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COVALENT BINDING *IN VITRO* OF POLYCHLORINATED BIPHENYLS TO MICROSOMAL MACROMOLECULES

INVOLVEMENT OF METABOLIC ACTIVATION BY A CYTOCHROME P-450-LINKED MONO-OXYGENASE SYSTEM

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Abstract—Incubation of ^{14}C-labeled polychlorinated biphenyls (PCBs) with rat, mouse or rabbit liver microsomes in the presence of an NADPH-generating system and molecular oxygen caused covalent binding of radioactive metabolites of PCBs to microsomal macromolecules. The binding was more pronounced with liver microsomes from animals pretreated with inducers of the microsomal mono-oxygenase system. The order of induction effect of the inducers used was KC-500 (a PCB preparation containing 55% chlorine) ≥ phenobarbital (PB) ≫ 3-methylcholanthrene (3-MC) in rats, PB ≫ KC-500 > 3-MC in mice, and PB ≫ KC-500 in rabbits. [^{14}C]KC-300 (a PCB preparation containing 42% chlorine) was more effective than [^{14}C]KC-500 as substrate for all the microsomal preparations. The binding reaction was dependent on both NADPH and oxygen, sensitive to carbon monoxide, glutathione, cysteine, hexobarbital, and aniline, and enhanced by EDTA, which inhibits lipid peroxidation. The addition of NADH, which was by itself a very poor electron donor, caused a synergistic increase of the NADPH-dependent binding of PCBs. It is concluded that the conversion of PCBs to active metabolites by the cytochrome P-450-linked mono-oxygenase system is prerequisite to the binding reaction. A survey of the effects of various inducers suggested that a cytochrome P-450 having a high aminopyrine *N*-demethylation activity is mainly responsible for the metabolic activation of PCBs in liver microsomes. Kidney and lung microsomes from untreated rats were virtually devoid of the PCB-binding capacity, but in kidney microsomes this capacity could be induced by pretreatment with 3-MC or KC-500, though not with PB.

Polychlorinated biphenyls (PCBs) are well-known environmental pollutants [1] and have been shown to cause liver injury in various animals including rat [2–5], mouse [6] and rabbit [7, 8]. However, the mechanism by which PCBs exert hepatotoxic effects is not clearly defined. Since PCBs themselves are chemically inert compounds, it is expected that they induce liver injury only after metabolic activation, as has been demonstrated for halogenated benzenes, polycyclic hydrocarbons, and other compounds [9]. In a preliminary communication, one of the authors (T. S.) reported that radioactive PCBs could bind covalently to rat liver macromolecules *in vivo* and to liver microsomes *in vitro*, and presented evidence that this binding requires metabolic activation of the compounds by the microsomal cytochrome P-450-containing mono-oxygenase system [10]. Since then, Wyndham *et al.* [11] and Seymour *et al.* [12] have reported metabolic activation and covalent binding of 4-chlorobiphenyl and 2,5,2′,5′-tetrachlorobiphenyl, respectively, by liver microsomes. In this paper, we now report a full account of our studies on the covalent binding of ^{14}C-labeled PCBs to rat, mouse and rabbit liver microsomes. The binding of PCB metabolites to rat kidney and lung microsomes is also reported.

MATERIALS AND METHODS

Two different types of ^{14}C-labeled PCBs were obtained from the Tokai Establishment of the Japan Atomic Energy Institute. One of them, called [^{14}C]KC-300* (2 μCi/mg) showed a gas chromatographic profile very similar to that of Kanechlor-300 (KC-300; chlorine content, 42 per cent), and the other, termed [^{14}C]KC-500 (1.6 μCi/mg), was similar in composition to Kanechlor-500 (KC-500; chlorine content, 55 per cent). Unlabeled KC-500 was obtained from Kanegafuchi Kagaku Co., Ltd., Tokyo. Benzo(a)pyrene, $NADP^+$, NADPH, NADH, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and horse-heart cytochrome *c* were purchased from Sigma Chemical Co., St. Louis, MO., sodium phenobarbital (PB) from Sanko Pure Chemical Co., Tokyo, and 3-methylcholanthrene (3-MC) from Katayama Chemical Co., Osaka. 3-Hydroxybenzo(a)pyrene was a generous gift from Dr. H. V. Gelboin. All the other chemicals and reagents were standard products of analytical grade and used without further purification.

Male Sprague–Dawley rats, weighing 100–150 g, male ICR mice, weighing 16–22 g, and male white rabbits, weighing about 2.5 kg, were used. They were allowed free access to standard laboratory chows

* Kanechlors (KCs) are the trade names of complex mixtures of polychlorinated biphenyls produced by Kanegafuchi Kagaku Co., Tokyo. The KC preparations also contain a trace amount of chlorinated dibenzofurans.

(Oriental Yeast Co., Tokyo) and water, and killed after starvation for 24 hr. The animals were pretreated with PB dissolved in saline, 3-MC dissolved in olive oil, and KC-500 dissolved in olive oil by injecting intraperitoneally for 3 consecutive days; the daily dose was 100 mg/kg of body weight for PB and KC-500 and 50 mg/kg of body weight for 3-MC. The animals were killed 24 hr after the last injection.

For preparation of microsomes, the liver or other tissues were homogenized with 4 vols of 0.25 M sucrose in a Potter-type homogenizer. The homogenate was centrifuged at 9000 *g* for 15 min, and the microsomal fraction was then sedimented from the resultant supernatant by centrifugation at 105,000 *g* for 90 min. The microsomal pellet was washed once with 0.15 M KCl and finally suspended in 0.25 M sucrose (microsomes equivalent to 1 g of wet tissue/2 ml).

