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in Vitro Generation of a Chemically Reactive Metabolite of 2,5,2',5'-Tetrachlorobiphenyl by Rhesus Monkey Liver Microsomes (39454)

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The family of compounds known as polychlorinated biphenyls (PCBs) has been used in industry since the 1930's as heat exchangers in transformers, plasticizers in paints and adhesives, electrical and hydraulic fluids, flame retardant coatings for woods, and vapor suppressants for insecticides, to name a few. The properties that make PCBs suitable for these uses, such as high temperature resistance to decomposition, also make them extremely stable compounds when accidentally freed into the environment. Recently, several reports have shown that PCBs are widespread global contaminants (1). PCBs consist of a large mixture of isomers, and it has only been recently that pure isomers were available for metabolic studies. One such isomer, 2,5,2',5'-tetrachlorobiphenyl (TCB), has been shown by Van Miller *et al.* (2) to be rapidly metabolized in the rat to 3-hydroxy-2,5,2',5'-tetrachlorobiphenyl. In other species, such as rabbits, Gardner *et al.* (3) have demonstrated that 2,5,2',5'-tetrachlorobiphenyl is metabolized through an arene oxide pathway to metabolic products consisting of trans-dihydrodiols and monohydroxy metabolites. Recently, Hsu *et al.* (4) have shown the presence of trans-dihydrodiols, monohydroxy, dihydroxy and transdihydrotriols in urine of infant and adult rhesus monkeys given an acute dose of the pure isomer. Dihydrodiols and dihydrotriols are thought to be derived exclusively from an arene oxide intermediate. Many members of this class of intermediates have been shown to be alkylating agents with carcinogenic potential (5). In many species the liver microsomal fraction has been shown to be the principle site of generation of arene oxides. The presently reported study demonstrates that a metabolite of 2,5,2',5'-TCB capable of covalently binding to macromolecules is formed *in vitro* by monkey liver microsomes.

Materials and methods. Tritiated 2,5,-2',5'-TCB ([^{3}H]TCB) was prepared by the method of Hutzinger and Safe (6), and liver microsomes were obtained from an adult female rhesus monkey (7). The basic incubation mixture consisted of 5 μmoles of NADP, 3 μmoles of ATP, 10 μmoles of glucose-6-phosphate, 25 μmoles of $MgCl_2$, and 2.5 IU of glucose-6-phosphate dehydrogenase in phosphate buffer (pH 7.4). To this NADPH generating system was added 0.3 ml of a 33% microsomal suspension (0.3 ml of microsomes from 0.1 g of liver) either active or as heat-deactivated (100° for 10 min) controls. The microsome-cofactor system was divided into four groups, each of which had heat-inactivated microsomes serving as a control. In Group 1 water was added; in Group 2 glutathione (GSH) (1 μmole) was added; Group 3 received 0.2 ml of postmicrosomal supernate (0.3 ml from 0.1 g of homogenized liver); and Group 4 received both 0.2 ml of postmicrosomal supernate and 1 or 10 μmoles of GSH. Substrate was added as [^{3}H]TCB (1.25 μCi, 12 μCi/mg) in 10 μl of methanol. The final volume was 3.7 ml. The flasks were stoppered and incubated at 37° for 1.0 hr in a shaking water bath (120 strokes/min).

The incubation was stopped by the addition of 1.25 ml of ice-cold 20% trichloroacetic acid (TCA). The resulting 5% TCA solution was held at 4° for 1 hr. The precipitate (Fig. 1) was collected by centrifugation (800*g*) and washed three times with ice-cold 5% TCA. TCA washes of each group were combined (Fraction I) for later testing for the presence of TCA soluble metabolite(s). The resulting protein-RNA pellet was washed (8×) with methanol (60°) until all extractable radioactivity was removed and the last wash had only background levels. The methanol extracts were combined and saved for later metabolite determination

