EPA 307(a)

J. C. Weber - B2SK (St. Louis)

November 16, 1976

DR. WOLFGANG MUELLER'S AFFIDAVIT
EPA 307(a)

D. R. Bishop
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Attached is Dr. Wolfgang Mueller's affidavit for the EPA 307(a) hearings on PCBs. His testimony is very favorable on Aroclor 1016. Dr. Mueller also has experimented with di- and trichlorobiphenyls in Rhesus Monkeys and rodents.

Cole Weber

J. Coleman Weber

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Enclosure

UNITED STATES
ENVIRONMENTAL PROTECTION AGENCY
BEFORE THE ADMINISTRATOR

In the Matter of:

PROPOSED TOXIC POLLUTANT EFFLUENT STANDARDS FOR POLYCHLORINATED BIPHENYLS (PCBs)	)	FWPCA (307) Docket No. 4

AFFIDAVIT OF DR. WOLFGANG F. MUELLER

DR. WOLFGANG F. MUELLER, being duly sworn, deposes and says:

1. I am currently employed at the Institut fuer Oekologische Chemie der Gesellschaft fuer Strahlen- und Umweltforschung m.b.H., (GSF) in Munich, Germany. I received a degree of Diplomchemiker in 1967, and the title of Doctor rer. nat. in 1969 from the University of Bonn, Germany. From 1969 to 1971, I was head of the pesticide metabolism laboratory and instructor in analytical and preparative organic chemistry at the Chemical Institute of Bonn University. In 1971 I joined the GSF Institut fuer Oekologische Chemie to install and direct a research laboratory for the study of dispersion tendencies of environmental chemicals. In 1973, I came to the primate center of the International Center for Environmental Safety at Holloman Air Force Base, New Mexico, to direct a research group studying the metabolism and toxicokinetics of PCB and other environmental chemicals in

non-human primates. ICES is a division of the Albany Medical College's Institute of Comparative and Human Toxicology. I attach as Exhibit A a more detailed curriculum vitae.

2. My primary professional interest has been in the field of metabolism and toxicokinetics of chlorinated hydrocarbon compounds (including PCB) in mammals, and their behavior in the environment (e.g., persistence, dispersion tendencies, bioaccumulation). I have personally conducted, as well as supervised, numerous studies in this field, through which I have gathered experience both in the analysis of PCB, their transport and accumulation in the environment and their metabolic and toxicokinetic behavior in mammals including primates. I have been asked to review the Criteria Document prepared by the Environmental Protection Agency in connection with this proceeding, as well as those witness statements relevant to mammalian toxicology, and to comment on the evidence compiled by the EPA concerning the alleged toxicity of Aroclor 1016.

3. In cooperation with ICES toxicologists, I have also studied the effects of PCB on the structure and function of target organs, e.g., liver, thymus, adrenals and gonads. The behavior of Polychlorinated Biphenyls depends very much on their degree of chlorination. Vapor pressure and water solubility are significantly higher for di- and trichloro BPs than for penta- and hexachloro-BPs, as shown in various

papers reviewed in the Criteria Document.[1/] These factors will influence the distribution of different PCB mixtures in the environment.

4. More significant for the evaluation of any possible biological hazard is the distribution coefficient between water and organic solvents as a measure for water/lipid distribution. This coefficient is an important factor for the degree of biological accumulation. It decreases with increasing chlorine content, which results in stronger enrichment of more highly chlorinated isomers in the lipids of plants and animals. The data compiled in the Criteria Document under IV.2[2/] show clearly that di- and trichlorobiphenyls are accumulated less than penta-, hexa-, or heptachloro isomers.

5. The fact that only very small amounts of di-, tri- and tetra-CB are found in living organisms, however, cannot be explained by selective enrichment alone. It must be assumed that those quantities of lower chlorinated isomers which are taken up by organisms, can be actively eliminated. It has indeed been shown in numerous studies, reviewed in Appendix E to the Criteria Document, that bacteria, invertebrates, fish, birds, and mammals are able to metabolize PCB, i.e. alter their structure to make them more water

soluble and thus excretable. The ability of PCB to be metabolized decreases in all investigated organisms with increasing chlorine content: mono- and dichloro BP are generally metabolized very rapidly, trichloro BP somewhat more slowly. Although the metabolism rate for tetrachloro BP is considerably lower in all species than for mono- to trichloro BP, residue data presented by Nisbet and Sarofim[3/] indicate that it is still sufficient to prevent accumulation under environmental conditions. Biphenyls with five or more chlorine atoms, however, are metabolized so slowly that they are found to be accumulated in many species, including mammals and man.

6. Elevated body burdens of these isomers have been shown to have adverse effects on the structure and function of certain organs in various species.[4/] The most serious changes caused by PCB are found in liver. Structurally, liver enlargement as well as enlarged hepatocytes, cytoplasmic vacuolisation and inclusions and fatty degeneration have been reported. Additionally, with high does of Aroclor 1260 and 1254, adenofibrosis was found (c.f. Affidavit of Dr. Kimbrough).[5/] Kimbrough[6/] also reported that upon feeding of 100 ppm Aroclor 1260 for 21 months to female rats, 170 out

of 184 rats developed hepatic nodules, 26 of which she classified as hepatocellular carcinoma. It is very important to point out that all these structural liver changes depend on the degree of chlorination of the PCB.

7. Tables III. 9.4 and III. 9.5 in the Criteria Document[7/] show that upon feeding of 100 ppm Aroclor 1016 for 8 months to rats, 3 out of 4 developed enlarged hepatocytes, which is the most sensitive and also the mildest parameter of liver changes listed in these tables. No other signs of hepatotoxicity of 1016 is shown for this dose level and duration. The same exposure to Aroclor 1242 produced enlarged hepatocytes in all treated rats, in addition cytoplasmic vacuolisation and inclusions were found in 3 out of 4, brown pigment in 2 out of 4 and hemorrhage or foci of necrosis in 1 out of 4 rats. No increase in lipids, adenofibrosis or foamy cytoplasm was reported for either of these Aroclors. At the same dose level and duration, Aroclor 1254 produced all these damages at a high incidence in female rats, somewhat less pronounced in males. Under the same experimental conditions, Aroclor 1260 was found to be less hepatotoxic than 1254: no adenofibrosis was found in male rats, and only in 1 out of 10 females; foamy cytoplasm was not found at all. This comparison demonstrates not only that the mixtures with a lower degree of chlorination are by far less hepatotoxic than the more highly

chlorinated products which have been used in almost all of the studies in which effects of PCB on the liver were investigated, it also indicates a significant difference between the Aroclors 1242 and 1016.

8. Taking into account the available information on metabolism and toxicokinetics of PCB, it is not surprising to find Aroclor 1016 to be less hepatotoxic in chronic studies than 1242. Whereas 1016 contains only 1 percent pentachloro BP and a trace of hexachloro BP, 1242 has been reported to contain between 8 and 14 percent pentachloro-, up to 1 percent hexachloro-, and a trace of heptachloro BP. (Table II. 3.1, of the Criteria Document).[8/] The tetrachloro BP content of 1016 is reported to be 21 percent and between 25 and 42 percent in 1242. A total of 78 percent of 1016 consists of mono-, di-, and trichloro BP, which are rapidly metabolized and show little accumulation tendency, whereas this group in 1242 amounts to only 43 to 66 percent. Due to the high accumulation tendency and slow metabolism of the hexa-, penta-, and to some degree also the tetrachloro-PB, these components will build up to much higher levels in the test animals upon chronic treatment with 1242 than would be expected with 1016. Consequently, prolonged intake of Aroclor 1242 will result in elevated liver concentrations of tetra-, penta- and hexachloro

BP, which will eventually cause similar hepatic changes as have been found with lower levels of Aroclor 1248 or 1254. Due to its lower content of tetrachloro BP and the virtual absence of higher chlorinated isomers, 1016 will not produce these effects.

9. The same considerations apply principally to the aspect of enzyme induction. The ability of PCB to induce hepatic mixed function oxidase systems depends on the degree of chlorination. (c.f. Criteria Document Tables III. 102 and III. 10.3)[9/] Comparing enzyme induction by the Aroclors 1016 and 1242, the accumulation of higher chlorinated isomers in the liver upon treatment with 1242 explains the stronger MFO induction by this mixture as compared to 1016.

10. In connection with the enzyme induction studies reviewed in the Criteria Document, the question must be asked: How relevant are they for the toxicological evaluation of PCB, especially of Aroclor 1016? The test animals have to be treated with relatively high does of PCB to produce unequivocal induction effects. Enzyme induction at the level of actual environmental exposure can only be minute. D.B. Peakall in a comprehensive review "PCBs and Their Environmental Effects" (CRC Critical Reviews in Environmental Control, Sept.

1975, pp. 469-508) arrives at this conclusion:

> "The minimum dose causing an effect should be compared with the calculated intake to ascertain if an effect is likely. In the case of man, the normal exposure to PCBs is too low for an effect on hepatic enzymes to be observed."

The argument has been used that enzyme induction by PCB might alter the human response to therapeutic drugs. Any such effect due to environmental PCB levels could only be so small that it falls well within the limits of individual variation of drug susceptibility.

11. The role of enzyme induction, especially in chemical carcinogenesis, is not as yet very well understood. In his own work,[10] Dr. Gelboin has demonstrated the enhancement of AAF carcinogenicity by phenobarbital as well as the reduction of the carcinogenicity of AAF by hydrocarbon type inducers. Moreover, he has shown that mixed function oxidases play a central role in the conversion of benzo-(a) pyrene to its active carcinogenic form. On the other hand, he reports that induction of the enzyme system with hydrocarbon type inducers reduces carcinogensis by polycyclic hydrocarbons in mammary gland or in lung.[11] In view of these apparent contradictions, it must remain highly speculative to deduce enhancement of chemical carcinogenesis by enzyme induction by PCBs. Of course, Dr. Gelboin is aware of this, since in his affidavit he uses the words "alter"[12] and "change"[13] rather than "enhance"

or "increase" to describe the influence of PCBs on tumor formation by chemical carcinogens, leaving open the possibility that, in theory, the presence of PCBs might even be beneficial.