Protein was determined by the method of Lowry *et al.* [13] using bovine serum albumin as standard. The contents of cytochromes P-450 and b_5 in microsomes were measured as described by Omura and Sato [14]. NADPH–cytochrome *c* reductase activity was assayed by the method of Masters *et al.* [15]. Aminopyrine *N*-demethylase activity was determined as described by Mazel [16]. Aryl hydrocarbon [benzo(a)pyrene] hydroxylase activity was determined in a reaction mixture containing 100 mM potassium phosphate buffer, pH 7.4, 3 mM $MgCl_2$, an NADPH-generating system consisting of 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate and 1 unit of glucose 6-phosphate dehydrogenase, 100 μM benzo(a)pyrene (added in 20 μl acetone), and a microsomal preparation derived from 50 mg tissue in a final volume of 1.0 ml. The mixture was incubated at 37° for 10 min and the product was measured by the method of Nebert and Gelboin [17]. Lipid peroxidation was measured by the thiobarbituric acid (TBA) method [18].

Covalent binding of metabolites of [^{14}C]PCBs to macromolecules of microsomes was determined as follows. The standard reaction mixture (final volume, 1.0 ml) contained 0.1 M potassium phosphate buffer, pH 7.4, microsomes (usually about 1 mg protein), 10.4 μg [^{14}C]KC-300 (20.6 nCi) or 13.0 μg [^{14}C]KC-500 (20.7 nCi) in 20 μl acetone, and the NADPH-generating system described above. The reaction was carried out at 37° for a suitable period of time with constant shaking in air and stopped by adding 3 ml of 10% trichloroacetic acid. The resultant precipitate was collected by centrifugation and washed with 3 ml of 10% trichloroacetic acid. The washed pellet was then extracted with 3 ml each of 80% methanol (twice), methanol–ether (1:1, v/v, twice) and 80% methanol (twice). No radioactivity was removed from the pellet by the last extraction, indicating that all the PCBs and their metabolites that were not in covalent linkages with macromolecules had already been extracted. The throughly extracted precipitate was dissolved in 1 ml Soluene-350 or 1 N NaOH and transferred to a counting vial. The radioactivity of the sample was measured in 10 ml of Permablend III solution by using a Packard Tricarb scintillation spectrometer. The value obtained was corrected for quenching by internal and external standardization. The radioactivity thus determined was taken as the amount of PCB metabolites covalently bound to macromolecules.

RESULTS

Covalent binding of PCB metabolites to liver microsomes; effect of induction. In a previous preliminary communication [10] it was shown that liver microsomes prepared from KC-500-pretreated rats could bind a significant amount of radioactive substances upon incubation with [^{14}C]PCBs in the presence of an NADPH-generating system and molecular oxygen. Evidence was also presented to suggest that this binding required metabolic activation of the compounds by the cytochrome P-450-containing mono-oxygenase system. In the present study, this was further examined by using liver microsomes from untreated rats and the animals that had been pretreated with inducers of hepatic microsomal cytochrome P-450. As is evident from Figs. 1 and 2, the covalent binding of PCB metabolites was more pronounced with liver microsomes from rats pretreated with the inducers

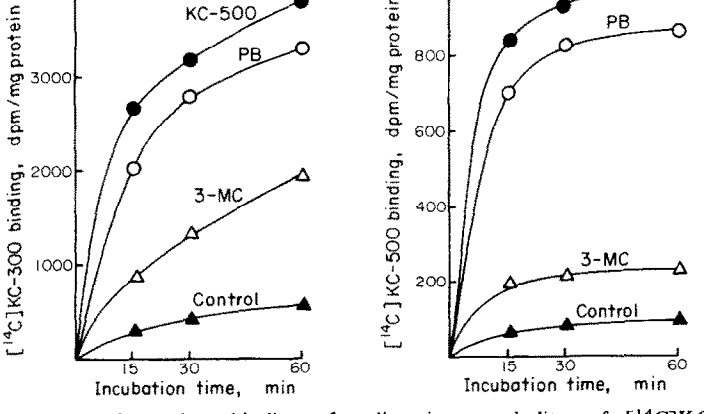

Fig. 1. Time course of covalent binding of radioactive metabolites of [^{14}C]KC-300 (a) and [^{14}C]KC-500 (b) to microsomal macromolecules. Liver microsomes from PB-, 3-MC- and KC-500-pretreated rats, and control rats were incubated for various periods of time with [^{14}C]KC-300 (10.4 μg) or [^{14}C]KC-500 (13.0 μg) in the presence of an NADPH-generating system under air at 37°. Points represent the mean for six experiments.

