Metabolic Activation of [^{14}C] Polychlorinated Biphenyl Mixtures by Rat Liver Microsomes

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Brodie et al. (1971) have reported that halogenated benzenes and other chemicals are converted to active metabolites by liver microsomes and suggested that liver injury by these chemicals results from such metabolic activation. It is also known that active metabolites formed from a variety of carcinogens and toxic compounds bind covalently to intracellular proteins, nucleic acids, and lipids and this binding is a more direct cause of carcinogenesis and cytotoxicity (Miller, 1970; Gillette et al. 1974).

Recent evidence has further suggested that arene oxides are the active formed from such chemicals as carcinogenic polycyclic hydrocarbons and halogenated benzenes (Jerina and Daly, 1974). The arene oxides thus produced have been shown to be readily hydrolyzed to inactive dihydrodiol derivatives by the action of epoxide hydrase which is also present in liver microsomes (Jerina and Daly, 1974). That an arene oxide of 3-methylcholanthrene is the active metabolite has been supported by Bürki et al. (1974a,b) who showed that administration of 1,1,1-trichloro-2-propene oxide, an inhibitor of epoxide hydrase, enhances both the binding of labelled methylcholanthrene to DNA and the methylcholanthrene-induced carcinogenesis.

Polychlorinated biphenyls (PCBs) are important environmental pollutants and shown to cause liver injury in many animals including man, monkey, rat and mouse (Nishizumi, 1970; Kuratsune, 1972; Kimbrough, 1972; Fishbein, 1974). However, the mechanism by which PCBs induce liver injury is not yet well understood. Since Gardner et al. (1973) have isolated a dihydrodiol metabolite of 2,5,2',5'-tetrachlorobiphenyl from the urine of rabbits given the parent compound, it is likely that epoxide metabolites are formed from PCBs as active intermediates.

This paper reports that PCBs can bind covalently to liver macromolecules in vivo and to microsomes in vitro and that this binding requires metabolic

Bulletin of Environmental Contamination & Toxicology,
Vol. 16, No. 1 © 1976 by Springer-Verlag New York Inc.

activation of the compounds by the microsomal monooxygenase system.

MATERIALS AND METHODS

Two preparations of [^{14}C] polychlorinated biphenyls were obtained from the Tokai Research Establishment of the Japan Atomic Energy Institute. One, called [^{14}C] KC-300 (2 μCi/mg), showed a gas chromatographic pattern practically identical with that of Kanechlor-300 (chlorine content, 42 %), and the other, called [^{14}C] KC-500 (1.6 μCi/mg), was similar in composition to Kanechlor-500 (chlorine content, 55 %).

For studies of the covalent binding of PCB metabolites in vivo, male Sprague-Dawley rats (100-120 g) were administered with a single dose of 25 mg of [^{14}C] PCBs dissolved in olive oil per kg of body weight by gastric intubation. The animals were killed under light ether anaesthesia after suitable periods of time, and the livers were excised. The excised liver was homogenized with 10 volumes of 0.25 M sucrose. A portion of the homogenate was used to determine the total liver radioactivity. To another 1 ml portion of the homogenate was added 3 ml of 10 % trichloroacetic acid to precipitate macromolecules. The resultant precipitate was collected by centrifugation and washed once with 3 ml of 10 % trichloroacetic acid. The washed precipitate was extracted successively with 3 ml each of the following solvents; 80 % methanol (two times), methanol-ether (1:1,v/v) (two times), and 80 % methanol (two times). No radioactivity was removed from the precipitate by the last extraction, indicating that all the PCBs and their metabolites that were not in covalent linkage with the hepatic macromolecules had been removed completely. The radioactivity in the throughly extracted precipitate was measured in a Packard Tri-Carb liquid scintillation spectrometer and taken as the amount of covalently bound metabolites.

