254mCi/mmole specific activity and 99.9+% purity as determined by gas chromatography was dissolved in absolute ethanol to give a 0.034 M dosing solution.

Two male Rhesus monkeys (2.60 and 2.85kg) and two female Rhesus monkeys (3.15 and 3.28kg) were placed in individual metabolism cages. Each monkey received one liter of water (usually not entirely consumed) and 110g of Purina monkey chow biscuits per day. This diet was supplemented with a daily ration of fresh fruit, usually one half of an apple or banana. The monkeys were allowed to acclimatize to their metabolism cages for three days before dosing was commenced. Each monkey received 62.5ul of the dosing solution in a piece of fruit each day. This dose is equal to 550ug or 5ppm of the daily diet.

Elimination

Every 24 hours urine from each monkey was collected and the volume measured. An aliquot was withdrawn and the radioactivity determined in a Packard 2425 Tri-Carb liquid scintillation counter. The urine was acidified to pH 1 with concentrated sulfuric acid and extracted for twenty-four hours with diethyl ether in a continuous liquid extractor. Ether was removed from the urine extracts by rotary evaporation and the concentrated extracts were plated on silica gel plates which were developed in benzene/ethanol/formic acid (90/9/1). Radioscans of the TLC plates with a Berthold

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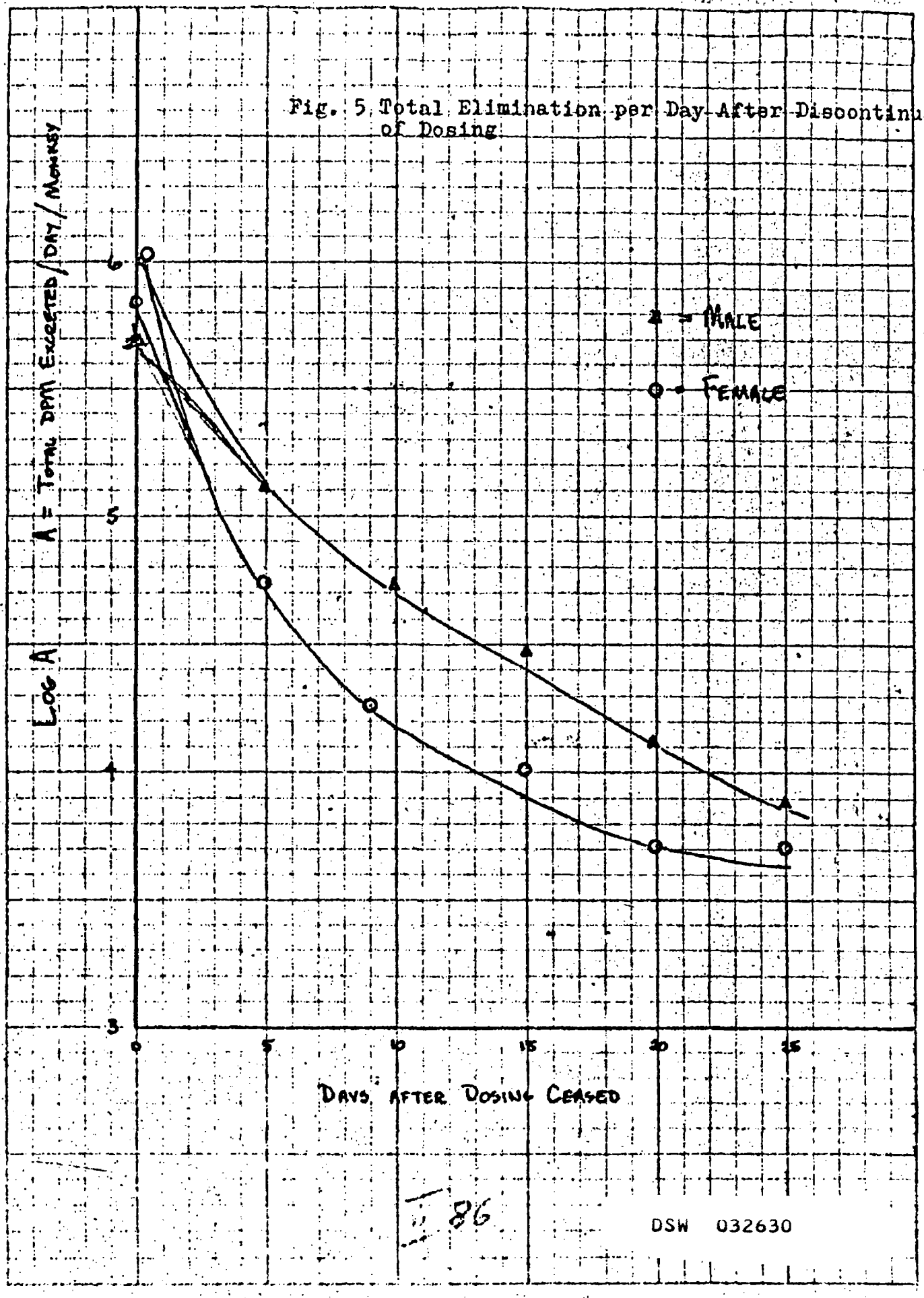
LB 2723 thin layer scanner revealed three zones of activity as were obtained in the single oral dose of Rhesus monkeys with 2,5,4'-TCB. After 85 days of dosing one male and one female monkey was sacrificed. Dosing of the remaining two monkeys was discontinued after the 85th day. On the 90th day of the experiment the urinary excretion of the two remaining monkeys had decreased to a level which could not be determined directly. Therefore the urine from the 90th day to the conclusion of the experiment was acidified to pH 1 with concentrated sulfuric acid and extracted with diethyl ether for 24 hours in a continuous liquid extractor. Most of the other was removed by rotay evaporation and the concentrated ether extract was quantitatively transferred to a 25 ml volumetric flask. Methylene chloride was added to the mark and two 0.5 ml aliquots were withdrawn for determination of the radioactivity by liquid scintillation counting.