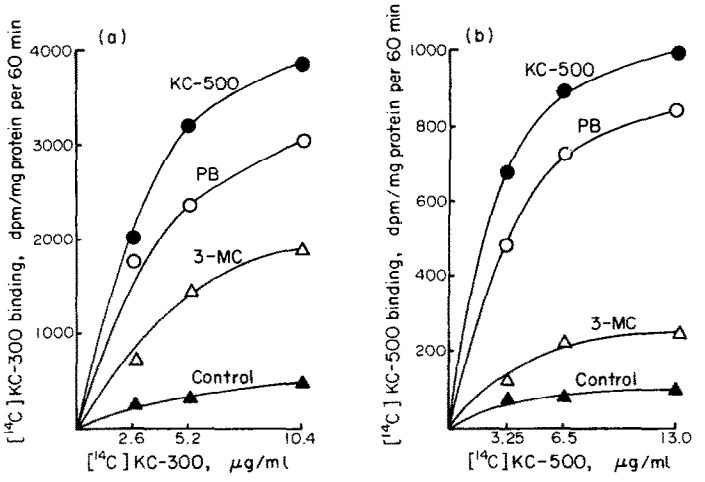

Fig. 2. Effect of substrate concentration on covalent binding of radioactive metabolites of [^{14}C]KC-300 (a) and [^{14}C]KC-500 (b) to microsomal macromolecules. Rat liver microsomes used were as in legend of Fig. 1. Points represent the mean for six experiments.

than with those from control animals. Among the inducers employed, KC-500 was the most effective in increasing the PCB-binding activity of liver microsomes, followed by PB and 3-MC in this order. With [^{14}C]KC-500 (13.0 μg) as substrate, for instance, the radioactivities bound to KC-500-PB- and 3-MC-induced rat liver microsomes after incubation for 60 min were 12-, 10- and 2-fold higher than the radioactivity bound to control microsomes. It was also found that the binding was much more extensive with [^{14}C]KC-300 (less chlorinated biphenyls) as substrate than with [^{14}C]KC-500 (highly chlorinated biphenyls). The radioactivities bound to liver microsomes from KC-500-pretreated rats after incubation for 60 min represented 12 and 4 per cent of the [^{14}C]KC-300 and [^{14}C]KC-500 added, respectively, in the incubation mixture. Figure 1 shows further that the time course of binding was linear only for short periods of time in all cases, though there was a tendency that the linearity was somewhat better with [^{14}C]KC-300 as substrate than with [^{14}C]KC-500.

Figures 3 and 4 show the corresponding results obtained with liver microsomes prepared from control and variously pretreated mice. In contrast to the case of rats, PB was by far the most effective inducer of the PCB-binding activity of mouse liver microsomes, and the induction by KC-500 and 3-MC was not very extensive, especially when the binding was measured with [^{14}C]KC-500 as substrate. With [^{14}C]KC-500 (13.0 μg) as substrate, the radioactivities bound to PB- and KC-500-induced mouse liver microsomes after incubation for 60 min were about 6- and 2-fold higher, respectively, than the radioactivity bound to control microsomes, whereas the binding activity of 3-MC-induced microsomes was practically the same as that of control microsomes.

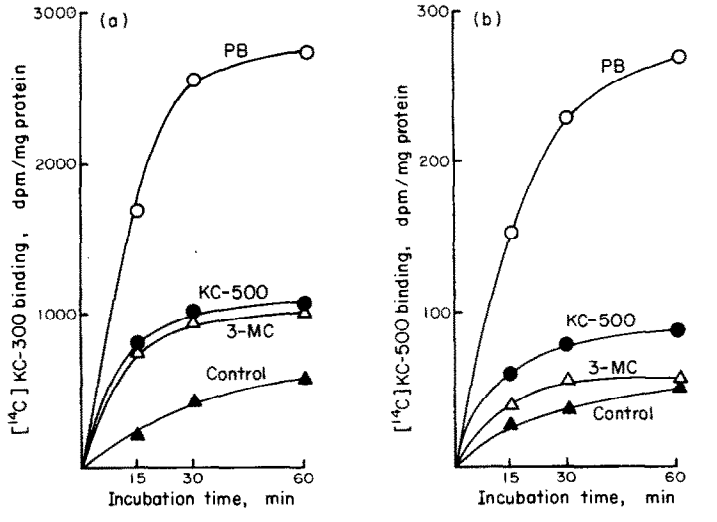

Fig. 3. Time course of covalent binding of radioactive metabolites of [^{14}C]KC-300 (a) and [^{14}C]KC-500 (b) to microsomal macromolecules. Liver microsomes from PB-, 3-MC- and KC-500-pretreated mice, and control mice were incubated for various periods of time with [^{14}C]KC-300 (10.4 μg) or [^{14}C]KC-500 (13.0 μg) in the presence of an NADPH-generating system under air at 37°. Points represent the mean for six experiments.

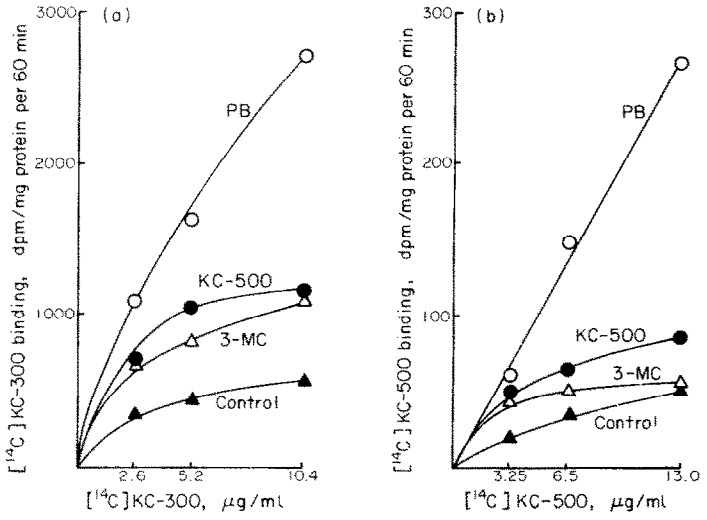

Fig. 4. Effect of substrate concentration on covalent binding of radioactive metabolites of [^{14}C]KC-300 (a) and [^{14}C]KC-500 (b) to microsomal macromolecules. Mouse liver microsomes used were as in the legend to Fig. 3. Points represent the mean for six experiments.