For in vitro experiments, the animals were pretreated for 3 days with a daily dose of 100 mg of unlabelled KC-500 (dissolved in olive oil) per kg of body weight (by intraperitoneal injection). One day after the last treatment, the rats were killed and liver microsomes were prepared by the method of Omura and Sato (1964). The incubation mixture for in vitro binding studies contained, in a final volume of 1.0 ml, an NADPH-generating system (0.5 μmole of NADP, 10 μmoles of glucose 6-phosphate, 25 μmoles of $MgCl_2$, and 5 units of glucose 6-phosphate dehydrogenase) 10.4 μg of [^{14}C] KC-300 or 13 μg of [^{14}C] KC-500 dissolved in 20 μl of acetone, 0.1 M phosphate buffer, pH 7.4, and microsomes

(0.75 or 1.5 mg of protein). The reaction was started by addition of [^{14}C] PCBs and run at 37°C for suitable periods of time. The reaction was stopped by adding 3 ml of 10 % trichloroacetic acid and amount of covalently bound metabolites was determined as described above.

RESULTS

Fig.1 and 2 show the time courses of covalent binding of PCB metabolites to the total liver macromolecules after oral administration of [^{14}C] KC-300 and [^{14}C] KC-500, respectively. The total liver radioactivity became maximum 8 h after administration of both [^{14}C] KC-300 and [^{14}C] KC-500, and the maximal radioactivity attainable was higher with KC-500 than with KC-300. The radioactivity covalently bound to macromolecules after [^{14}C] KC-300 administration also became maximal about 8 h after the treatment and decreased thereafter at a slower rate than that for the total liver radioactivity.

After [^{14}C] KC-500 administration, on the other hand, the increase in the covalently bound radioactivity was slower reaching a maximal value only about

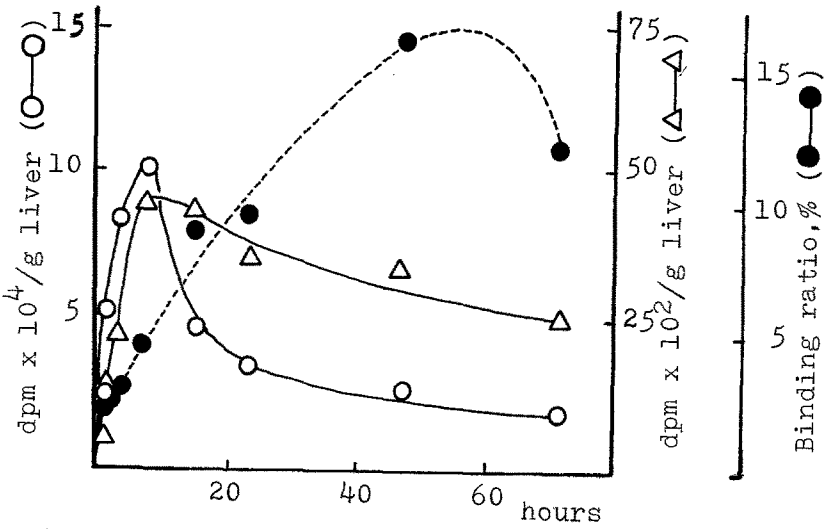

Fig.1 Time course of covalent binding of [^{14}C] KC-300 to rat liver macromolecules *in vivo*. Total liver radioactivity (O—O), covalently bound radioactivity (△—△) and ratio of covalently bound radioactivity to tatal liver radioactivity (●—●) were determined at various intervals after [^{14}C] KC-300 (25 mg/kg; 0.05 mCi, p.o.) Points represent means of two rats.