12. Dr. Gelboin's view offered in his affidavit in this proceeding that any degree of enzyme induction represents "a violation of the integrity of the human organism"[14/] is at least somewhat unusual. It stands to reason that inducible mixed function oxydases have developed to provide the organism with a tool to handle varying amounts of a variety of chemicals to which it is exposed in its environment. Consequently, MFO activities changing within reasonable limits may be considered as a physiologically normal reaction to the challenge by any chemical, including PCB.

13. It should also be noted that experiments involving MFO induction by PCBs have generally been conducted using higher chlorinated mixtures. It has been shown that with decreasing chlorine content of the isomers, their ability to induce MFO activity also decreases.[15/] Since Aroclor 1016 contains practically no isomers with more than four chlorine atoms, the results of induction studies with A 1248, 1254 or 1260 are not directly applicable to A 1016.

14. Similarly, the studies in which Dr. Kimbrough[16] demonstrated production of neoplastic lesions in rodent livers, confirmed by Dr. Squire,[17] have all been conducted with Aroclor 1254 and 1260 at relatively high dose levels. These mixtures contain mainly isomers with five or more chlorine atoms per molecule which are only very slowly metabolized and show a strong accumulation tendency, resulting in high body burdens and liver concentrations. Aroclor 1016, on the other hand, contains only isomers with one to four chlorine atoms per molecule which have been shown to be metabolized and excreted rapidly and show little accumulation tendency. Dr. Kimbrough's results, which are highly valuable in general for the evaluation of the impact of higher chlorinated PCBs on man and the environment, cannot be used for the assessment of toxic effects of Aroclor 1016.

15. Dr. Allen's affidavit[18] seems to contain the most severe scientific evidence against continued use of PCB. Most of his studies have been done with Aroclor 1248, which cannot be as easily disregarded as non-pertinent to A 1016 as could the studies with A 1254 and 1260. The difference in content of higher chlorinated isomers between 1016 and 1248, however, is marked enough

to point out that accumulation of 1016 components must be expected to be much lower than accumulation of 1248. 1248 contains an appreciable amount of penta- and hexachloro-isomers which are probably responsible for residual tissue concentrations as well as for the effects which Dr. Allen found upon long-term low-level exposure. Unfortunately, he shows only very little analytical data for the characterization of the residual PCBs found in animals after 1248 feeding. One table[19/] in his Affidavit in this proceeding with gaschromatographic retention times of PCB peaks from tissues of rhesus monkeys, shows that only those isomers with long retention times, i.e. high chlorine content, are found in adipose and liver tissue examined in that study. Since Aroclor 1016 does not contain penta- and hexachloro-isomers at all, or only in minute percentages, Dr. Allen's findings with 1248 are of questionable value for the toxicological evaluation of Aroclor 1016.

16. I have certain reservations concerning the conclusions which Dr. Allen drew from his findings on the metabolism of 2.5.2'5'.tetra-chlorobiphenyl in the infant rhesus monkey where he postulates an arenoxide pathway to the dihydro-dihydroxy metabolite. Our own metabolism studies with chronic feeding of low levels (5 ppm of the daily diet) of dichlorobiphenyls and

trichlorobiphenyl (all ^{14}C - labeled) in adult rhesus monkeys have so far not revealed any dihydro-dihydroxy metabolities and their conjugates. I attach as Exhibit B, C, D and E the results of these studies, two of which have been published, and the other two publications are in preparation at this time. A lactation transfer study, in which the Bayer product Clophen A 30 was fed to mothers of nursing rhesus infants, has shown some accumulation in the infants. It appears that the mixed function oxidase system responsible for direct hydroxylation in the rat and the adult rhesus monkey is not yet sufficiently developed in infants to metabolize all of the ingested PCBs by direct oxidation. In case of acute intoxication with a relatively high amount of PCB, as simulated by a single oral dose of 500 mg/kg TCB in Dr. Allen's experiment, the alternative pathway to the dihydro-diol via an arenoxide may be the only route available to the infant primate organism to detoxify the PCB. It remains to be shown whether this pathway is also used under the more relevant conditions of daily intake of small amounts of PCB with the mother's milk.

17. In any case, the results obtained from studies with infant rhesus monkeys should not be generalized for the non-human primate. Our studies indicate that adult rhesus monkeys, like rats, form mono- and dihydroxy metabolites and their conjugates from di- and trichlorobiphenyl. Furthermore, being aware of Dr. Allen's findings with 2.2'5.5' TCB in infant rhesus monkeys, we dosed rhesus infants

(5-7 month age) with 5 mg/kg of 2.5.4' Trichlorobiphenyl ^{14}C p. os. and examined urine and feces for metabolites. Under our experimental conditions, we did not find any dihydro-diol metabolite formed even by rhesus infants, only monohydroxy metabolites and conjugates. The identification of the dihydro-diol as described in Exhibit 9 of Dr. Allen's Affidavit is indirect and therefore open to criticism; there should be at least mass spectroscopic proof of the existence of the dihydro-diol. For the 2.5 and 5 ppm feeding study, from which Dr. Allen derived his data on the high toxicity of A 1248 to rhesus monkeys especially nursing infants, he should be able to show positive proof that cross-contamination from studies with higher PCB levels was safely avoided.

18. Dr. McNulty's affidavit does not serve to establish any solid scientific conclusions, due to the small number of animals used. The death of the single animal used for the 3 ppm dose[20/] may have been accidental or connected to the repeated stomach biopsies; only one control does not rule out the possibility that the change in the stomach mucosa were caused by parameters other than the low level of PCB. Dr. McNulty should show convincing proof to rule out the possibility of cross-contamination from other test diets, or even food contamination from higher chlorinated PCBs from the environment, either of which might be a source of a higher PCB level in the test animals' food than 3 ppm of A 1242, which could be the cause of the single death at 3 ppm. In any event, since Aroclor 1016 contains only 1/10

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of the percentage of penta- and hexachlorobiphenyls found in Aroclor 1242, its toxicity to rhesus monkeys should be expected to be lower than that of 1242.

19. In summary, it is my opinion that for the correct evaluation of ecologic-toxicological effects of Aroclor 1016 the following facts must be considered:

a) Aroclor 1016 differs in its chemical composition significantly from more highly chlorinated mixtures including 1242 in that it consists to nearly 80 percent of mono-, di-, and trichlorobiphenyls, and contains only 1 percent of pentachloro BP and a trace of hexachloro BP.

b) Due to its chemical composition, Aroclor 1016 can be metabolized relatively rapidly and much more completely than the products with higher chlorine content and 1242. Therefore its overall biological accumulation in the environment will be much lower than that of those other products.

c) Particularly in mammals, continued intake of small amounts of Aroclor 1016 will not result in elevated total body burdens or organ concentrations of PCB. Results from studies which have been carried out with Aroclors 1260, 1254, 1248 and 1242 cannot be directly applied for the toxicological evaluation of 1016, since all these products contain higher chlorinated isomers which are accumulated and stored in mammalian tissues.

d) The results on hepatotoxicity which are derived from studies with Aroclors 1254 and 1260 are of no practical value for the evaluation of the hepatotoxicity of 1016. The Criteria Document contains evidence that feeding 100 ppm of 1016 in the diet to rats for up to 10 months produces only the mildest form of structural liver changes, whereas 1254 under the same experimental conditions produces the full range of severe damage. There is absolutely no evidence that Aroclor 1016 at intake levels of actual environmental contamination or even at 10-100 times higher food concentration could cause liver carcinoma.

e) Although the enzyme inducing effects of low chlorinated biphenyls are far less marked than those demonstrated for higher chlorinated isomers, a certain (although small) effect of Aroclor 1016 on mixed function oxidases does exist. It cannot be said with assurance, however, that some small degree of induction of mixed function oxidases should be regarded as undesirable or injurious.

DR. WOLFGANG F. MUELLER

Subscribed and sworn to before me this __ day of November, 1976.

Notary Public

My Commission expires:

REFERENCES

1. EPA Criteria Document, EPA 440/9-76-021 at pages 43-53.
2. Id. at pages 282-330.
3. Panel on Hazardous Trace Substances (P.B. Hammond, I.C.T. Nisbet, and A.F. Sarofim). 1972. Polychlorinated Biphenyls--environmental impact. Environmental Research. 5(3): 249-362; Nisbet, I.C.T. 1976. Environmental Transport and occurrence of PCBs in 1975. P.N.C. PCB: 245-247.
4. See generally, EPA Criteria Document at pages 172 et seq.
5. Testimony of Renate Kimbrough, EPA Exhibit J at page 4.
6. Id. at pages 8-9 and Exhibit H therein.
7. EPA Criteria Document at pages 195-196.
8. Id. at page 11.
9. Id. at pages 210-211.
10. Testimony of Harry V. Gelboin, EPA Exhibit O, at pages 5-6.
11. Id. at page 6.
12. Id. at page 6.
13. Id. at page 6.
14. Id. at page 6.
15. Criteria Document at pages 220 et seq.
16. Testimony of Renate Kimbrough, EPA Exhibit J at pages 5-11.
17. Testimony of Robert A. Squire, EPA Exhibit N at page 3.
18. Testimony of James R. Allen, EPA Exhibit Q with attached exhibits.
19. Exhibit 3 to EPA Exhibit Q at page 447.
20. Testimony of Wilbur P. McNulty, EPA Exhibit I at page 3.