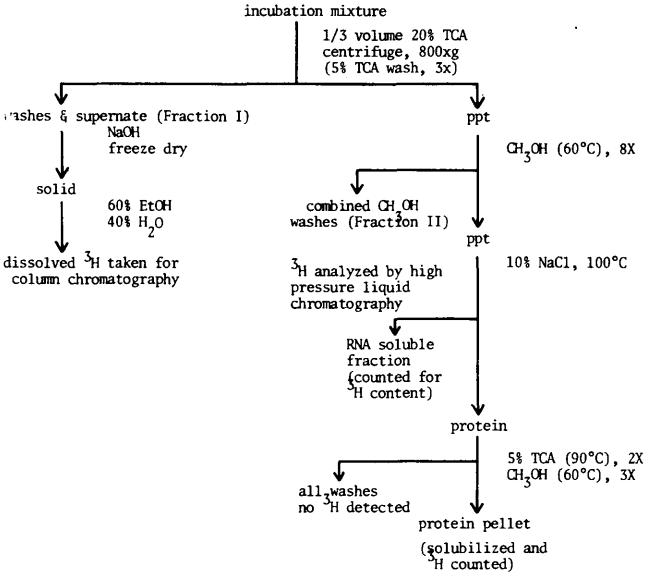

FIG. 1. Flow chart for the extraction of liver microsomes incubated with [^{3}H]2,5,2′,5′-tetrachlorobiphenyl.

(Fraction II). The pellet was dried with ether and extracted with 10% NaCl (1 hr, 100°) followed by two extractions with 10% NaCl (1/2 hr, 100°) to dissolve RNA (8, 9). The sodium chloride extracts were combined and sampled for ^{3}H content. The protein pellet was further extracted (2×) with 5% TCA (90°) to extract any remaining RNA and wahsed three times with methanol (60°) to eliminate any trapped [^{3}H]TCB that might have been released with the RNA extraction. The resulting protein pellet was dissolved in Unisol and the radioactivity counted after the addition of Unisol-Complement. The NaCl, TCA, and methanol washes were sampled or added whole to 10 ml of Aquasol and counted on a Packard Tri-Carb 3375 liquid scintillation spectrometer with an automatic external standard. This complete procedure when conducted with 5 mg of bovine serum albumin substituted for microsomes showed no statistical difference between normal and heat inactivated protein in the adsorption of [^{3}H]TCB.

Chromatography of the initial methanol extract (Fraction II) of the TCA insoluble pellets was carried out on a Waters Associates 6000 psi high-pressure liquid chromatograph (C_{18}-corasil, 80% CH_3OH–20% H_2O) equipped with two model 6000A pumps, solvent programmer, and ultraviolet and refractive index detector. The initial TCA washes (Fraction I) of the protein-RNA pellet were neutralized with NaOH and freeze-dried. The resulting solid was extracted serially with hexane, ether, methanol, and finally ethanol:water (3:2). The ethanol:water soluble material was chromatographed on silica gel 60 (70–230 mesh) (E. Merck) using ethanol:water (3:2) as the solvent at a flow rate of 1.0 ml/min.

Results. Distribution of the [^{3}H]TCB in homogenate fractions. The exhaustive methanol extraction (Fraction II) removed greater than 98% of the [^{3}H]TCB from the initial TCA precipitates of the normal microsomes and the heat inactivated controls in all groups. Approximately 0.2% of the [^{3}H]TCB remained bound to macromolecules in Group 3 with 0.05% represented as adsorbed material (control homogenates). Introduction of GSH into an active generating system (Groups 2 and 4) increased the TCA soluble [^{3}H]TCB by 1.0 to 0.5% above a standard level of approximately 0.4% in all other groups (Table I). The low conversion rate is due to the stability of the TCB molecule. *In vivo* conversion to excretable metabolites takes place at only approximately 1%/day (10).

In vitro generation of PCB metabolite capable of binding to macromolecules. As can

be seen in Table II, homogenates of heat inactivated microsomes bound some radioactivity that was not extracted. This material was considered to be adsorbed to the macromolecules and was used as an adsorption control. The water incubation (Group 1) in which only the microsomes, substrate, and NADPH generating system were present, produced a significant quantity of tritium bound to the protein fraction compared to the heat inactivated controls. When GSH (Group 2) was added to this system, no significant change in bound tritium could be detected. When microsomal supernate (Group 3) was added, a significant increase in the amount of tritium bound to protein was noted. The addition of either 1 μmole or 10 μmoles of GSH to the incubation medium containing microsomal supernate (Group 4) eliminated the significant increase in tritium bound to proteins. The incubations with microsomal supernate strongly increased binding of tritium to the RNA fraction. A reduction of tritium bound to RNA was noted by the addition of either 1 or 10 μmole of GSH to the reaction flask (Table III). In both instances the reduced level was similar to that occurring in Group 2.

TABLE I. THE AMOUNT OF [^{3}H]TCB (METABOLIZED AND UNMETABOLIZED) APPEARING IN THE TCA SOLUBLE FRACTION (FRACTION I) OF MICROSOMES INCUBATED WITH [^{3}H]TCB.