In mouse liver microsomes, too, [^{14}C]KC-300 was a much better substrate for the binding reaction than [^{14}C]KC-500. It was further noticed that the binding capacity of control mouse liver microsomes was not very much different from that of control rat liver microsomes. However, the effect of induction on this capacity was considerably more pronounced in rats than in mice.

Effect of induction on microsomal components. Since pretreatment of rats and mice with PB, 3-MC or KC-500 enhanced the PCB-binding capacity of their liver microsomes as described above, we then examined the effects of these treatments on the contents of microsomal redox components, especially that of cytochrome P-450, and on the drug hydroxylase activities. In confirmation of previous findings [19, 20], it was found that cytochrome P-450 in liver microsomes from animals pretreated with 3-MC showed a Soret peak at 448 nm, instead of 450 nm, in the carbon monoxide difference spectrum. This was also the case for the hemoprotein in liver microsomes from KC-500-treated animals. The pigment in control and PB-induced microsomes had a Soret peak at 450 nm in the carbon monoxide difference spectrum. The former hemoprotein is usually called cytochrome P-448 or cytochrome P_1-450 [19].

As shown in Table 1, significant increases in the levels of cytochrome P-450 and NADPH–cytochrome *c* reductase activity in liver microsomes were observed by pretreatment of rats with PB, although no increase in cytochrome b_5 was observed. In 3-MC-treated rats, on the other hand, the levels of both cytochrome P-450 (in the form of cytochrome P-448) and cytochrome b_5 were elevated, but only a slight increase was detected in NADPH–cytochrome *c* reductase activity, as reported previously [21]. Among the three inducers employed, KC-500 exhibited the highest induction effect, increasing the content of cytochrome P-450 (in the form of cytochrome P-448) about 2.5-fold. This latter inducer also caused significant increases in the contents of cytochrome b_5 and NADPH–cytochrome *c* reductase. Furthermore, 3-MC caused only a 1.5-fold induction of aminopyrine *N*-demethylase whereas both PB and KC-500 caused 4-fold induction of the *N*-demethylase. PB had a slight effect on the activity of benzo(a)pyrene hydroxylase, but KC-500 treatment resulted in a significant enhancement of the hydroxylase activity, though to a lesser extent than that observed in rats treated with 3-MC.

In mice, on the other hand, PB was most effective in increasing the content of cytochrome P-450 in liver

Table 1. Effects of pretreatment with various inducers on liver microsomal contents of cytochrome b_5 and P-450, and activities of NADPH–cytochrome *c* reductase, aminopyrine *N*-demethylase and benzo(a)pyrene hydroxylase in rats*

	Cytochrome b_5 (nmoles/mg protein)	Cytochrome P-450 (nmoles/mg protein)	NADPH cytochrome *c* reductase (nmoles/mg protein/min)	Aminopyrine *N*-demethylase (nmoles HCHO/mg protein/min)	Benzo(a)pyrene hydroxylase [nmoles 3-hydroxybenzo(a)pyrene/mg protein/60 min]
Control	0.365 ± 0.014	0.593 ± 0.032	32.1 ± 1.8	1.04 ± 0.02	5.22 ± 0.42
PB	0.363 ± 0.036	0.963 ± 0.005†	49.5 ± 7.0†	4.68 ± 0.45†	5.58 ± 0.48
3-MC	0.460 ± 0.017†	1.221 ± 0.005†	39.3 ± 6.5	1.86 ± 0.08†	20.34 ± 0.18†
KC-500	0.440 ± 0.032†	1.504 ± 0.191†	46.3 ± 2.7†	4.67 ± 0.41†	10.08 ± 1.32†

* Pretreatment of rats with various inducers and analysis of microsomal components were performed as described in Materials and Methods. Each value represents the mean for six rats and S. D.

† Significantly different from corresponding controls, $P < 0.05$.

Table 2. Effects of pretreatment with various inducers on liver microsomal contents of cytochrome b_5 and P-450, and activities of NADPH–cytochrome *c* reductase, aminopyrine *N*-demethylase and benzo(a)pyrene hydroxylase in mice*

	Cytochrome b_5 (nmoles/ mg protein)	Cytochrome P-450 (nmoles/ mg protein)	NADPH cytochrome *c* reductase (nmoles/ mg protein/ min)	Aminopyrine *N*-demethylase (nmoles HCHO/ mg protein/ min)	Benzo(a)pyrene hydroxylase [nmoles 3-hydroxybenzo(a)pyrene/ mg protein/ 60 min]
Control	0.414 ± 0.058	0.741 ± 0.012	30.4 ± 0.6	2.24 ± 0.47	3.06 ± 0.54
PB	0.388 ± 0.009	1.415 ± 0.194†	37.6 ± 1.7†	9.66 ± 0.61†	4.98 ± 1.08†
3-MC	0.432 ± 0.016	0.900 ± 0.086†	31.9 ± 2.2	3.43 ± 0.66†	21.6 ± 5.34†
KC-500	0.411 ± 0.032	1.068 ± 0.117†	35.5 ± 5.6†	3.94 ± 0.81†	3.24 ± 0.72

* Pretreatment of mice with various inducers and analysis of microsomal components were performed as described in Materials and Methods. Each value represents the mean for six mice and S. D.

