Metabolic Fate of ^{3}H 2,5,2′,5′-tetrachlorobiphenyl in Infant Nonhuman Primates

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During the past 40 years environmental contamination by polychlorinated biphenyls (PCBs) has become a serious problem because of their extensive uses in industry. Severe PCB intoxication in humans was reported in Japan following consumption of contaminated rice oil (KURATSUNE 1969). The majority of the research to date on the biological effects of the PCBs has been conducted on commercially prepared mixtures of these compounds. PCBs are a mixture of many chlorinated isomers; hence, the utilization of a pure individual isomer was more appropriate for the evaluation of their metabolic fate and biological effects. Recently, VAN MILLER et al. (1975) have reported that over 76% of a single dose of tritiated 2,5,2',5'-tetrachlorobiphenyl (TCB) was excreted by rats within 72 hours. In addition, most of the radioactivity in the urine was monohydroxy TCB. Because the primate is far more susceptible to the toxic effects produced by PCBs than the rat (ALLEN et al. 1974) it was deemed important to evaluate the metabolic fate, site of localization, and excretion rate of the PCB isomers in the primate. In the presently reported experiment it was determined that the metabolic fate of TCB in the infant nonhuman primate was decidedly different than in the rat. At 72 hours a large percentage of the TCB was retained in the body in an unmetabolized form and was primarily associated with the serum proteins and cellular macromolecules. That portion of the TCB excreted in the urine was metabolized to dihydro-TCB diol which is not the case in rats.

MATERIALS AND METHODS

Tritiated TCB was prepared from 2,5,2',5'-tetrachlorobenzidine by the method of HUTZINGER and SAFE (1972). Specific activity of the tritiated TCB was adjusted to 6.1 μci/mg with TCB. Seven infant rhesus monkeys initially weighing 600-800 g were divided into two groups and housed individually in metabolism cages. The 4 monkeys of the experimental groups were given a single dose of tritiated TCB (500 mg/kg) dissolved in 1.5 ml of corn oil by gastric intubation. The 3 monkeys of the control group were given 1.5 ml corn oil. The animals had free access to water throughout the experiment and were intubated with 20 ml 5% glucose solution at 24 and 48 hours. Feces and urine were collected at 24 hour intervals. The animals were anesthetized, perfused intercardially with physiological saline, and sacrificed at 72 hours.

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Feces, blood, and urine (100-200 mg) samples were oxidized in a Packard Model 306 sample oxidizer with Monophase 40 (Packard) as the scintillation cocktail. The radioactivity was measured in a Packard Tri-Carb scintillation counter. Representative tissue samples were fixed in neutral buffered formalin and processed for light microscopic examination in a manner previously described (ALLEN et al. 1973).

Gas liquid chromatography (GLC) and thin layer chromatography (TLC) were employed to evaluate the metabolites in the extracts of urine and tissue homogenates. Samples for TLC were applied to silica gel, flexible plates (J.T. Baker Chemical Co.) and developed with methylene chloride. The plates were then scanned on a Packard Model 7201 radiochromatogram scanner. GLC was done on a Hewlett-Packard Model 7620A gas liquid chromatograph fitted with an electron capture detector. Two microliters of sample solutions were injected into a glass column (1/8" x 6') containing 3% SE-30 on Gaschrom Q (100-200 mesh) at 200°C.

The presence of trans-3,4-dihydro-3,4-dihydroxy-2,5,2',5'-tetrachlorobiphenyl (dihydro TCB diol) in the urine was determined by the method of GARDNER (personal communication). Urine was extracted twice with equal volumes of ether. The ether extracts were concentrated and chromatographed by TLC. Dihydro-TCB diol was converted to monohydroxy tetrachlorobiphenyl in methylene chloride containing 2 to 3 drops of concentrated H_2SO_4.

For determination of binding between TCB or its derivatives and tissue components (REID et al. 1973), 1 g liver was homogenized in a Potter-Elvehjem tube with 4 ml of water. Skin was homogenized in a Polytron (Brinkman Instruments) with 4 volumes of lytic solution (STEWART and FARBER 1973). The homogenates were extracted with hexane (5 ml x 3) and then an equal volume of 20% trichloroacetic acid was added to the aqueous phase at 4°C for 1 hour. The resulting precipitates were washed with methanol (5 ml x 4). The residues were air dried and weighed. About 100 mg of the residue was oxidized in the sample oxidizer, and the radioactivity was measured as described above. Aliquots of each fraction were added with 10 ml Scintisol Complete (Isolab) for measuring radioactivity.

Aliquots of blood serum (0.2 ml) or liver solution (0.1 ml liver homogenate + 0.2 ml lytic solution) were chromatographed by application to a Sephadex G-25 column (1 x 26 cm). The samples were eluted with distilled water with about 2 ml of the eluent collected in each fraction. The amount of nucleic acid and protein in the eluent was determined by measuring the UV absorption at 260 or 280 nm. One ml of solution from each fraction was added with 10 ml Scintisol Complete for counting the radioactivity.