Group		^{3}H as a percentage of substrate ±SD	
1	H_2O	0.52 ± 0.11	(7)[a]
	H_2O boiled	0.31 ± 0.06	(5)
2	GSH (0.31 mg)	1.24 ± 0.23	(7)
	GSH boiled	0.40 ± 0.12	(5)
3	MS[b]	0.43 ± 0.10	(7)
	MS boiled	0.35 ± 0.08	(5)
4	MS + GSH (0.31 mg)	1.09 ± 0.19	(7)
	MS + GSH boiled	0.37 ± 0.06	(5)

[a] Number of incubations.
[b] Microsomal supernate.

In vitro generation of polar PCB metabolites. The methanol extracts (Fraction II) of the 5% cold TCA precipitate were chromatographed on corasil-C_{18} column (1.0 ml/min, 100 psi) using 20% H_2O–80% CH_3OH as the solvent. This system has been used in our laboratory to separate unmetabolized TCB from its hydroxylated metabolites (4). Mono-, di-, and trihydroxylated metabolites all elute with the solvent front in this system while TCB elutes several fractions later. Essentially no metabolized TCB could be found in the methanol extract of the incubation with microsomal supernate (limit of detection about 0.5%).

The chromatography of the TCA soluble material from Group 2 (Fraction I) on silica gel using ethanol:water (60:40) as the solvent showed an elution pattern different from free [^{3}H]TCB indicating a possible metabolic product. The elution volume for free [^{3}H]TCB mixed with GSH and TCA was 45 ml while the [^{3}H]TCB extracted from the TCA soluble freeze-dried material had an elution volume of 58 ml on the same

TABLE II. [^{3}H]TCB BOUND TO PROTEIN RESULTING FROM INCUBATION OF [^{3}H]TCB AND MONKEY LIVER MICROSOMES IN A NADPH GENERATING SYSTEM.

Substrate added to homogenate		dpm Bound to protein ±SD	
Group 1	H_2O	2,255 ± 548[a]	(7)[i]
	H_2O (heated microsomes)	1,218 ± 246[a]	(5)
Group 2	GSH	2,872 ± 258[b,g]	(7)
	GSH (heated microsomes)	1,520 ± 77[b]	(5)
Group 3	MS[h]	4,782 ± 539[c,g]	(7)
	MS (heated microsomes)	1,260 ± 76[c]	(5)
Group 4	MS + GSH (.31 mg/flask)	2,061 ± 486[d,f]	(7)
	MS + GSH (3.1 mg/flask)	1,934 ± 238[e]	(7)
	MS + GSH (.31 mg/flask) (heated microsomes)	1,084 ± 138[d,e]	(5)

Note: [a] $p \leq .005$; [b] $p \leq .0001$; [c] $p \leq .0001$; [d] $p \leq .001$; [e] $p \leq .0001$; [f] $p \leq .0001$; [g] $p \leq .0001$ (Student's t test used to determine p value); [h] microsomal supernate; [i] number of incubations.

TABLE III. ^{3}H-TCB BOUND TO MICROSOMAL RNA AFTER INCUBATION OF ^{3}H-TCB WITH RHESUS MONKEY LIVER MICROSOMES IN A NADPH GENERATING SYSTEM.

Substrate added to homogenate		dpm Bound to microsomal RNA ±SD
Group 1	H_2O	302 ± 47[a] (7)[i]
	H_2O (heated microsomes)	107 ± 13[a] (5)
Group 2	GSH	201 ± 71[b,g] (7)
	GSH (heated microsomes)	68 ± 34[b] (5)
Group 3	MS[h]	468 ± 88[c,f,g] (7)
	MS (heated microsomes)	41 ± 17[c] (5)
Group 4	MS + GSH (.31 mg/flask)	154 ± 79[d,f] (7)
	MS + GSH (3.1 mg/flask)	195 ± 93[e] (7)
	MS + GSH (.31 mg/flask) (heated microsomes)	57 ± 13[d,e] (5)

Note: [a] $p \leq .0001$; [b] $p \leq .005$; [c] $p \leq .0001$; [d] $p \leq .02$; [e] $p \leq .01$; [f] $p \leq .0001$; [g] $p \leq .0001$; Student's t test used to determine p value; [h] microsomal supernate; [i] Number of incubations.

column (Fig. 2). Exhaustive extraction of the freeze-dried TCA soluble material with hexane (60°) and ether (35°) failed to extract an appreciable quantity (10%) of tritium, indicating that the [^{3}H]TCB is not a mono-, di-, or triol, all of which are freely soluble in ether. The remaining tritium (90%) was solubilized by using methanol (60°) (but not methanol, 20°) or with ethanol:water (60:40). No such [^{3}H]TCB could be found in the freeze-dried TCA fraction of the heat-inactivated GSH incubation (Group 2).