† Significantly different from corresponding controls, $P < 0.05$.

microsomes (about 2-fold increase), as shown in Table 2. In contrast to the case of rats, however, the increase in the content of cytochrome P-450 (in the form of cytochrome P-448) by KC-500 treatment was only about 1.4-fold. 3-MC was a poorer inducer in this respect. Both KC-500 and PB treatments, but not 3-MC treatment, caused moderate increases in NADPH–cytochrome *c* reductase activity, whereas no elevation of the cytochrome b_5 level was observed after all three treatments. Moreover, PB caused a 4-fold induction of aminopyrine *N*-demethylase activity, whereas 3-MC or KC-500 caused only a 1.5-fold induction of the *N*-demethylase. Among the three inducers employed, only 3-MC treatment resulted in significant enhancement of benzo(a)pyrene hydroxylase activity in mice.

Effect of induction in rabbits. Table 3 summarizes the effects of pretreatment of rabbits with PB, 3-MC and KC-500 on the composition and PCB-binding activity of their microsomes. As in rats and mice, PB caused marked enhancements of cytochrome P-450 content, aminopyrine *N*-demethylase activity, and the amount of bound PCB metabolites, but had a weak effect on benzo(a)pyrene hydroxylase activity. 3-MC treatment of rabbits, on the other hand, increased the liver microsomal content of cytochrome P-448 and the activity of benzo(a)pyrene hydroxylase, but it decreased slightly the PCB-binding activity, in contrast to the cases of rats and mice. Furthermore, while the KC-500-mediated increases in the PCB-binding and benzo(a)pyrene hydroxylase activities of liver microsomes in rabbits were almost to the same extent as in mice, aminopyrine *N*-demethylase activity was not increased by this treatment in rabbits.

Requirements for and inhibition of PCB binding. As reported briefly in a previous paper [10], the binding of metabolites of both [^{14}C]KC-300 and [^{14}C]KC-500 to liver microsomes from KC-500-pretreated rats showed almost absolute dependence on both molecular oxygen and $NADP^+$, which was added as a component of the NADPH-generating system (Table 4). This finding suggested that the activity of the cytochrome P-450-linked mono-oxygenase system was required for the binding process. In fact, the reaction was inhibited strongly when the atmosphere was replaced by CO–O_2 (8:2, v/v), whereas 1 mM KCN stimulated the binding slightly. It was further found that both glutathione and cysteine, at a concentration of 1 mM, caused 33–74 per cent inhibition of the binding reaction. At a concentration of 1 mM aniline, a type II substrate of the mono-oxygenase system, and hexobarbital, a type I substrate, were both strongly inhibitory. As shown in Table 5, the PCB-binding capacity of liver microsomes from PB-treated mice also required both NADPH and molecular oxygen and was sensitive to CO. It was also inhibited by glutathione, cysteine, aniline and hexobarbital in similar ways to the case of KC-500-induced rat liver microsomes.

NADH synergism in PCB binding. Since Cohen and Estabrook [22–24], Hildebrandt and Estabrook [25], and Correia and Mannering [26, 27] have shown that NADH possessed a synergistic action on NADPH-dependent oxidation of various drugs catalyzed by liver microsomes, it was of interest to study whether a similar NADH synergism can be observed in the PCB-binding reaction. As shown in Fig. 5, NADH was a much poorer electron donor than NADPH in

Table 3. Effects of pretreatment with various inducers on liver microsomal content of cytochrome P-450, activities of aminopyrine *N*-demethylase and benzo(a)pyrene hydroxylase, and amount of metabolites of [^{14}C]PCBs covalently bound to microsomal macromolecules in rabbits*

	Cytochrome P-450 (nmoles/ mg protein)	Aminopyrine *N*-demethylase (nmoles HCHO/ mg protein/ min)	Benzo(a)pyrene hydroxylase [nmoles 3-hydroxybenzo(a)pyrene/ mg protein/ 60 min]	Amount covalently bound (dis./min/mg protein/60 min)	
				With [^{14}C]KC-300	With [^{14}C]KC-500
Control	0.74	1.32	1.38	346	42
PB	2.02	3.53	1.74	3063	331
3-MC	2.71	1.14	6.27	133	28
KC-500	2.21	1.26	3.42	837	142

* Pretreatment of rabbits with various inducers and analysis of microsomal components were performed as described in Materials and Methods. Each value represents the mean for two rabbits.

Table 4. Inhibition of PCB binding in liver microsomes from KC-500-pretreated rats*

	Amount covalently bound (dis./min/mg protein/60 min)			
	With [^{14}C]KC-300	Per cent inhibition	With [^{14}C]KC-500	Per cent inhibition
Basic system	3058 ± 165		701 ± 47	
Under nitrogen	166 ± 46	95	23 ± 10	97
Under CO:O_2 (8:2)	1049 ± 100	66	74 ± 10	89
NADP$^+$ omitted	32 ± 5	99	51 ± 11	93
Glutathione (1 mM) added	1092 ± 13	74	265 ± 17	62
Cysteine (1 mM) added	1590 ± 65	48	473 ± 63	33
Aniline (1 mM) added	320 ± 10	90	85 ± 25	88
Hexobarbital (1 mM) added	1705 ± 221	44	349 ± 21	50
KCN (1 mM) added	4146 ± 138	(+38)	1043 ± 98	(+49)

* The basic system (1 ml) contained rat liver microsomes (1 mg protein/ml); phosphate buffer, pH 7.4 (100 mM); [^{14}C]KC-300 (10.4 μg) or [^{14}C]KC-500 (13.0 μg); and the NADPH-generating system. Reactions were started by the addition of substrate in 20 μl acetone and incubated under air for 60 min with shaking at 37°. Each value represents the mean of three experiments and S. D.

supporting the binding of metabolites of [^{14}C]KC-300 to both PB-induced rat and mouse liver microsomes. With 0.5 mM NADH as donor, for example, the rat and mouse microsomes bound only 7 and 9 per cent, respectively, of the PCB metabolites that could be bound in the presence of 0.5 mM. NADPH. As can be clearly seen in Fig. 5, however, the addition of NADH to the NADPH-containing reaction mixture resulted in the binding of PCB metabolites to an extent which was much higher than the sum of the activities attainable on separate addition of both nucleotides. The synergistic increase obtained at a concentration of the nucleotides of 0.5 mM was 2.8- and 2.3-fold for the rat and mouse liver microsomes respectively.