EXHIBIT A

CURRICULUM VITAE

NAME:	Wolfgang F. Mueller
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EMPLOYED BY:	Institut fuer Oekologische Chemie der Gesellschaft fuer Strahlen-und Umweltforschung mbH Munich, W.-Germany Head of Primate Metabolism Research Laboratory
TRAINING:	Studied Chemistry at University of Bonn W.-Germany Diplom Examen 1966 Dr. rer. nat. 1969
WORKING EXPERIENCE:	Head of Research Group "Metabolism of chlorinated hydrocarbon-pesticides in mammals", Univ. Bonn 1969-1971
	Installation and direction of research group "Dispersion tendencies of environmental chemicals" GSF Inst. f. Oekologische Chemie Munich 1971-1973
	Installation and direction of research group "Comparative metabolism of environmental chemicals in non-human primates and other laboratory animals"
	GSF Institut fuer Oekologische Chemie in cooperation with the Institute of Comparative and Human Toxicology (Albany Medical College) at the International Center of Environmental Safety Holloman AFB, N.M. 1973-

VEROEFFENTLICHUNGEN

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(3) Klein, W., W. Mueller and F. Korte. Insektizide in Stoffwechsel XVI. Ausscheidung Verteilung and Stoffwechsel von Endrin ^{14}C in Ratten. Liebigs Ann. Chem. 713 (1968) 399 GSF - Oe.

(4) Korte, F., W. Klein, I. Weisgerber, R. Kaul, W. Mueller, A. Djirsarai. Recent results in studies on the fate of chlorinated insecticides. Repr.: Inter-American Converrence on Toxicology and Occupational Medicine. August 1970. 51-6. GSF - Oe 19.

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(21) W.F. Mueller, F. Coulston, F. Korte. Metabolism and Toxicokinetics of Chloralkylene-9 in Rhesus Monkeys. (In press or preparation)

EXHIBIT B

Chemosphere No. 4, pp 143 - 146, 1973. Pergamon Press. Printed in Great Britain.

EXCRETION RATES OF PURE DI- AND TRICHLOROBIPHENYL-^{14}C IN THE RHESUS MONKEY

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The chemical properties of polychlorinated biphenyls (PCB's) which make them industrially useful are the same properties which cause them to persist in the environment. These include thermal stability, resistance to chemical oxidation and hydrolysis, water insolubility, solubility in organic solvents, and compatibility with many types of macromolecules. Accumulation in fatty tissues of animals and men has been extensively described, but little is known about the pharmacokinetics of PCB's. Body distribution has been studied in rats[1-3], quails[4], cockerels[5], hens[6], and boars[7] with commercial mixtures, e.g. Aroclor 1245 or 1242. Information upon excretion is available only from a few of those long term studies in which mostly high dose levels were investigated. Highly chlorinated biphenyls can be detected easily in natural samples. Residues of lower chlorinated mixtures, however, have not been reported in spite of their predominant industrial use. This may be due to more rapid metabolism or excretion of these compounds. The present study was designed to determine whether lower chlorinated biphenyls are stored in the body and how fast they are excreted when administered at low single doses to non-human primates.

The PCB's investigated were 2,4'-dichlorobiphenyl, a major constituent of Aroclor 1221 (13.6 %)[8], and 2,2',5-trichlorobiphenyl which is considered to be a major component of Aroclor 1232 and 1242. Both of these were ^{14}C-labelled compounds. The 2,4'-dichlorobiphenyl was synthesized in the German laboratory (spec. activity 8.1 mCi/mM) and the 2,2',5-trichlorobiphenyl was obtained from Mallinckrodt Chem. Corp. St. Louis (Lot Nr. 4449 m).

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Considering the levels of PCB's found in human fatty tissues (USA: 1 ppm, Germany: 5.6 ppm) and considering that the amounts of major components of PCB mixtures are about 10 % of the total weight of the mixture, doses corresponding to about 80 µg/kg body weight of both PCB-isomers as well as a high dose of 566 and a lower dose of about 17 µg/kg of the dichlorobiphenyl were administered. Since the fatty tissue accounts for 20 % of the total body weight of the monkey, the medium dose of the single PCB isomer corresponds, for example, to a value of approximately 4 ppm of the PCB mixture found in natural fat samples. Anticipating fatty tissues to be of special significance female monkeys were chosen for these studies because their abdominal fat allowed a much easier biopsy, although the actual difference in total amount of fat between males and females is very small. Four female animals weighing between 3.1 and 5.56 kg were housed in metabolic cages with free access to Purina monkey chow and water. The diet fed did not contain chlorinated hydrocarbons and all animals had no previous drug treatment so that enzyme induction or drug interaction could be excluded. Each animal was administered once intravenously in the left forearm vein. The carrier used consisted of 0.2 ml ethanol, 20 mg Tween 80, and 0.2 ml water. Urine and feces were collected daily and radioactivity was measured by liquid scintillation counting (Packard Modal 3380) after addition of 0.5 ml urine to 10 ml of Patterson Greene[9] solution. Feces were homogenized in a mortar and an aliquot sample was burned in an automatic combustion apparatus (Oximat, Intertechnique) determining the $^{14}CO_2$ contents. Experimental data are shown in Table I.

	3,4-Dichlorobiphenyl			2,4,5,2',5'-Pentachlorobiphenyl
Monkey	I	II	III	IV
dose in Ci	2.75	0.32	50.02	14.02
dose in µg	94.4	554.55	1969.8	566.3
body weight (kg)	3.34	5.28	5.1	5.6
µg/kg body weight	28.2	77.1	386	82.8
recovered urine (ml)	1000	1240	1140	1357
recovered feces (g)	330.1	322.5	330.8	339.5
urinary excreted radioactivity (% of admin.)	44.8	30.91	30.88	42.02
fecal excreted radioactivity	30.27	39.68	38.44	48.79
totally excreted activity after 14 days (in % of admin.)	84.07	76.83	90.1	93.02
half-life (days)	8.8	7.1	7.8	8.1

Table I Exp. data on PCB application to monkeys

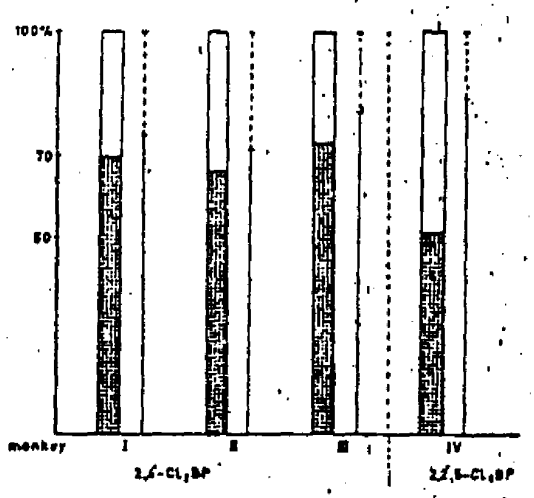

Fig. I: Rel. excretion of radioactivity in urine ▥ and feces □ and ratio urine | to feces |

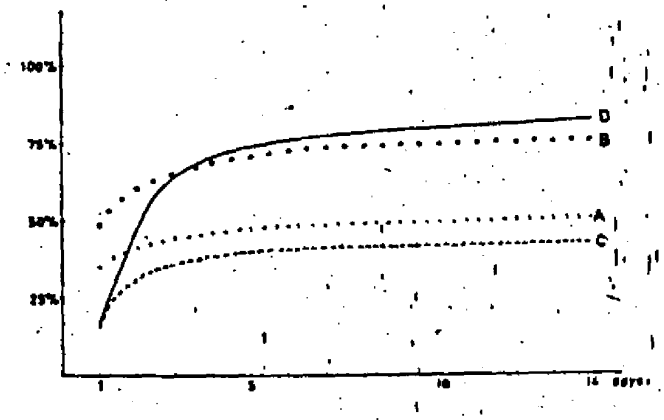

Fig. II: Excretion of radioactivity:
after 2,4'- dichlorobiphenyl appl., urinary A, total B
after 2,2',5 trichlorobiphenyl appl., urinary C, total D

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An average of 73.5 % of the administered radioactive 2,4'-dichlorobiphenyl was recovered during the first 14 days, of this 70 % was excreted in urine and 30 % via feces (Fig. I). The radioactivity excreted by either way corresponds to the excretions in ml or in grams. Although the dose level ranged from 1 to 33 times, there was no significant change in the total percentage eliminated. The dichlorobiphenyl was excreted very rapidly with the maximum amount of the first day after administration. The biological halflife differs within individual animals and with dose from 1.1 to 2.9 days. Continuation of the collection of urine and feces for another 7 days until day 21 resulted in an increase of eliminated radioactivity of only 0.58 % (0.35 % in urine, 0.23 % in feces). After 14 days about 7 % of the administered radioactivity could be detected in the fatty tissue, while the blood level was negligible.

A comparable medium dose of the 2,2',5-trichlorobiphenyl was given to one monkey and 82 % of the given radioactivity was recovered after 14 days. Fig. I shows that equal amounts of radioactivity were found in urine and feces, but the relative concentration in feces was much higher than in urine. Bilary excretion appeared to be more important for the elimination of the trichloro- than for the dichloro-compound. Excretion patterns (Fig. II) were also different: while the total excretion curve for the dichlorobiphenyl starts at a high level with slow increase, a high gradient exists for the trichlorobiphenyl curve. Radioactivity in feces reached high values on the second and third day. The halflife was 2.1 days in relation to 1.1 days for the comparable dose of the dichlorobiphenyl. The slower elimination of the trichlorobiphenyl from the body may be explained by a depot effect, a stronger plasma-binding, or by the fact that the dichlorobiphenyl is more easily metabolized.