Daily collections of combined male feces and combined female feces were performed. Weighed samples from the homogenized feces were oxidized as usual in a Packard 306 sample oxidizer and the liberated $^{14}CO_2$ was counted in a Packard 2425 Tri-Carb liquid scintillation counter. The feces were thoroughly mixed with anhydrous magnesium sulfate and extracted with diethyl ether for 24 hours in a Soxhlet extractor. Thin layer chromatography or column chromatography of the fecal extracts gave results similar to those discussed for the single oral dose of 2,5,4'-TCB in Rhesus monkeys.

After 85 days of dosing at a level of 5 ppm of the daily diet a male and female monkey was sacrificed. Of the total radioactivity recovered, 58% was excreted in the urine of the females and 42% in the female feces. The recovered radioactivity from the males was found to be 48% in the urine and 52% in the feces. Figure 4 shows the cumulative dose and

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Fig. 5 Total Elimination per Day After Discontinua of Dosing



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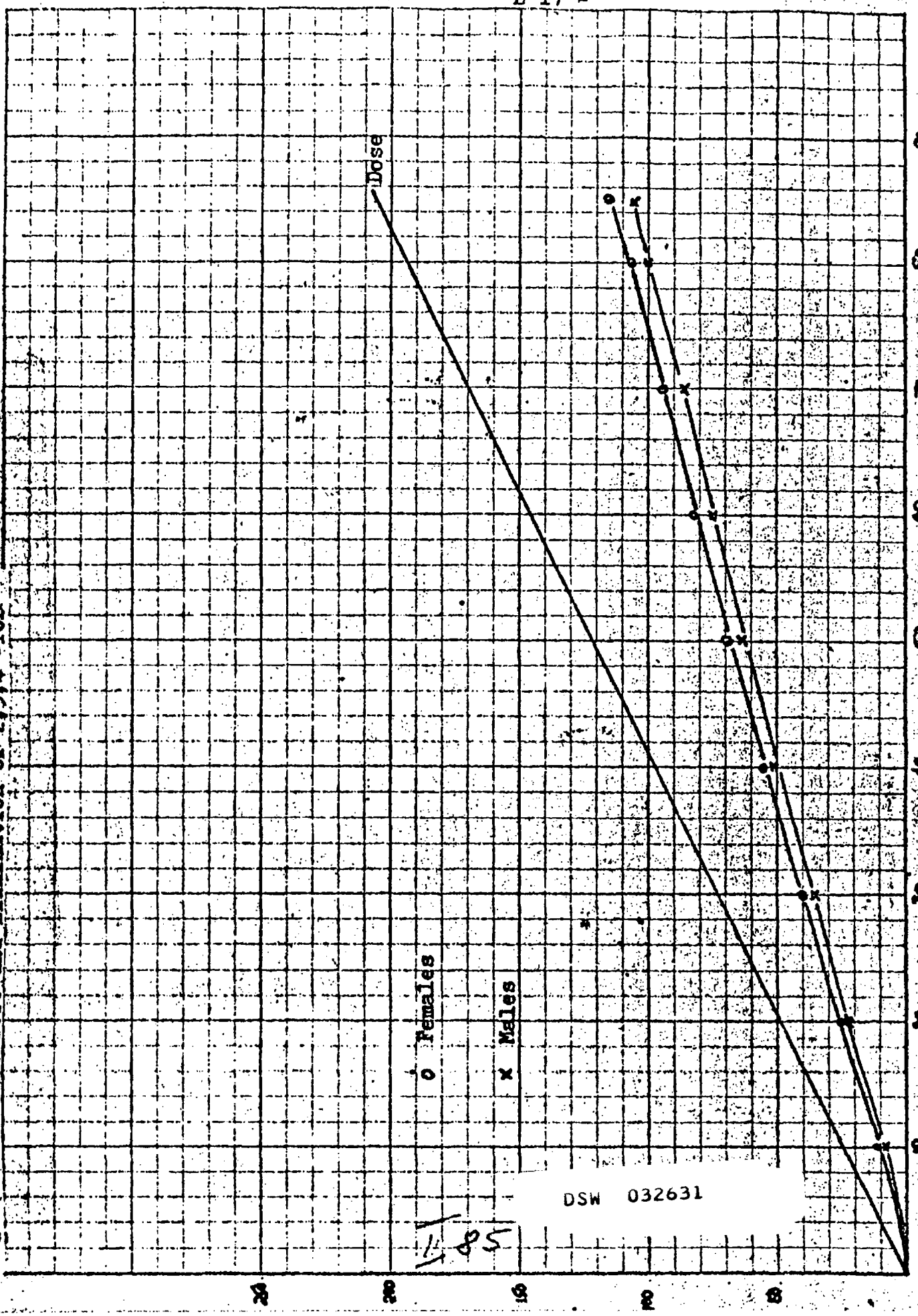


Fig. 4 Cumulative Dose and Elimination of 2,5,4'-TCB

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elimination of 2,5,4'-TCB. After the 85th day dosing of the remaining two monkeys was discontinued. Figure 5 shows the rapid decline in excretion of radioactivity after dosing ceased.

Body Distribution of 2,5,4'-TCB

Table 2 lists the tissues taken at necropsy from the two animals sacrificed on the 85th day of the experiment. All tissue samples were placed in Whirl-Pack plastic bags and stored at -18° until they were oxidized. Whenever sufficient tissue was available, two carefully weighed samples were burned in a Packard 306 sample oxidizer and then counted in a Packard 2425 Tri-Carb liquid scintillation spectrometer. Table 2 lists mean values for the concentration of ^{14}C-labelled material (as 2,5,4'-TCB) found in tissues.

The remaining two monkeys were allowed to recover for 27 days and then they too were sacrificed and necropsies performed. All of the tissues examined for the first two monkeys were also examined for these two. However, the only significant levels of radioactivity were found in the bile, fat (of which omental fat gives typical values), hair, and liver. Table 3 gives these results.