Relationship between PCB binding and lipid peroxidation. As reported previously [28, 29], unsaturated fatty acids of liver microsomal phospholipids readily undergo peroxidation in the presence of NADPH and oxygen, resulting in degradation of cytochrome P-450 heme and a lowered activity of drug oxidation reaction. Kamataki and Kitagawa [30] showed that the addition of EDTA to the incubation mixture inhibited the lipid peroxidation and thus stimulated the *N*-demethylations of ethylmorphine and aminopyrine. We examined the relationship between the PCB binding and lipid peroxide formation in PB-induced rat liver microsomes to further clarify the role of the cytochrome P-450 mono-oxygenase system in the binding reaction. As shown in Fig. 6, the addition of EDTA (10 μM) to the incubation mixture resulted in a marked decrease in lipid peroxide formation and in a significant enhancement in the covalent binding of radioactive metabolites of [^{14}C]KC-300 to macromolecules. Furthermore, when the concentration of EDTA added to the reaction mixture was increased from 0 to 10 μM, the PCB binding was increased in proportional to the decrease in lipid peroxidation.

PCB binding to kidney and lung microsomes. Table 6 compares the binding of metabolites of [^{14}C]KC-300 to liver, kidney and lung microsomes prepared from control rats and those pretreated with PB, 3-MC and KC-500. It is seen that kidney and lung microsomes from control rats were practically devoid of the PCB-binding activity, whereas a significant amount of PCB metabolites was bound by control liver microsomes. Pretreatment of rats with 3-MC and KC-500, however, caused induction of significant binding activities in kidney microsomes but not in lung microsomes, though the induced level of activity in kidney microsomes was much less than that induced in liver microsomes. PB treatment, on the other hand, showed no effect on the PCB-binding activities of kidney and lung microsomes, although it was very effective in inducing the activity in liver microsomes.

As is also shown in Fig. 6, PB had no effect on the benzo(a)pyrene hydroxylase activities of kidney and lung microsomes, whereas 3-MC treatment resulted in significant enhancement of the hydroxylase activity of both microsomes. Moreover, although the KC-500-mediated increase of benzo(a)pyrene hydroxylase activity in lung microsomes was less than

Table 5. Inhibition of PCB binding in liver microsomes from PB-pretreated mice*

	Amount covalently bound (dis./min/mg protein/60 min)			
	With [^{14}C]KC-300	Per cent inhibition	With [^{14}C]KC-500	Per cent inhibition
Basic system	2425 ± 48		237 ± 7	
Under nitrogen	38 ± 10	98	7 ± 2	97
Under CO:O_2 (8:2)	1055 ± 166	57	109 ± 25	55
NADPH omitted	5 ± 1	99.8	7 ± 2	97
Glutathione (1 mM) added	1061 ± 42	56	128 ± 10	46
Cysteine (1 mM) added	1566 ± 17	35	191 ± 31	20
Aniline (1 mM) added	182 ± 17	93	28 ± 7	88
Hexobarbital (1 mM) added	1330 ± 35	45	125 ± 22	47

* The basic system (1 ml) contained mouse liver microsomes (1 mg protein/ml); phosphate buffer, pH 7.4 (100 mM); [^{14}C]KC-300 (10.4 μg) or [^{14}C]KC-500 (13.0 μg); and the NADPH-generating system. Reactions were started by the addition of substrate in 20 μl acetone and incubated under air for 60 min with shaking at 37°. Each value represents the mean of three experiments and S. D.

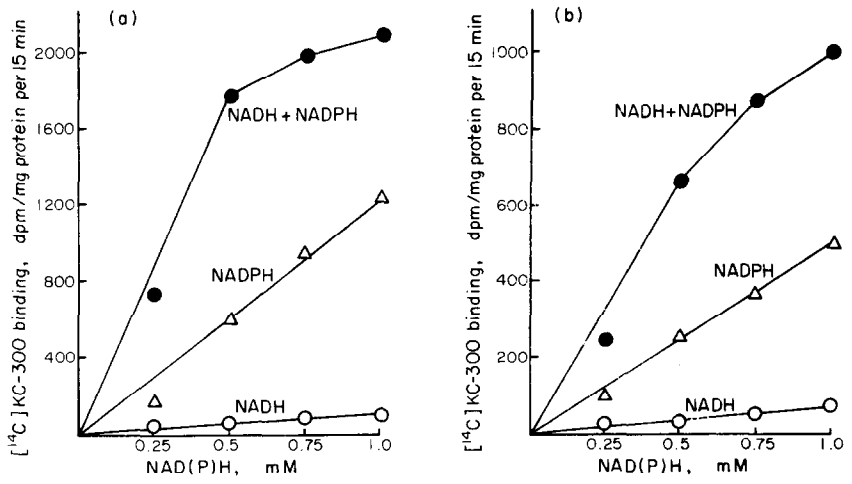

Fig. 5. NADH synergism in PCB binding. Liver microsomes from PB-pretreated rats (a) and mice (b) were used. The reaction mixture (1 ml) consisted of microsomes (1 mg protein/ml); phosphate buffer, pH 7.4 (100 mM); NADH and/or NADPH (0–1 mM) and [^{14}C]KC-300 (10.4 μg). Points represent the mean for two experiments.

that observed with 3-MC, KC-500 had a higher induction effect in kidney microsomes than 3-MC. It was thus suggested that the increase in PCB-binding capacity in kidney microsomes was somehow related to that in the benzo(a)pyrene hydroxylase activity, but no relationship was seen between the two enzyme activities in lung microsomes.