Discussion. From the findings of Gardner *et al.* (3) and Hsu *et al.* (4), it has become increasingly apparent that the lower chlorinated PCBs containing adjacent hydrogens can readily be metabolized through the chemically active arene oxide intermediate. Jensen *et al.* (11) has shown that even higher chlorine isomers are metabolized to

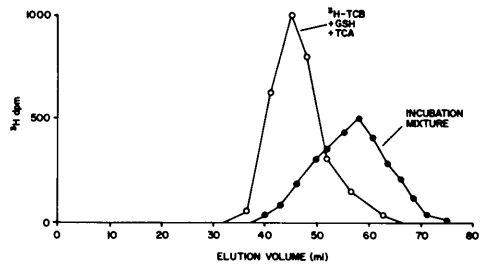

FIG. 2. Chromatography of [^{3}H]TCB, TCA, and GSH (○——○) and the TCA soluble material from the incubation (Fraction I, Group 2) (●——●) on a silica gel-60 column using 60% ethanol-40% water as the eluting solvent.

mono-oxygenated metabolites through a mechanism not yet fully characterized. Such metabolites can, and obviously do, form conjugated metabolites with other biological molecules. Proteins and RNA exposed to *in vitro* generation of a possible arene oxide intermediate of TCB have been shown here to covalently bind [^{3}H]TCB to a significant extent. Such reactions could conceivably lead to alterations in the macromolecular structure at the site of alkylation. The fact that hexane and ether were able to extract only 10% of the radioactivity from the TCA soluble material (Fraction I, Group 2) implies that the bulk of the radioactivity in the freeze-dried TCA soluble material (about 1% of the total substrate ^{3}H) was either more polar than the previously isolated triols or conjugated to very polar materials such as GSH. This would account for its initial solubility in the TCA soluble fraction. Comparatively, 0.2% of the total substrate was found to be covalently bound to macromolecules when GSH was not present (Group 3, Table II). This represents a 20% binding of metabolically converted products.

Kimbrough *et al.* (12) administered higher chlorine isomer mixtures of PCBs to 200 female rats for 2 years and observed a 14% incidence of tumors (vs 1% controls) and a 78% incidence of neoplastic nodules in the liver (vs 0% controls). It is conceivable that these tumors were due to the interaction of arene oxide-like intermediates with the genetic material at the cellular

level. Other investigators have also observed neoplastic liver nodules in rats (13) and mice (14). These arene oxides are usually extremely reactive as is evident by the fact that tumors related to PCB administration were observed only in the liver where the arene oxide is presumably generated. In our experiments, the active metabolite appears sufficiently nonreactive to leave the site of synthesis and react with supernate protein. This reaction was prevented by the addition of glutathione which presumably conjugates the active metabolite before it reacts with protein. The fact that none of the heat deactivated homogenates bound large quantities of radioactivity to proteins or GSH indicates that metabolism is required to achieve this binding capability. The only materials heated were the microsomal suspensions and not the glucose-6-phosphate or the microsomal supernate. Therefore, the microsomal supernate alone does not have the capacity to generate the reactive intermediate, and the NADPH generating system is completely intact in the homogenate mixture.

The fact that PCBs are so widespread in the environment makes it extremely important that we understand the mechanism by which these materials are toxic. When adult rhesus monkeys were fed 2.5 ppm PCB in the diet, skin lesions, abnormal menstrual cycles, difficulty in maintaining pregnancy, and abnormal offspring were observed (15). It is conceivable that certain segments of the human population are exposed to PCBs at levels that may well exceed 2.5 ppm. The presently reported study indicates that some PCB isomers may be metabolized through a reactive arene oxide intermediate which has the potential to bind to macromolecules and alter their character. These data suggest that PCBs will continue to be a significant health hazard for many years to come.

Summary. Incubation of tritiated 2,5,-2′,5′-tetrachlorobiphenyl with normal monkey liver microsomes in a NADPH-generating system results in the formation of active metabolite(s) of the [^{3}H]2,5,2′,5′-tetrachlorobiphenyl capable of covalently binding to RNA and protein isolated from the incubation mixture. The metabolite is not formed when the control microsomes are held at 100° for 10 min prior to incubation. The addition of microsomal supernate to the solution causes an increase in the binding of the active metabolite to macromolecules while the addition of glutathione to the incubation medium significantly inhibits this increase.

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