It is note worthy that different excretion rates already exist between these low chlorinated compounds, but with no sign of real storage as it is usually expected for PCB's. The general rapid excretion at these dose levels by the rhesus monkey might indicate that accumulation of these compounds in men will not occur. However, further investigations, especially elucidation and structural identification of metabolites, will be necessary to understand the toxicity of the PCB's.

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EXHIBIT C

Beiträge zur Ökologischen Chemie LXXXIV*
Metabolism of Lower Polychlorinated Biphenyls-^{14}C in the Rhesus Monkey

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INTRODUCTION

Metabolism studies on PCB are important for estimating the toxicity of metabolites after knowing their identity and biological pathway. Some pure PCB-isomers were shown to be converted to hydroxylated products by rat liver microsomes (GREB et al. 1974), rabbits (BLOCK and CORNISH 1959), rats (HUTZINGER et al. 1972, YOSHIMURA and YAMAMOTO 1973) and pigeons (YOSHIMURA and YAMAMOTO 1973). The purpose of this study was to identify all major metabolites after PCB-administration to Rhesus monkeys and to measure the amounts of different excreted metabolites. Correlation of conversions and elimination pattern of PCBs with different chlorine content might lead to conclusions about the behavior of other PCBs.

The excretion rates of 2,4'-dichlorobiphenyl and 2,5,2'-trichlorobiphenyl have been described elsewhere (GREB et al. 1973); we now wish to report the identification of their metabolites.

EXPERIMENTAL

The PCBs investigated were 2,4'-dichlorobiphenyl and 2,5,2'-trichlorobiphenyl, both major components of the lower AROCLOR series. They were labelled with ^{14}C. Female Rhesus monkeys were housed in metabolic cages and the PCB was injected once in the left forearm vein. Three animals received 2,4'-dichlorobiphenyl in doses of 16.8, 77.6 and 566 µg/kg, while 82.6 µg/kg of the 2,5,2'-trichlorobiphenyl were administered to one monkey. Urine and feces were collected daily. Conjugates were hydrolyzed by refluxing urine with an equal volume of 8 N H_2SO_4 for one hour and extracted with ether. Feces were mixed with anhydrous sodium-sulfate and extracted for two days in a Soxhlet apparatus with CH_2Cl_2 and then with methanol. The combined extracts were concen-

*LXXXIII. Communication: GREB, W., W. Klein, F.COULSTON, L.GOLBERG, and F.KORTE: Bull.Environ.Contam. Toxicol., in press.

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trated under a nitrogen stream and purified by preparative layer chromatography on silica gel Merck Nr.5765, 5766(solvent: CH_2Cl_2). After methylation by diazomethane (DE BOER and BACKER 1963) the metabolites were characterized by GLC (Packard 873, EC-detector, 1% OV-1, 2m) and identified by GLC-MS (LKB 9000 A).

RESULTS AND DISCUSSION

After 14 days about 77% of the administered radioactivity were recovered, and reaction of urine with Glusulase (Endo Laboratories) according to K.D. VOIGT (1965) showed that about 17% of metabolites were conjugated with sulfuric or glucuronic acid. Incubation of urine and feces in vitro with the original PCBs revealed no formation of metabolites due to reaction with bacteria from the intestines.

In vivo urinary and fecal metabolites were identical and the metabolic pattern in excreta was constant between the first and fifth day after application. Only metabolites, no parent compound, were detected in the excreta.

Fig. 1 gives a survey of the metabolites formed from both PCBs.

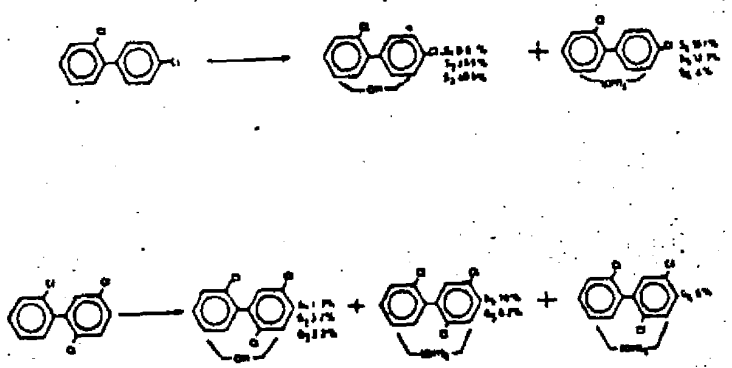

Fig.1. Metabolism of Polychlorinated Biphenyls by Rhesus Monkeys

TABLE 1

TLC -, GLC- and MS-Data of 2,4'-Dichlorobiphenyl Metabolites in Monkeys

Data	OH-Metabolite S_1	OH-Metabolite S_2	OH-Metabolite S_3	$(OH)_2$-Metabolite S_4	$(OH)_2$-Metabol. S_5	$(OH)_2$-Metabol. S_6
R_f before methylation (CH_2Cl_2)	0.66	0.50	0.25	0.13	< 0.1	< 0.1
R_f after methylation (CH_2Cl_2)	0.82	0.70	0.66	0.59	0.64	0.52
GLC after methylation R_t (min.)	10.2/179°C	12,1/185°C for 9 min. then temp. raised 5°C min.	11.4/185°C for 9 min. then temp. raised 5°C min.	14.8/200°C for 8 min., then temp. raised 5°C/ min.	13.1/200°C for 8 min. then temp. raised 5°C/ min.	11.6/200°C for 8 min., then temp. raised 5°C/ min.
MS-fragment after methylation M^+	252 s	252 s	252 s	282 s	282 s	282 s
M^+-CH_3	-	237 l	237 m	267 l	267 m	267 m
metastable	-	-	223 m	-	-	-
M^+-Cl	217 l	217 m	-	247 m	247 m	-
M^+-HCl	-	-	-	246 m	246 m	-
M^+-CH_3-CO	209 m	209 s	209 m	239 m	239 l	239 l
M^+-CH_3-Cl	-	-	202 l	-	-	-
$M^+-CH_3-CO-HCl$	173 l	-	173 m	-	-	-
$M^+-CH_3-CO-Cl_2$	-	-	139 m	204 l	-	-

Peak-Intensities: s=strong, m=medium, l=low

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TABLE 2

TLC-, GLC - and MS-Data of 2,5,2'-Trichlorobiphenyl Metabolites in Monkeys

Data	OH-Metabolite G_1	OH-Metabolite G_2	OH-Metabolite G_3	$(OH)_2$-Metabolite G_4	$(OH)_2$-Metabolite G_5	$(OH)_3$-Metabol. G_6
R_f before methylation (CH_2Cl_2)	0.49	0.25	0.25	0.11	< 0.1	< 0.1
R_f after methylation (CH_2Cl_2)	0.76	0.72	0.72	0.64	0.53	0.32
GLC after methylation R_t (min.)	13.8/180°C	15.4/185°C	16.2/185°C	17.0/200°C for 8 min., then temp. raised 5°C/min.	13.7/200°C for 8 min., then temp. raised 5°C/min.	17.8/200°C for 8 min., then temp. raised 5°C/min.
MS-fragment after methylation M^+	286 s	286 s	286 s	316 s	316 s	346 s
" M^+-CH_3	271 l	271 l	-	301 l	301 l	-
" M^+-Cl	251 m	-	251 l	281 l	-	-
" M^+-CH_3-CO	243 m	243 l	243 l	273 m	273 l	-
" M^+-CH_3-Cl	-	-	-	266 l	-	-
" M^+-Cl_2	186 l	-	-	246 l	246 s	276 l
" $M^+ CH_3-CO-Cl_2$	173 l	173	-	203 l	-	-

Peak-Intensities: s= strong, m = medium, l=low

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After methylation all purified metabolites showed in the mass spectra -OCH_3-groups indicating that the original metabolites were phenolic derivatives. In both cases a number of isomers had been formed. Isomers only differed in peak-intensities. All isolated compounds showed the typical isotopic distribution pattern corresponding to the number of chlorine atoms in the parent PCB. For 2,4'-dichlorobiphenyl there was no conversion exceeding the introduction of 2 OH-groups per molecule. For the 2,5,2'-trichlorobiphenyl we observed a metabolite with three OH-groups. Corresponding to its formation the monohydroxy-metabolite with the lowest concentration should be the precursor of the highest concentrated dihydroxy-compound. Only three monohydroxy-derivatives were formed although six (resp. seven for the trichlorobiphenyl) structures are possible. For each PCB a highly polar zone of 1 and 4% resp. of radioactivity could not be identified. Data of the metabolites are shown in Table 1 - 2.

Comparing the excreted amounts (in % of totally excreted radioactivity) of metabolites formed, the dichlorobiphenyl was mainly excreted as monohydroxy-derivatives, while the trichlorobiphenyl was mainly eliminated as dihydroxy-compounds (Table 3).

TABLE 3

Comparison of Metabolites formed of PCBs by Rhesus Monkeys

Metabolites	Dichloro-biphenyl	Trichloro-biphenyl
Monohydroxy-metabolites	66.6%	7.8%
Dihydroxy-metabolites	32.4%	82.2%
Trihydroxy-metabolites	-	6 %
Polar metabolites, unidentified	1 %	4 %

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The following might explain this different behavior. Due to the additional chlorine atom, the trichlorobiphenyl is more lipophilic than the dichlorobiphenyl. The monohydroxy-metabolites react in the same way: the monohydroxy-dichlorobiphenyl is excreted rapidly, while the water solubility of the monohydroxy-trichlorobiphenyl is still low. After further hydroxylation the trichlorobiphenyl-metabolites reach a polarity to be easier eliminated. A dihydroxy-trichlorobiphenyl thus should correspond to a monohydroxy-dichlorobiphenyl as regards speed of elimination. For higher chlorinated BPs, a higher degree of hydroxylation is necessary and will cause a longer retention time of the compound in the body, that is, a slow excretion rate. This might explain their accumulation.