DISCUSSION

The results presented above support the view that the cytochrome P-450-linked monooxygenase system is involved in the covalent binding of PCBs to liver microsomal macromolecules. The evidence for this conclusion can be summarized as follows: (a) the binding process requires both NADPH and oxygen and is inhibited by CO and by substrates of the mono-oxygenase system, (b) NADPH is a much better electron donor for the binding than NADH, but NADH exerts a synergistic effect on the NADPH-dependent binding, as observed for drug oxidations by the mono-oxygenase system [22–27], (c) EDTA, which stimulates drug oxidations by preventing the lipid peroxidation-induced destruction of cytochrome P-450 [29, 30], also enhances the binding activity and (d) pretreatment of animals with inducers of the mono-oxygenase system causes elevation of the PCB-binding capacity of liver microsomes, though the extent of induction is variable depending on the species of animals and the kind of inducer used.

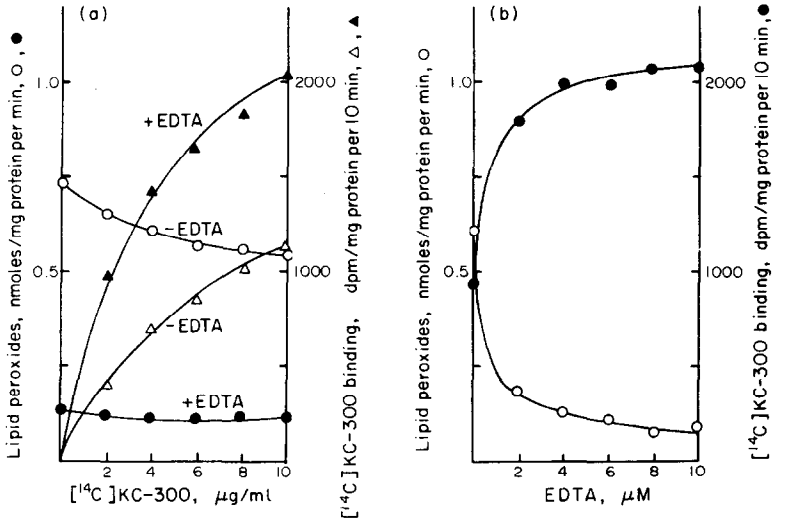

Fig. 6. Effects of EDTA on [^{14}C]KC-300 binding and lipid peroxidation in liver microsomes from PB-pretreated rats. The reaction mixture (1 ml) consisted of liver microsomes (1 mg protein/ml); phosphate buffer, pH 7.4 (100 mM); [^{14}C]KC-300 [(a) 0 to 10.4 μg; (b) 10.4 μg]; the NADPH-generating system; and EDTA [(a) 0 or 10 μM; (b) 0–10 μM]. Reactions were started by the addition of substrate under air for 10 min and were terminated by adding 2 ml of 10% trichloroacetic acid. Lipid peroxides in the resultant supernatant (0.5 ml) of trichloroacetic acid extract were measured colorimetrically with the thiobarbituric acid reaction [18], and the amount of metabolites of [^{14}C]KC-300 covalently bound to microsomal macromolecules was determined as described in Materials and Methods.

Table 6. [^{14}C]KC-300 binding in liver, kidney and lung microsomes, and activity of benzo(a)pyrene hydroxylase in kidney and lung microsomes from rats pretreated with PB, 3-MC, and KC-500*

	Amount covalently bound (dis/min/mg protein/60 min)			Benzo(a)pyrene hydroxylase [nmoles 3-hydroxy-benzo(a)pyrene/mg protein/60 min]	
	Liver	Kidney	Lung	Kidney	Lung
Control	324 ± 57	3 ± 3	4 ± 1	0.28 ± 0.04	0.08 ± 0.01
PB	2786 ± 312†	2 ± 2	5 ± 2	0.28 ± 0.05	0.08 ± 0.02
3-MC	1370 ± 197†	37 ± 5†	8 ± 4	3.79 ± 0.38†	2.32 ± 0.59†
KC-500	3560 ± 296†	114 ± 25†	6 ± 2	4.94 ± 0.41†	0.40 ± 0.04†

* Pretreatment of rats with various inducers and analysis of microsomal enzyme activities were performed as described in Materials and Methods. Each value represents the mean for three rats and S. D.

† Significantly different from corresponding controls, $P < 0.05$.