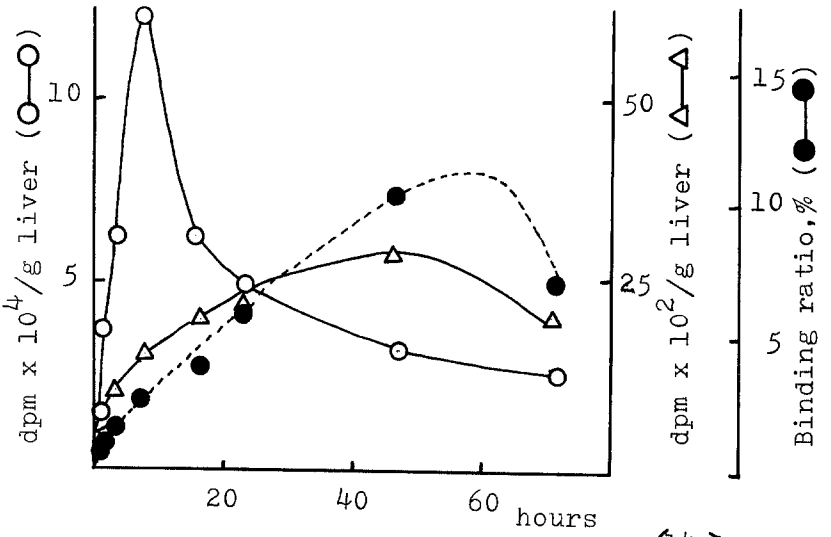

Fig.2 Time course of covalent binding of [^{14}C]KC-500 to rat liver macromolecules in vivo. Total liver radioactivity (O—O), covalently bound radioactivity (△—△) and ratio of covalently bound radioactivity to total liver radioactivity (●—●) were determined at various intervals after [^{14}C]KC-500 (25 mg/kg; 0.04 mCi, p.o.) Points represent means of two rats.

50 h after the treatment. At 50 h after the treatment, about 16 and 11 % of the total liver radioactivity were found in the covalently bound form for the animals administered with [^{14}C]KC-300 and [^{14}C]KC-500, respectively.

When liver microsomes from KC-500-pretreated rats were incubated with [^{14}C]PCBs in the presence of an NADPH-generating system, considerable radioactivities became covalently bound to microsomal macromolecules. As is evident from Fig.3, the amount of covalently bound radioactivity was dependent on the amount of microsomes used. Fig. 3 also shows that the covalent binding was more extensive with [^{14}C]KC-300 as substrate than with [^{14}C]KC-500.

As shown in Table 1, the covalent binding of radioactivity of both [^{14}C]KC-300 and [^{14}C]KC-500 required almost absolutely the presence of NADP which was added to the system as a component of the NADPH-generating system. Both types of binding reaction were also inhibited almost completely if nitrogen was used instead of air as the atmosphere. Furthermore, in an atmosphere of CO-O_2 (8:2, v/v) the bindings of [^{14}C]KC-300 and [^{14}C]

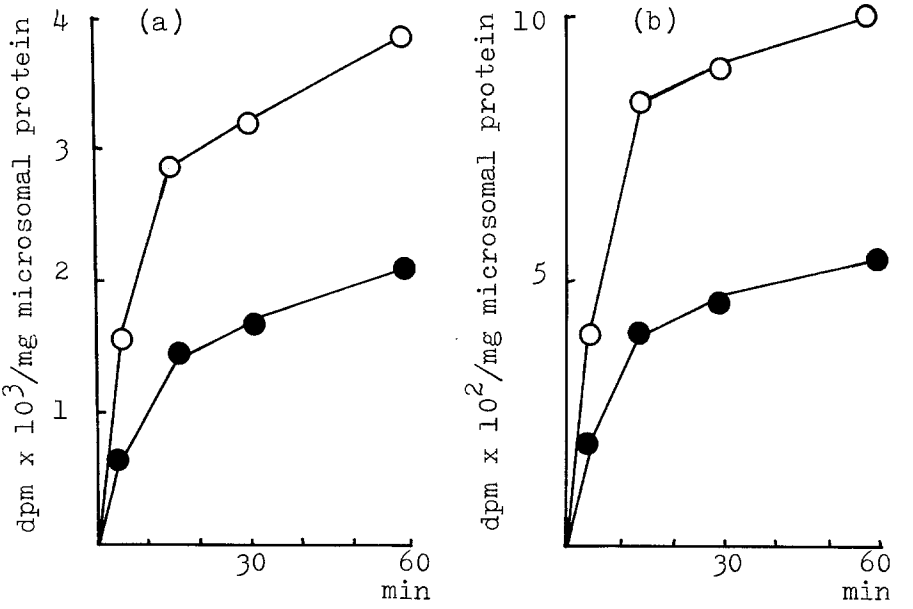

Fig.3 Covalent binding of [^{14}C]KC-300 (a) and [^{14}C]KC-500 (b) catalyzed by liver microsomes from KC-500-pretreated rats in the presence of an NADPH-generating system in vitro. Microsomal protein concentration were 0.75 mg (●—●) and 1.5 mg (○—○) per ml of incubation mixture. Points represent means of two experiments.