CONCLUSION

Metabolism of PCB with high chlorine content will be difficult and proceed slowly. Concerning lower chlorinated biphenyls, however, this study reveals that total degradation to hydroxylated metabolites and the rapid excretion of the PCBs investigated in primates indicate that accumulation of lower chlorinated biphenyls at low doses will not occur.

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EXHIBIT D

EXPERIMENTS WITH 2,2'-DICHLOROBIPHENYL

A. Milling, R. Case

INTRODUCTION:

Due to new restrictions regarding the usage of highly chlorinated biphenyls, lower chlorinated biphenyl mixtures will be used to a greater extent in the future. The environmental impact of these mixtures is unknown; therefore, it becomes desirable to study the rates of metabolism, tissue storage levels, toxicity and other important pharmacological properties of these compounds in laboratory animals. This requires radioactive isomers which are as pure as possible. In the past, studies of this type which involved dichlorinated biphenyls have been few in number (1).

This report describes experiments, some of which are still in progress, performed during the past two years concerning the metabolism, storage levels after long term exposure, microsomal oxidation in the liver and kidney, possible fecal bacterial metabolism and toxicity to skeletal muscle and nerve of 2,2'- dichlorobiphenyl-^{14}C in Rhesus monkey and rodents.

SINGLE ORAL DOSE:

Materials and Methods: ^{14}C - labelled 2,2'- dichlorobiphenyl (2,2'-DCB), at a radiochemical purity of greater than 99.5% was dissolved in sesame oil and administered to the animals as a single oral dose by stomach tube. The dose for rats (2 males, 2 females), mice (4 males, 4 females) and Rhesus monkeys (2 adult males) was 20 mg/kg of body weight. The animals were kept in metabolic cages to allow

for separate collection of urine and feces. The radioactivity in the urine was determined by direct liquid-scintillation counting in a Packard Instruments Tri-Carb, Model 2425. Feces of rats and mice were homogenized in a mortar and feces of the monkeys in a blender. Fecal samples in triplicate were burned in a Packard 306 sample oxidizer; the $^{14}CO_2$ content was determined by counting in the scintillation counter. The feces were extracted with methanol for 24 hours using a soxhlet apparatus, while the urine samples were extracted with ether in liquid extractors after acidification with hydrochloric acid (pH 1-2).

Fecal and urine extracts were analyzed for metabolites by thin-layer chromatography on Silica Gel G (methylene chloride or hexane-ethyl acetate 9:1). Localization and quantitation of radioactive zones was carried out on a Berthold thin-layer scanner. The isolated metabolites were identified by GC-mass spectrometry on a Finnigan 3000 D instrument, using a 1.3 m x 2 mm 3% OV-1 on chromosorb W-HP column.

Six and nine days after dosing, the mice and rats, respectively, were euthanized (ether) and all their organs were removed. The radioactivity in these organs was determined by combustion and subsequent counting in the scintillation counter. The remainder of the carcasses was ground in a meat grinder, homogenized and also examined for residual activity. The monkeys were not sacrificed; thus no values for residual activity in carcasses are reported.

Results and Discussion: Male mice excreted a total of

47% of the fed radioactivity within 6 days in both the urine (18.5%) and feces (28.5%), whereas the females excreted a total of 45.2% of the radioactivity in feces (24.9%) and in urine (20.3%). For both males and females the major quantity (85%) of recoverable radioactivity was excreted within 24 hours after dosing. Subsequent to sacrificing, the carcasses, including organs of both males and females contained less than 0.5% of applied dose.

Within nine days, male rats excreted a total of 51.5% of the fed radioaotivity in the feces (48.9%) and urine (2.6%); the females excreted 56% of the applied dose in feces (48.5%) and urine (7.5%). For both males and females, the major quantity (75%) of recoverable radioactivity was excreted within 24 hours after dosing. The combined activity in organs of males was found to be no greater than 0.1% and 0.3% in the carcasses; for the females, 0.86% of applied radioactive dose was found in the organs and 0.6% in the carcasses.

Within ten days, an average of 68.6% (57.1% and 80.0%) of the radioactivity was excreted in feces (14.6%) and urine 54.0%) of monkeys. Moreover, greater than 90% of recoverable radioactivity was excreted within 48 hours after dosing.

Table I lists the relative amounts of major metabolites of 2,2'-DCB for mice, rats and Rhesus monkeys excreted within 24 hours after dosing. As shown in Table I, the major quantity of applied 2,2'-DCB was rapidly (24 hours) metabolized and excreted by mice, rats and Rhesus monkeys. Whereas rats

TABLE I

Excretion (% Administered Dose) of Radioactive Material During 24 hrs. after Application of 2,2'- DCB

	A	B	C	D	E	Total
Mice:						
Males	n.d.[a]	n.d.	11.7	n.d.	30.8	42.5
Females	n.d.	n.d.	9.3	n.d.	29.4	38.7
Rats:						
Males	n.d.	27.9	4.0	n.d.	8.0	39.9
Females	n.d.	27.0	5.0	n.d.	9.6	41.6
Monkeys:						
Males	5.0	trace	1.0	1.0	43.0[b]	50.0

A = 2,2'-DCB-^{14}C

B = Monohydroxy Metabolite A · R_f = 0.75

C = Monohydroxy Metabolite B R_f = 0.45

D = Dihydroxy Metabolite R_f = 0.35

E = Polar R_f = less than 0.1

R_f values are ± 10 % on silica gel (methylene chloride)

a = not detected

b = glucuronides of monohydroxy A (3 parts), monohydroxy B (5 parts) and dihydroxy - 2,2'- DCB (2 parts)

excreted the radioactivity mainly in feces (app. 90%), monkeys, in contrast, utilized the urinary path (app. 80%). Mice in intermediary fashion, excreted the radioactive substances somewhat more in feces than in urine. For all three species conjugate metabolites predominated in urinary excretions.

Chromatography (TLC and GC) and mass spectral data Table II confirmed that 2,2'- DCB was metabolized in mice, rats and monkeys to a common monohydroxy derivative B (see Table I). Although positive proof will depend on independent synthesis, metabolite B, was suggested by Goto, et al. (2), to be 3-hydroxy-2,2'-dichlorobiphenyl.

TABLE II

Mass Spectrum of Monohydroxy 2,2'- DCB Common to Rats, Mice and Monkeys

m/e	% Rel. Intens.
240	22.8
238	29.7
205	6.9
203	16.9
175	6.3
168	45.3
140	11.0
139	40.6
71	46.9
69	78.1
57	100.0
55	81.3

Excreted material obtained from mice contained approximately 25% of metabolite B, whereas with monkeys only a few per cent of free metabolite B was detected. However, cleavage with Ketodase^{R} (Warner - Chilcott Laboratories) of polar material obtained from monkeys gave monohydroxy metabolite B and a dihydroxy metabolite of 2,2'- DCB (M^{+} 254) of undetermined structure. The mass spectrum of the dihydroxy metabolite is shown in Table III. Moreover, acid cleavage of polar material obtained from monkeys gave, in addition to dihydroxy-

2,2' - DCB (2 parts) and monohydroxy metabolite B (5 parts), monohydroxy metabolite A (3 parts). The same monohydroxy metabolite A was the major constituent obtained from rat feces (Table I), but could not be detected in mice excretion. The mass spectrum of metabolite A is represented in Table IV and was suggested by Goto et al. (2) to be 5-hydroxy-2,2'-DCB.

TABLE III

Mass Spectrum of Dihydroxy-2,2'- DCB Obtained from Monkeys

m/e	% Rel. Intens.
256	68.0
254	100.0
221	12.0
219	28.0
193	17.0
191	38.0
184	86.0
155	80.0
129	19.0
128	43.0
127	62.0
126	60.0
125	20.0
77	80.0
55	95.0

Minor quantities of unchanged starting material (from TLC and GC) were also recovered from feces of monkeys, but not from rats or mice.

Conclusion: The rapid metabolism of 2,2'-DCB in three species studied has been observed. Based on these results, a build-up of high concentrations of 2,2'-DCB due to prolonged low level intake is not to be expected.

TABLE IV

Mass Spectrum of Monohydroxy-2,2'- DCB
(Metabolite A) common to Rats and Monkeys

m/e	% Rel. Intens.
240	22.0
238	33.6
205	6.0
203	13.2
168	22.4
139	60.0
69	76.0
67	32.0
63	34.0
57	100.0
55	88.0

LONG TERM FEEDING STUDY:

Materials and Methods: Four Rhesus monkeys (2 males, 3.21 kg and 2.67 kg; 2 females, 3.1 and 2.81 kg) were placed in separate metabolism cages each of which was equipped with collection devices for urine and feces. Each of the monkeys was fed a daily diet of monkey chow (120 g), 1/2 apple and 1/4 banana which had been dosed with 99.5% pure 2,2'- dichlorobiphenyl-^{14}C, specific activity 0.18 mC/mMole. The daily oral dose for each monkey was 600 micrograms which is 5 ppm of the daily food intake. The urine and feces were collected daily and analyzed for radioactivity.