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For polyacrylamide gel electrophoresis 8%-3% polyacrylamide gel was prepared. After serum samples were applied directly on the 3% gel which was covered with only a buffer layer, electrophoresis was performed at 4°C, pH 9.0 with 0.065 Tris borate as tank buffer and was continued for about 50 minutes at 280 volts (capacitance 1.0, pulse rate 200 pulse/second, Ortec 4100 pulsed constant power supply). The gels were stained for 1 hour in 7.5% acetic acid solution with 1% amido black 10B and destained overnight in 7.5% acetic acid solution. For measuring binding of TCB with serum proteins the gels were sliced into 0.5 cm sections which were then oxidized by a sample oxidizer for scintillation counting.

RESULTS

During the 72 hours following administration of TCB the animals were inactive, the skin was flushed and the hair had an oily appearance. At necropsy the livers of the experimental animals were enlarged and pale. Microscopically, there was a decided hypoplasia of the bone marrow, a decrease in the size of the Malpighian corpuscles of the spleen, a regression of the cortex of the thymus and a moderate fatty infiltration of the liver. Less than 2% of the tritium was eliminated in the urine and 1% in the feces by 72 hours. Skin, adrenal and bone marrow had the highest concentration of radioactivity (Table I). The concentration in the blood was low as opposed to relatively high concentrations reported by VAN MILLER et al. (1975) for rats. In addition to the blood, the radioactivity in the lungs, spleen, heart, thymus, brain, testes, lymph node and bladder were low; therefore, the results are not listed in the Table.

TABLE I

Tissue Concentrations[a] of ^{3}H in Infant Monkeys Following A Single Oral Dose of ^{3}H TCB

Tissue	Animal No. 301	302	303	304
Liver	3.5	3.5	8.4	1.6
Kidney	2.1	3.8	9.0	2.1
Large Intestine	0.9	2.7	5.2	15.3
Adrenal gland	5.4	1.4	18.3	10.6
Skin	8.9	6.8	7.5	14.8
Muscle	2.0	1.6	1.2	2.4
Pancreas	1.8	0.4	2.9	5.4
Salivary gland	1.5	6.6	---	4.8
Bone marrow	6.7	12.6	4.9	16.3
Blood[b]	0.2	0.7	0.2	0.3
Fat	---	---	---	3.0
Stomach	0.6	2.2	2.2	2.9

[a] Percent dose per g tissue (x100); [b]Percent dose per ml blood

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There were two radioactive bands with R_f values of 0.02 and 0.05 (Fig. 1) on TLC of the ether extracts of urine (30-40% radioactivity of urine). The methanol eluted substances from the band with R_f 0.5 did not show any significant peak on GLC for 30 minutes even when the oven temperature was set at 160 or 240°C. The labeled compound in this band is a new, unidentified metabolite. The second band with R_f 0.02 was eluted with methanol. Following the injection of the methanol eluent, a single peak with a relative retention time (RRT) of 3.09 (retention time of this compound [251 sec]/retention time of TCB [81 sec]) was seen on GLC. When the methanol solution was dehydrated, dried and dissolved in 1 ml methylene chloride containing 2 to 3 drops of concentrated sulfuric acid, the metabolite was converted to another substance with R_f 0.8 on TLC and RRT 1.94 (retention time 161 sec) on GLC. The ratio of retention time of this compound before and after dehydration was 1.54. GARDNER et al. (personal communication) reported similar values for dihydro TCB diol. These values were R_f 0-0.1 before dehydration with R_f values after dehydration of 0.9 and 0.8 for 3-OH TCB and 4-OH TCB, respectively. In addition, the reported value for the ratio of retention time is 1.52, agreeing well with the value given above.

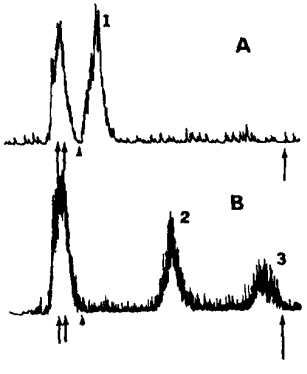


Fig. 1. TLC radiochromatogram of urine ether extracts from rats (A) and infant nonhuman primates (B) given a single oral dose of ^{3}H-2,5,2',5'-tetrachlorobiphenyl (TCB). (1) 3-hydroxy TCB, (2) Unknown, (3) dihydro TCB diol. Δ = solvent front, + = sample application, ++ = tritium marker.

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For evaluation of the interaction of TCB and cellular components, Sephadex G-25 column chromatography and polyacrylamide gel electrophoresis were employed. Most of the radioactive labeled material was found to be associated with the cellular macromolecules and the serum proteins. As shown in Figure 2 more than 90% of the tritium was eluted from the Sephadex G-25 column, with most eluted with the protein and nucleic acid fractions of the liver homogenate. Further binding studies of serum by polyacrylamide gel electrophoresis showed (Fig. 3) that the radioactivity was primarily associated with serum albumin. When the liver homogenates were extracted with hexane, about 57% of the radioactivity was removed, 2.6% by trichloroacetic acid, and 27% by methanol, leaving only 1.1% of the radioactivity in the residue. A similar result was seen in the extraction of skin homogenates (Table II). It was estimated by GLC that more than 95% of the chlorinated compounds in the hexane extracts was unmetabolized TCB.