Examination of Table 2 reveals some interesting distribution patterns. The female has high levels in the ovaries and fallopian tubes, the levels in the testes of the male are relatively low. Also very interesting is the fact that the concentrations found in the female's fat are significantly higher than in the male's. And perhaps most significant of all are the low levels found in the lymph nodes of the male in comparison to the much higher levels found in the lymph nodes of the female.

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Table 2 Mean Concentrations (ppm) of 2,5,4'-TCB in Tissues of Rhesus Monkey after 85 Days of Dosing

	ppm 2,5,4'-TCB	
Tissue	Male	Female
Liver	1.16	1.45
Bile	60.90	28.83
Urinary Bladder	0.17	0.59
Pancreas	0.28	0.77
Spleen	0.10	0.11
Adrenal Cortex	1.04	1.55
Kidney	0.86	0.71
Heart	0.24	0.51
Lungs	0.20	0.20
Thymus	0.18	0.30
Thyroid	0.33	0.10
Pituitary	0.94	0.11
Ovaries		2.20
Fallopian Tubes		1.86
Uterus		0.20
Vagina		0.75
Testes	0.23	
Prostrate	0.78	
Skin	1.08	0.97
Skeletal Muscle	0.18	0.11
Hair	0.40	0.48
Bone	<0.10	<0.10
Bone Marrow	<0.10	1.30
Blood Plasma	0.41	0.20
Tongue	0.20	0.19
Parotid	0.41	0.45

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Table 2 Continued

Tissue	ppm 2,5,4'-TCB Male	Female
Mammary Glands	1.86	0.37
Eye		
Cornea	<0.10	<0.10
Vitreous Humor	<0.10	<0.10
Optic Nerve	1.99	0.76
Esophagus	0.34	0.14
Stomach	0.25	0.15
Duodenum	0.62	0.44
Jejunum	0.29	0.48
Small Intestine	0.12	1.41
Cecum	0.70	1.21
Colon	0.31	0.23
Lymph Nodes		
Sublinqual	0.21	1.15
Mesenteric	0.20	3.92
Hilar	0.29	0.67
Axillary	0.17	1.24
Inquinal	0.28	2.96
Fat		
Mesenteric	3.00	8.13
Omental	5.03	9.79
Inquinal	5.02	7.98
Subcutaneous	3.53	5.50
Brown	2.97	5.35
Sciatic Nerve	0.44	0.37
Meninges	0.22	0.10

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Table 2 Continued

Tissue	ppm 2,5,4'-TCB Male	Female
Central Nervous System		
Motor Cortex	0.12	0.17
Sensory Cortex	0.13	0.16
Corpus Call.	0.11	0.21
Thalamus	0.11	0.24
Hypothalamus	0.16	0.19
Caloarine Fissure Cortex	0.15	0.15
Mesencephalon	0.18	0.18
Pons	0.17	0.24
Cerebellum	0.15	0.12
Cervical Spinal Cord	0.19	0.26

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Table 3 Mean Concentrations (ppm) of 2,5,4'-TCB in Tissues of Rhesus Monkey after 85 Days of Dosing and 27 Days Recovery

Tissue	ppm 2,5,4'-TCB	
	Male	Female
Bile	0.35	0.10
Omental Fat	0.22	0.24
Hair	0.11	0.05
Liver	0.09	0.09

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Summary

2,5,4'-TCB is excreted rapidly from rats and more slowly from Rhesus monkeys. Most of the excreted material has been metabolized to a monohydroxy compound which is then excreted free or as a conjugate. The body distributions of 2,5,4'-TCB in rats and Rhesus monkeys show that the female reproductive system accumulates particularly high levels of the compound. Furthermore, a sex difference in the way Rhesus monkeys cope with 2,5,4'-TCB appears to have been observed. Bone marrow and lymph node levels in the female Rhesus monkey are ten times or more greater than in the corresponding male tissues.

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