The radioactivity in the urine was determined directly by liquid scintillation counting in a Packard Instruments Tri-Carb, Model 2425. The male feces were combined and mixed thoroughly with anhydrous magnesium sulfate. Triplicate

samples were combusted in the Packard 306 sample oxidizer; the $^{14}CO_2$ content was determined by liquid scintillation. The female feces was treated in similar fashion. The isolation of metabolites from the feces was accomplished by soxhlet extraction with ether. The urine was acidified (pH 1-2) with sulfuric acid and extracted with ether via liquid-liquid extractor. Urine and fecal extracts were concentrated and then analyzed by thin-layer chromatography on Silica Gel G (chloroform:ether, 8:1). Radioactive zones were detected by a Berthold thin-layer scanner. Identification of the isolated metabolites is still in progress. Authentic samples of the possible metabolites are being synthesized so as to establish the identity by comparison with various methods including mass spctra, infrared spectra and if possible nuclear magnetic resonance spectra.

After 83 days dosing, one male and one female were sacrificed. During the autopsy various tissue samples were removed and frozen for storage and future analysis. The tissue samples were analyzed for radioactivity by the combustion process just described. The remaining male and female were sacrificed on the 110th day, that is, 27 days after dosing had ceased. Tissue samples were again taken and analyzed in the same manner.

Results and Discussion: On the 83rd day after dosing had begun, 70.8% of the total administered dose had been recovered in the males. Of this total, 55.4% was recovered in the urine and 15.4% was recovered in the feces. In the

females, 67.9% of the administered dose had been recovered; 50.3% in the urine and 17.6% in the feces. The distribution of the various detectable metabolites is listed in Table V.

TABLE V

Distribution of the Urinary Metabolites of 2,2'- DCB in Rhesus Monkey

Metabolite	R_f	%
1	0.88	2.7
2	0.71	2.7
3	0.57	34.9
4	0.50	5.9
5	0.39	13.1
6	0.23	8.2
7	0.17	22.2
8	0.06	10.4

As can be seen from Table V, approximately 60% of the excreted metabolites are in the form of monohydroxylated isomers of 2,2'- DCB. The remaining 40% is excreted in the form of either dihydroxylated isomers or highly polar conjugates. The distribution of metabolites in the feces is still being investigated; however, preliminary results have shown that of the 16% average excretion found in the feces, approximately 6% is unmetabolized starting material 2,2'- DCB, and the remaining is mostly dihydroxymetabolites. Final verification of these results will be available shortly.

The recovery of radioactive material from the male and female Rhesus monkeys became negligable after 10 days. The tissue levels comparing the male sacrificed at 83 days of dosing with those of the male in which dosing ceased at 83 days but sacrificing delayed for 27 more days, i.e., at 110 days is listed in Table VI. The same data is listed for the females.

TABLE VI

Tissue Levels of Rhesus Monkeys Dosed 83 Days with 2,2'- Dichlorobiphenyl

Tissue:	Male 2.86kg 83rd	Male 3.37kg 110th	Female 3.48kg 83rd	Female 2.95kg 110th
Adrenal Cortex	0.25	-	0.32	-
Medulla	0.68	2.85	0.29	-
Bile	2.68	-	2.09	-
Blood	0.46	-	-	-
Bone	-	-	-	-
Bone Marrow	1.42	-	-	-
Brain (Residual)	-	-	-	-
Calcarine fissure cortex	0.17	-	-	-
Caudate nucleus	0.26	ns	ns	ns
Cecum	-	-	-	-
Cerebellum	-	-	-	-
Cerebral spinal fluid	0.54	ns	ns	ns
Choroid of eye	-	-	-	-
Choroid plexus	12.20	0.38	-	0.60
Colon	-	-	-	-
Cornea	-	-	-	-
Corpus callosum	0.15	-	-	-
Cortex of brain:				
Motor	0.30	-	-	-
Sensory	ns	-	-	-
Diaphragm	-	-	-	-
Duodenum	-	-	-	-
Esophagus	-	-	-	-
Fat:				
Brown	0.91	0.10	-	-
Inquinal	2.49	0.16	0.14	0.10
Mesenteric	0.82	0.11	0.14	-
Omental	3.12	0.12	0.11	-
Pancreatic	ns	ns	ns	ns
Pericardial	ns	-	ns	ns
Subcutaneous	1.15	0.10	0.10	-
Fallopian tubes	ns	ns	ns	-
Hair	0.52	0.11	0.25	-
Heart	-	-	-	-
Hypothalamus	-	-	-	-
Ileum	-	-	-	-
Iris of eye	0.47	-	-	-
Jejunum	-	-	-	-
Kidney:				
Left	0.12	-	0.10	-
Right	0.12	-	0.11	-
Lens	-	-	-	-

TABLE VI cont.

Tissue:	Male 83rd	Male 110th	Female 83rd	Female 110th
Liver: (Lobe)				
Left Lobe	0.18	0.18		-
Lateral	ns	ns	0.14	ns
Central	ns	ns	-	ns
Medial Lobe	0.21	0.12	0.12	-
Right Lobe	0.17	0.10	0.15	-
Lung: (Lobe)				
Left Lower	-	-	-	-
Left Medial	-	-	-	-
Left Upper	-	-	-	-
Right Cardial	-	-	-	-
Right Lower	-	-	-	-
Right Medial	-	-	-	-
Right Upper	-	-	-	-
Lymph Nodes:				
Axillar	1.33	-	-	0.37
Hilar	0.70	-	0.10	0.10
Inquinal	0.74	-	0.79	0.10
Large Intestinal	-	-	-	-
Pancreatic	-	-	-	-
Small Intestinal	0.91	0.10	-	-
Mammary Gland	0.21	-	-	-
Medulla of Brain	-	-	-	-
Meninges	0.22	-	-	-
Mesencephalon	-	-	-	-
Muscle	-	-	-	-
Nerve:				
Optic	0.16	-	0.10	-
Sciatic	0.13	-	-	-
Ovaries	ns	ns	-	-
Pancreas	-	-	-	-
Pituitary	12.10	0.31	0.29	-
Pons	0.14	-	-	-
Prostate Gland	0.26	0.37	ns	ns
Salivary Glands:				
Parotid	-	-	-	-
Sublingual	-	-	-	-
Submandibular	-	-	-	-
Sclera of Eye	-	-	-	-
Skin	-	-	-	-
Spinal Cord	-	-	-	-
Spleen	-	-	-	-
Stomach	-	-	-	-
Testes	-	-	ns	ns
Tongue	-	-	-	-
Thalamus	0.17	-	-	-
Thymus	-	-	-	-
Thyroid	0.39	-	-	-
Trachea	0.23	-	-	-

TABLE VI cont.

Tissue:	Male 83rd	Male 110th	Female 83rd	Female 110th
Urinary Bladder	-	-	-	-
Urine	ns	ns	ns	-
Uterus	-	-	-	-
Vagina	-	-	-	-
Vitreous Body of Eye	0.13	-	-	-

ns = no sample available

- = values less than 0.1 ppm

Conclusion: In general, Rhesus monkey does not seem to store particularly high levels of 2,2'- dichlorobiphenyl in the tissues following a long-term dosing in the food. In one of the males, the levels were seemingly high in the pituitary gland and in the choroid plexus. The female which was sacrificed after 83 days of dosing had relatively low tissue levels. It was noted during the course of the experiment that this particular monkey had a poor appetite for both food and water.

BACTERIAL METABOLISM:

Materials and Methods: Brain-heart infusion and thioglycolate media were inoculated with urine (inoculating needle) and incubated at 37°C for 42 hours. Urine from male and female was incubated separately. Feces samples were treated in a similar manner. After the incubation period, 2,2'- DCB-^{14}C (600 micrograms, sp. act. 0.18 mC/mMole) was added to each of the inoculated incubation tubes. Incubation at 37°C was continued for another 48 hours. After this period, each sample was acidified to pH 1-2 and extracted

overnight by liquid-liquid extraction (ether). The extract was concentrated and analyzed by thin-layer chromatography (Silica Gel G - Chloroform:Ether, 8:1).

Results and Discussion: Recovery of only starting material suggested that during the time that the urine and feces were in contact in the collection pans, no bacterial metabolism produced any of the metabolites which were discovered in the urine.

Conclusion: During the collection period, bacteria do not produce any of the isolated metabolites.

Acknowledgement: The tissue samples at autopsy were kindly provided by Dr. M. Iatropoulos. The equipment and chemicals for the bacterial metabolism was provided by Mr. Jim Bryan. Throughout the investigation technical assistance was provided by Miss Jan Jennings. Miss Lupe Gaston provided technical assistance for the feeding study.

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EXHIBIT E

EXPERIMENTS WITH 2.5.4'-TRICHLOROBIPHENYL

R. Felt

2/27/76

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 3.6×10^7kg 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.