KC-500 radioactivities were inhibited by 66 and 92 %, respectively. Since the requirements for NADPH and molecular oxygen and the sensitivity to CO are known to be characteristic features of the cytochrome P-450-containing microsomal monooxygenase system, it could be concluded from these results that this monooxygenase system was involved in the conversion of PCBs to metabolically active derivatives capable of covalently binding to macromolecules. It was further found that both reduced glutathione and cystein inhibited the binding reactions significantly.

DISCUSSION

The in vivo experiments descrived above indicate that the radioactivity of [^{14}C]PCBs orally administered to rats are bound covalently to macromolecules in hepatocytes. Since PCBs themselves are chemically inert compounds, it is likely that metabolic activation of PCBs is required for the covalent binding reaction. The present study has shown that liver microsomes contain an enzyme system responsible for such metabolic

TABLE 1

Inhibition of Covalent Binding of [^{14}C] KC-300 and [^{14}C] KC-500 to Liver Microsomes from KC-500-Pretreated Rats.

	^{14}C PCBs bound(dpm/mg protein/60 min)			
	^{14}C KC-300	% inhibition	^{14}C KC-500	% inhibition
Complete system	3,078	-	1,067	-
NADP omitted	31	99	75	93
Under N_2	92	97	43	96
Under $CO:O_2$(8:2)	1,034	66	83	92
Glutathione (1 mM) added	1,108	64	715	33
Cysteine (1 mM) added	1,601	48	406	62

Rat liver microsomes was prepared and incubated with substrates as described in MATERIALS AND METHODS. Values represent means of three experiments.

activation, although the possibility can not yet be excluded that the other subcellular fractions can also activate PCBs. The microsomal system involved in the activation requires both NADPH and molecular oxygen and is sensitive to CO. These findings indicate clearly that the microsomal monooxygenase system containing cytochrome P-450 is actually involved.

Although the nature of the activated metabolites of PCBs is not known, it seems likely that arene oxides are the activated products in view of the previous findings on metabolic activation and covalent binding of halogenated benzenes, carcinogenic polycyclic hydrocarbons, and some other chemicals (Jerina and Daly, 1974; Gardner et al., 1973). The isolation of dihydrodiol derivative of a tetrachlorobiphenyl from rabbit urine (Gardner et al.,1973) also supports this possibility.

Mitchell et al. (1973) have reported that pretreatment of mice with diethylmaleate, a drug known to deplete hepatic glutathione, potentiated acetaminophene-induced hepatic necrosis, whereas pretreatment with cysteine ,a precursor of glutathione, prevented the hepatic damage. In the present study, both glutathione and cysteine have been shown to cause significant inhibition of the covalent binding of PCB metabolites to macromolecules in the in vitro microsomal system. Although the significance of these inhibition is still unclear, it seems that glutathione and cysteine play a role in preventing the cytotoxic effects of activated

metabolites of foreign compounds.

In both *in vivo* experiments, the metabolites of KC-300 can bind more efficiently to macromolecules than those of KC-500. This is compatible with the report of Jensen and Sundström (1974) that lowly chlorinated PCBs are more readily metabolized than highly chlorinated PCBs.

Jollow *et al*. (1974) have reported that the amount of covalently bound radioactivity in mouse liver reached maximum about 2 h after administration of labelled acetaminophene. In this study, the maximum covalent binding has been observed 8 and 50 h after administration of [^{14}C]KC-300 and [^{14}C]KC-500, respectively. Such delayed incorporation of PCB radioactivity appears to be related to the longer storage of PCBs in the body. It is expected that highly chlorinated PCBs can be stored in the body for longer periods of time than lowly chlorinated PCBs. Kimbrough *et al*.(1973) have reported that lesions of the liver of rats fed 500 ppm of Aroclor 1254 (a preparation of PCBs) persist for a long time even after the cessation of exposure to PCBs. It is, therefore, likely that *in vivo* PCBs are stored in the body and then gradually metabolized to activated products in the liver inducing chronic liver injury.

ACKNOWLEDGEMENT

I am grateful to Professor Ryo Sato, Institute for Protein Research, Osaka University , for his valuable advices during the course of this work. I wish to thank Mr. Yoshiki Nunoura, Mr. Shozo Iwagami and Mrs. Fusako Shinto for their valuable assistance. I also wish to thank Miss Mayumi Bando for preparing this manuscript.

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