TABLE II

Percent of Radioactive Material in Various Solvent Extracts of Skin, Liver and Serum

Tissue	Percent of Total Radioactivity			
	hexane extract	TCA supernate	methanol extract	residue
Liver	57.0	2.6	27.0	1.1
Skin	85.1	4.2	6.2	0.38
Serum	50.3	---	----	----

DISCUSSION

The metabolic fate and toxicity of aromatic hydrocarbons have recently been reviewed by JERINA and DALY (1974). Direct hydroxylation (DALY et al. 1968) and hydroxylation through the arene oxide intermediate are the two major metabolic pathways of the aromatic ring. Metabolic formations of arene oxides which are capable of alkylating with a variety of nucleophils, including cellular macromolecules such as DNA, RNA and protein, explain many toxic and carcinogenic properties of aromatic hydrocarbons. Because of the identification of dihydro TCB diol (GARDNER et al. 1974) as one of the metabolites in rabbits after TCB administration the arene oxide 2,5,2',5' tetrachlorobiphenyl-3,4 oxide (TCB oxide) has been suspected of being a precursor of the hydroxylated metabolites and the active metabolites which cause biochemical and pathological

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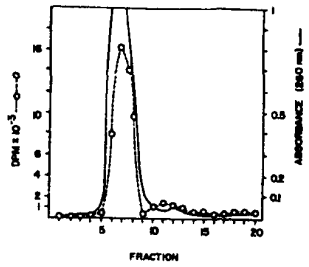


Fig. 2. Sephadex G-25 column chromatography of liver solution from infant nonuman primates given a single oral dose of ^{3}H 2,5,2',5'-tetrachlorobiphenyl. ——— = absorbance at 260 nm; -o-o- = DPM per ml.

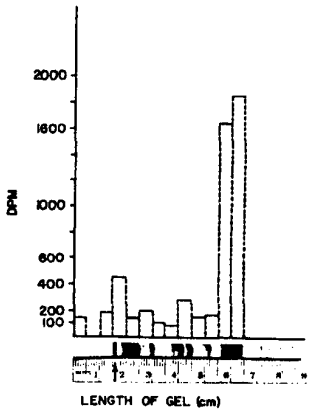


Fig. 3. Polyacrylamide gel electrophoresis of infant monkey serum 72 hr following a single oral dose of ^{3}H 2,5,2',5'-tetrachlorobiphenyl. ↑ = junction of 3% and 8% gel (ORTEC 1973).

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changes in experimental animals. In the present study, a substantial fraction of the radioactivity in the urine is in the form of dihydro TCB diol. This result suggests that at least some of the TCB is metabolized through an arene oxide intermediate and further suggests that alkylation of the macromolecules should occur. Due to the slow rate of metabolism, as evidenced by the low level of radioactivity in the excreta and the high level of unmetabolized TCB in the tissues, it is likely that the amount of TCB covalently bound to the macromolecules is low. This is substantiated by the low levels of radioactivity in the residues of extracted tissue (Table II). This metabolic pathway may not cause significant acute toxic effects in monkeys. However, during long term exposure this arene oxide may be responsible for toxicity and possible tumor induction in monkeys. Indeed, induction of hepatomas by PCBs has recently been reported by KIMBROUGH and LINDER (1974). Since man and nonhuman primates show similar symptoms and lesions resulting from PCB exposure, they likely metabolize PCBs in the same manner. Due to the carcinogenic and toxicological potential of PCBs and their widespread presence in the environment, the human health significance of these compounds is of great importance.

It is of interest that there is a decided difference in the metabolism of TCB by rats and nonhuman primates (Fig. 1). Over 12% of the tritium was recovered in the urine of rats within 72 hours, most of which was metabolized as monohydroxylated TCB (VAN MILLER et al. 1975). The criteria of arene oxide formation are (1) occurrence of NIH shift; (2) appearance of glutathione conjugates; (3) formation of trans-dihydro diols or catechols (JERINA and DALY 1974). Arene oxides of halogenated compounds are usually more stable thus allowing the formation of glutathione conjugates or trans-dihydrodiol (LINDSAY et al. 1972). However, only monohydroxy TCB can be found in the urine extracts of rats. Therefore, in rats hydroxylation of TCB appears unlikely through an arene oxide intermediate but rather through direct hydroxylation. The hypothesis is further supported by the evidence that direct hydroxylation of 2,4,5,2',4',5'-hexachlorobiphenyl occurs in rats (JENSEN and SUNDSTROM 1974). Since rats showed no deleterious effects from doses of TCB capable of producing distinct lesions in the nonhuman primate, direct hydroxylation in rats seems to be responsible for the detoxification and rapid excretion of these compounds.

The high specific activity of radioactive labeled material in the adrenal gland and skin of monkeys in this experiment is noteworthy because acne, skin lesions, and disturbance of steroid hormone metabolism, which occur in long term experiments (BARSOTTI and ALLEN 1975) may relate to the localization of TCB in those tissues.

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