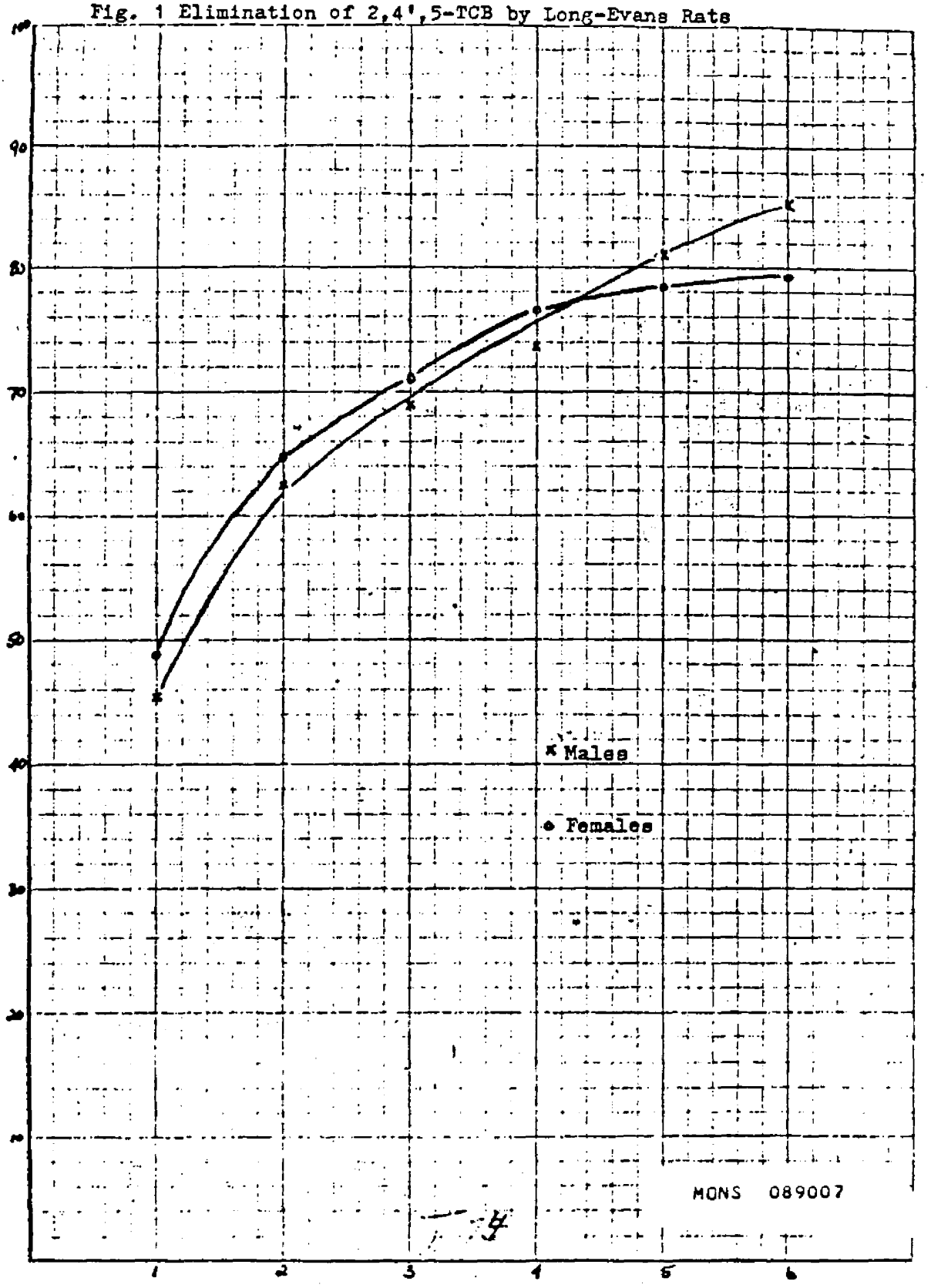
Feces from each rat were collected daily, homogenized and weighed samples prepared. Three fecal samples per rat per day were burned in a Packard 306 sample oxidizer and the liberated $^{14}CO_2$ counted in a Packard 2425 Tri-Carb liquid scintillation spectrometer. All male fecal samples for a day were 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

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



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
Subcutaneous 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

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 chromatography was dissolved in acetone to give a 0.240M 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

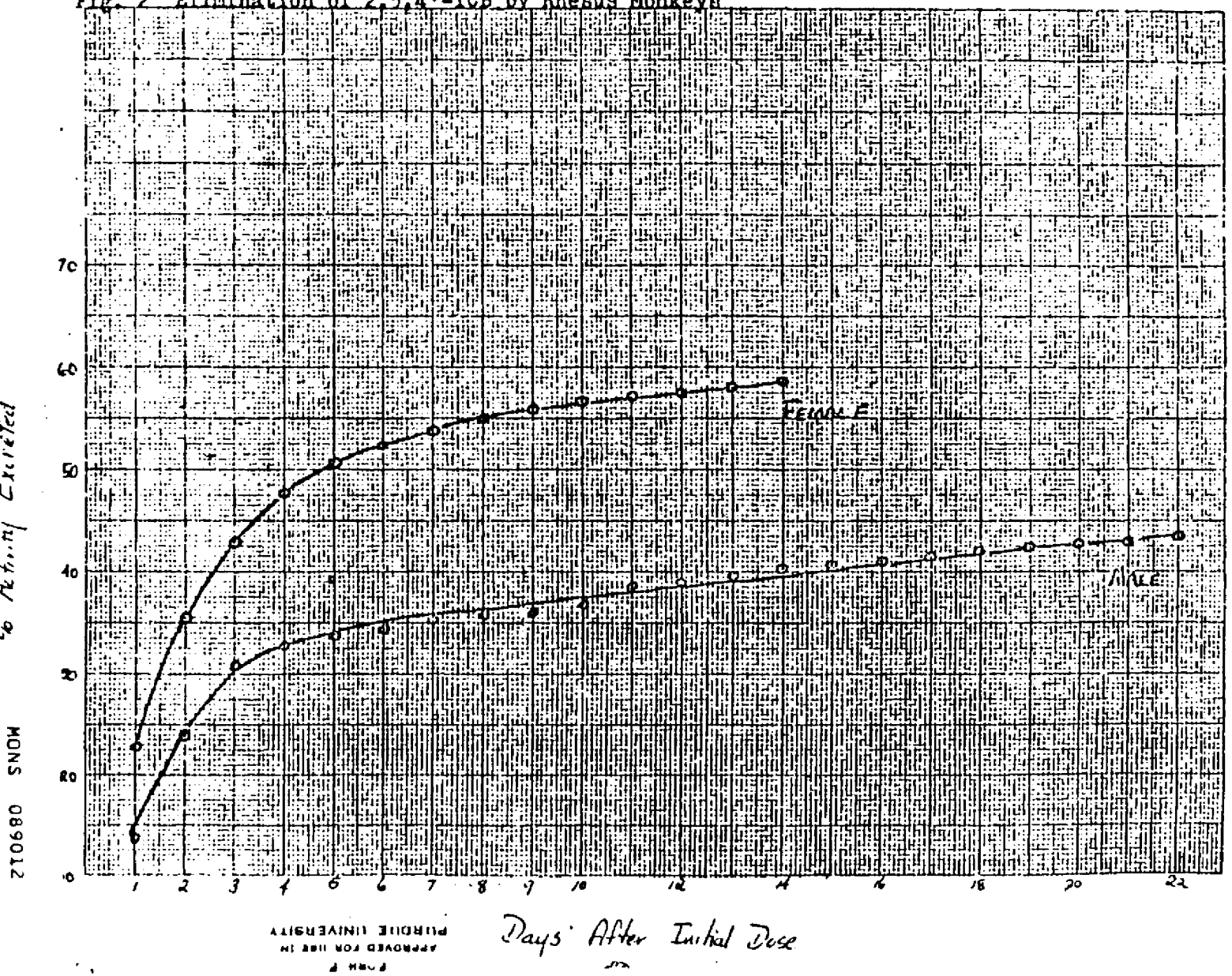
layer scanner revealed three zones of activity. Most of the activity (>85%) resided in the starting zone. The bulk of the remaining activity was in a peak of R_f 0.35. The remaining radioactive peak of high R_f disappeared after the second day.

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.

I 72

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.

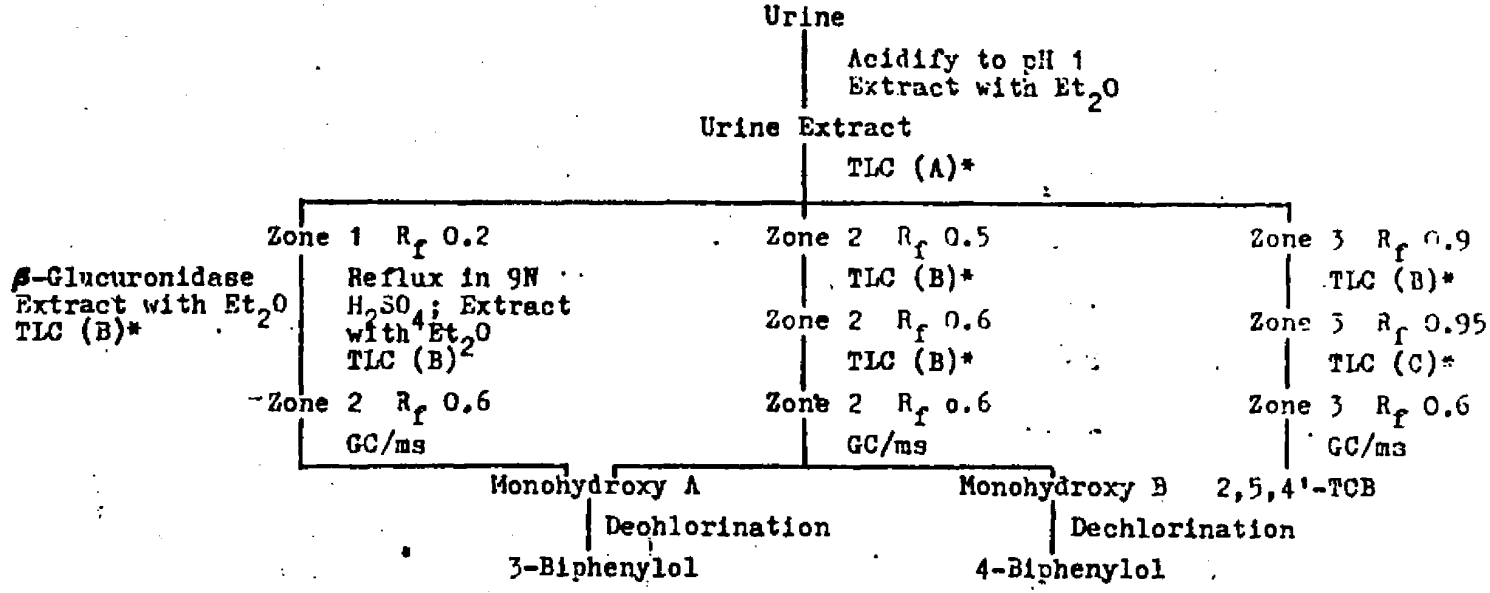
The zone of highest R_f value from the first plating of the urine extracts was further purified by two successive platings on silica gel. Development with methylene chloride/ethyl acetate (95/5) was followed by TLC on silica gel with hexane development. Verification of the identity of this compound was 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.