It is likely that the mono-oxygenase system converts PCBs to chemically activated products which subsequently bind to microsomal macromolecules, though the nature of the products is not yet known. It is tempting to assume that PCB epoxides are the activated forms, since halogenated benzenes, polycyclic hydrocarbons, etc. have been shown to be converted to their epoxides before undergoing covalent binding to macromolecules [9, 31, 32]. The formation of PCB epoxides seems especially likely because a dihydrodiol, the hydrolysis product of an arene oxide, of a tetrachlorobiphenyl has been isolated from the urine of animals treated with the tetrachlorobiphenyl [33]. This possibility is also supported by the finding that KC-300, a less chlorinated biphenyl mixture, is a much better substrate for the binding than KC-500, which is a highly chlorinated mixture. Since epoxide formation requires the presence of adjacent unchlorinated carbon atoms, it is expected that KC-300 can form more epoxides than KC-500. The inhibition of PCB binding by glutathione and cysteine may also be due to inactivation of the epoxides by the thiol compounds.

Recent work has revealed that liver microsomes contain multiple species of cytochrome P-450, and treatment of animals with an inducer results in selective induction of one or more species of the cytochrome. It has been shown that cytochrome P-450 purified from PB-induced liver microsomes differs in molecular and catalytic properties from the cytochrome (called cytochrome P-448) obtained from 3-MC-induced microsomes [20, 34–36]; while the former is much more active in aminopyrine *N*-demethylation than the latter, the reverse is the case for benzo(a)pyrene hydroxylation [19, 21]. There is also evidence that PCB treatment of rats induces both types of the cytochrome simultaneously [37, 38]. It is, therefore, interesting to know which of the multiple forms of cytochrome P-450 is functional in the PCB-binding process.

A survey of the induction data obtained in this study shows that the PCB-binding activity of liver microsomes is always induced in parallel to the activity of aminopyrine demethylase, but not that of benzo(a)pyrene hydroxylase, activity in all three species of animals examined. It may, therefore, be concluded that a P-450 cytochrome or cytochromes having high aminopyrine demethylase activity are principally, if not solely, responsible for metabolic activation of PCBs for the binding reaction. It is also likely that, in all the animal species, this type of cytochrome or cytochromes is efficiently induced by PB treatment.

In rats, however, KC-500 can also induce a very high PCB-binding activity. It is likely that KC-500 can induce two types of cytochrome P-450 simultaneously, one with a high aminopyrine demethylase activity and the other with a high benzo(a)pyrene hydroxylase activity. In fact, treatment of rats with KC-500 leads to concomitant induction of the demethylase, hydroxylase, and NADPH–cytochrome *c* reductase activities, as reported previously [37, 38]. In contrast to the case of rats, the induction effect of KC-500 on PCB binding and aminopyrine demethylation in mouse liver microsomes is very low and this inducer causes no increase in benzo(a)pyrene hydroxylase activity. Since 3-MC can induce a high benzo(a)pyrene hydroxylase activity in the mouse strain used, this lack of response to KC-500 does not imply that the mouse strain is aromatic hydrocarbon nonresponsive [39]. Like mice, rabbits also respond to KC-500 only weakly with respect to the induction of PCB-binding and aminopyrine demethylase activities. Moreover, 3-MC treatment of rabbits decreases slightly, rather than increases, the PCB-binding capacity of liver microsomes. This finding may be related to a recent report by Kawalek *et al.* [40] that cytochrome P-448 from 3-MC-induced rabbit liver microsomes differs in molecular weight and immunological reactivity from rat liver cytochrome P-448. Rabbit cytochrome P-448 has also been reported to have lower hydroxylation activities for several substrates than the rat counterpart [41].

As discussed above, the effect of KC-500 treatment on liver microsomal drug-metabolizing activities is considerably variable depending on the species of animals. Although KC-500 seems to induce both aminopyrine-demethylating and benzo(a)pyrene-hydroxylating cytochromes P-450 (or P-448) simultaneously in rat liver microsomes, the situation appears to be different in mice and rabbits, in which KC-500 does not induce the demethylating cytochrome. It may induce a new type of cytochrome P-450 in these animals.

The KC-500 preparation used in this study has been reported to contain a trace amount (4 ppm) of polychlorinated dibenzofurans [42]. However, it is very unlikely that this impurity has anything to do with induction because the amount of this impurity in a daily dose of KC-500 is only 0.4 μg/kg of body weight.

PB treatment of animals has been reported to cause only slight or no induction of drug-metabolizing activities in microsomes of extrahepatic tissues, whereas 3-MC can induce benzo(a)pyrene (aryl hydrocarbon) hydroxylase activity in various tissues including kidney and lung [43]. It has been confirmed in this study that 3-MC, but not PB, strongly induces benzo(a)pyrene hydroxylase activity in rat kidney and lung microsomes. KC-500 also induces a strong benzo(a)pyrene hydroxylase activity in kidney microsomes, though only very weakly in lung microsomes. An unexpected finding is that in kidney microsomes, but not in lung microsomes, both 3-MC and KC-500 cause significant induction of PCB-binding capacity concomitant with increases in benzo(a)pyrene hydroxylase activity. This is in sharp contrast to the case of liver microsomes, where the increase in the PCB-binding activity occurs in parallel to aminopyrine demethylase, but not benzo(a) pyrene hydroxylase, activity. It is likely that the nature and inducibility of cytochrome P-450 hemoprotein in liver, kidney and lung microsomes are profoundly different from one another.

Finally, it should be mentioned that both KC-300 and KC-500 are complex mixtures of variously chlorinated biphenyls. It has been reported that metabolic behavior of PCBs is affected by the positions and number of chlorine substituents [44–46]. As already mentioned, covalent binding is more pronounced with PCBs having fewer chlorine substituents. It is also likely that the same factors affect the action of PCBs as an inducer. Therefore, the results obtained in this study should be viewed as representing the average behavior of many PCB isomers. In this respect, it is desirable to examine the induction effects and covalent binding activities of structurally defined PCBs.

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