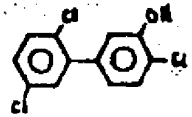
The material in zone 2 was purified by TLC on silica gel using methylene chloride/ethyl acetate (95/5) as the developing solution. Gas chromatography-mass spectrometry of this material revealed two monohydroxy-2,5,4'-TCB's in an approximate ratio of

T 30

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

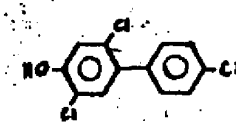


*TLC Developing Solutions : A Benzene/Ethanol/Formic Acid (90/9/1)
B Methylene Chloride/Ethyl Acetate (95/5)
C Hexanes



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

Monohydroxy A



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

Monohydroxy B

MONS 089014

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.

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 pi[illegible] 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

LB 2725 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 ether 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

T 84

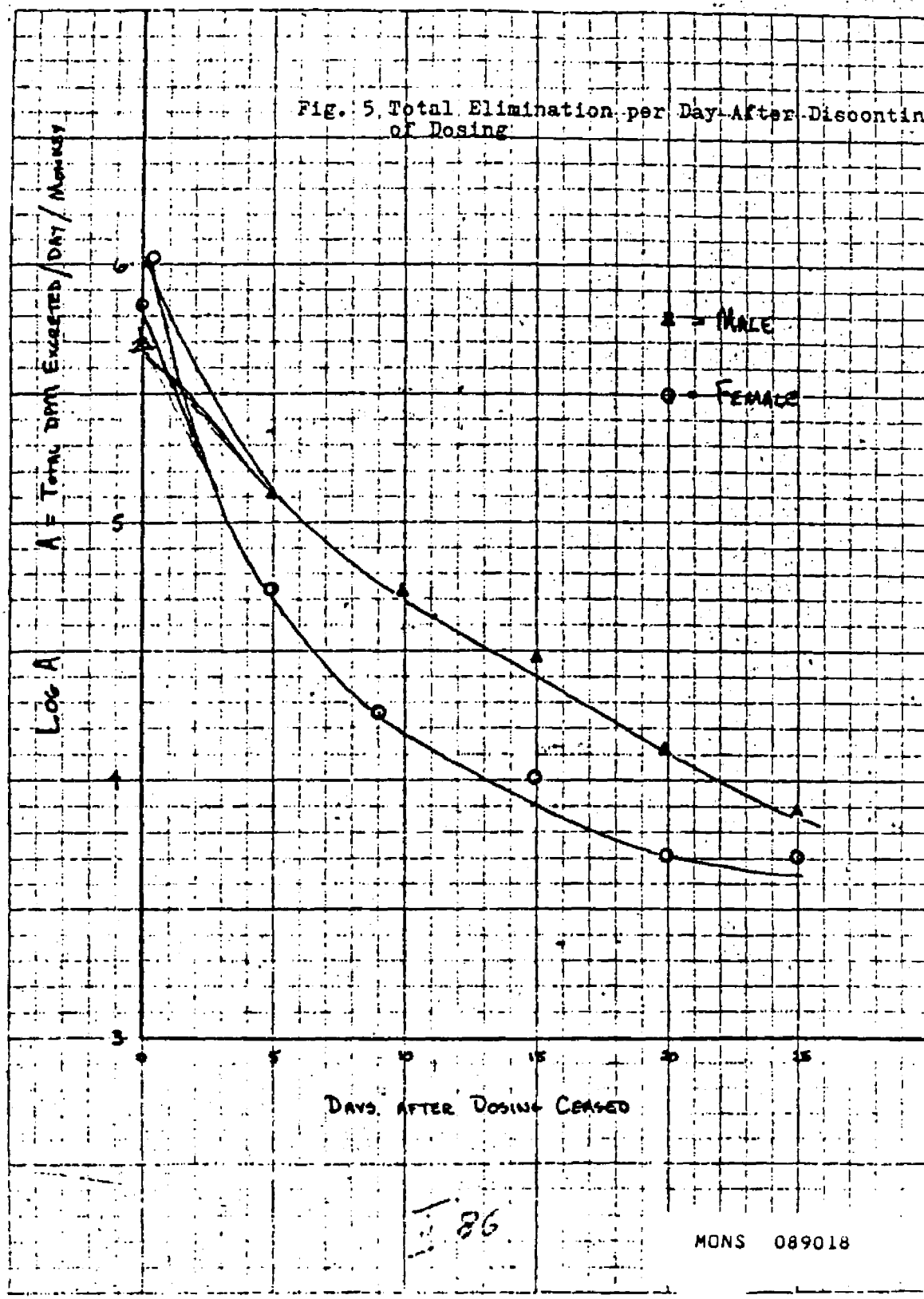
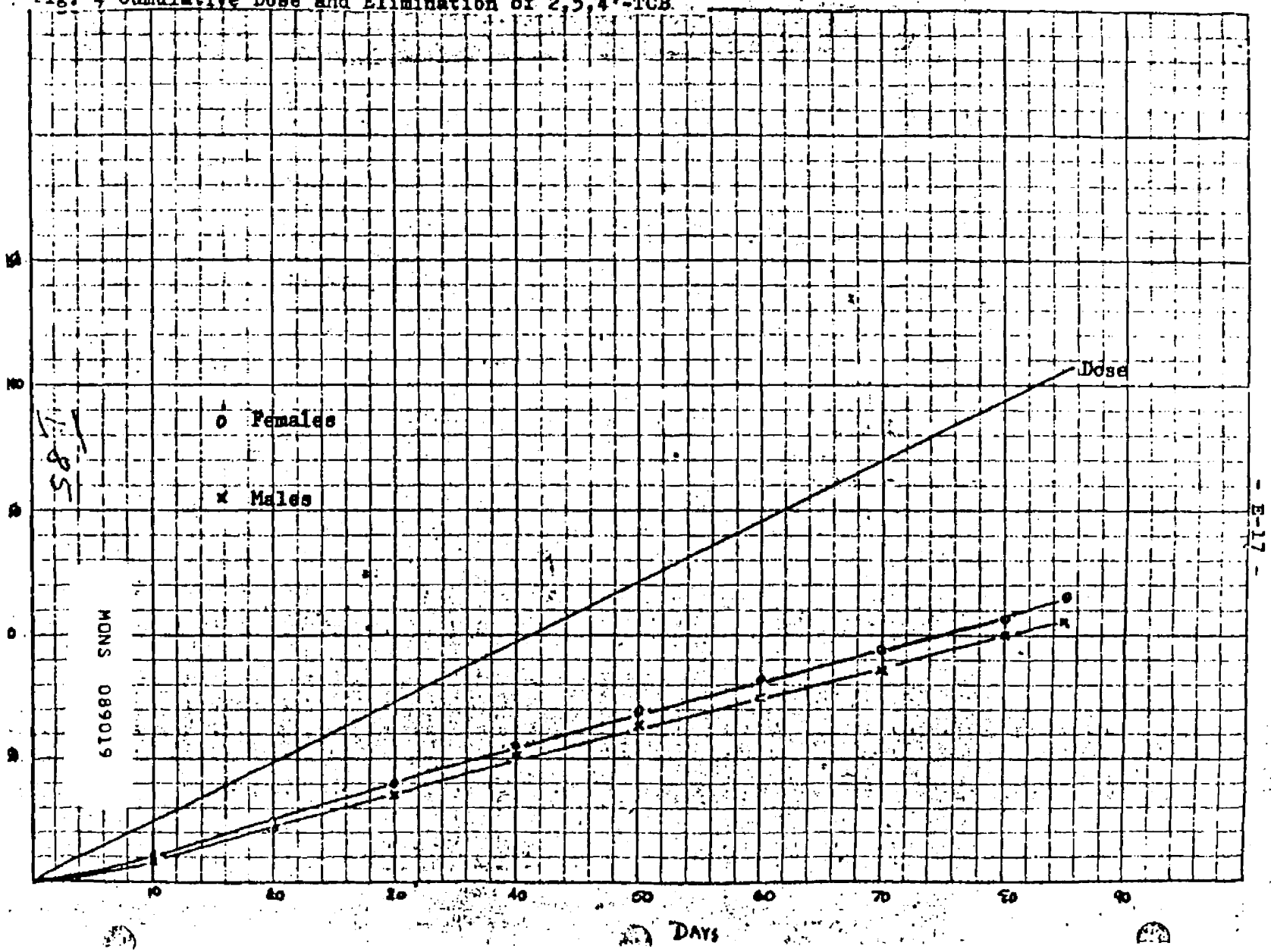


Fig. 5 Total Elimination per Day After Discontinue of Dosing

186

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



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.

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

Tissue	ppm 2,5,4'-TCB 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

II 88

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	0.25
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	5.00	8.15
Omental	5.03	9.79
Inquinal	5.02	7.98
Subcutaneous	3.53	5.50
Brown	2.97	5.55
Sciatic Nerve	0.44	0.37
Meninges	0.22	0.10

II.89

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
Calcarine 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

T 90

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

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

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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