Environmental Health Perspectives
Vol. 59, pp. 113-119, 1985

Inductive Effect on Hepatic Enzymes and Toxicity of Congeners of PCBs and PCDFs

by Hidetoshi Yoshimura,* Shin'ichi Yoshihara,*† Nobuyuki Koga,* Kiyoshi Nagata,* Ikuo Wada,* Jun Kuroki* and Yumiko Hokama*

The present paper describes a marked induction of liver microsomal cytochrome P-450 and cytosolic DT-diaphorase to cause possible disorder of steroid homeostasis and promotion of carcinogenicity of 4-nitroquinoline *N*-oxide (4-NQO) in rats by pretreatment with 3,4,5,3′,4′-pentachlorobiphenyl (PenCB) or 2,3,4,7,8-pentachlorodibenzofuran (PenCDF). The animals were sacrificed 5 days after the pretreatment.

These induction experiments showed that 7α-hydroxylation of both progesterone and testosterone in liver microsomes was selectively increased to a great extent, but hydroxylations at the 2α-, 6β- and 16α-positions were depressed, together with 5α-reduction. From the same microsomes, three of the strongly induced P-450 isozymes, i.e., high- and low-spin P-448s and P-452, were purified. The last isozyme was most responsible for 7α-hydroxylation of testosterone.

The pretreatment, also increased activity of DT-diaphorase and reduction of 4-NQO about 10-fold in liver 9000*g* supernatants. This reduction of 4-NQO was solely catalyzed by DT-diaphorase and the only product was 4-hydroxylaminoquinoline *N*-oxide, a proximate carcinogen, indicating that the pretreatment strongly increased production of a proximate carcinogen from 4-NQO. Such an enhancement of the metabolic activation of 4-NQO by the pretreatment was also observed to some extent in the lung and the skin. Persistency of PenCB and PenCDF in the liver of rats was also discussed.

Introduction

Polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) are well known to be major causal agents of Yusho and also widespread environmental contaminants that have a potent inductive effect on liver microsomal mixed-function oxidases (MFO) (*1*). In a series of studies on these polychlorinated aromatic hydrocarbons, we found that some congeners that were categorized as 3-methylcholanthrene (MC)-type, but not phenobarbital (PB)-type in terms of inductive effects, all exerted severely toxic effects in rats, including decrease of the body weight, atrophy of the thymus and spleen and lipid accumulation in the liver (*2,3*). In addition, pretreatment of rats with these congeners resulted in preferential induction of not only microsomal cytochrome P-448, but also cytosolic DT-diaphorase, and the toxic potencies were correlated well with the activities of benzo[a]pyrene 3-hydroxylase and DT-diaphorase induced (*2,3*). As the representatives for such PCBs and PCDFs possessing extremely high toxicity and strong ability for MC-type induction, 3,4,5,3′,4′-pentachlorobiphenyl (PenCB) and 2,3,4,7,8-pentachlorodibenzofuran (PenCDF) would be the best candidates.

Presently, the mechanism responsible for such a correlation has not been identified. However, this could be explained at least in part by the following two ways. The first is a direct toxic effect through a disturbance of certain intermediary metabolic paths that may be caused by the marked increase of above enzymes over a long period, because these enzymes have an important role in intermediary metabolism as well as in drug metabolism. For instance, the steroid metabolism might be disturbed by abnormal changes of the content ratios of multiple forms of cytochrome P-450 that were induced by pretreatment with PenCB and PenCDF. In Yusho patients, quite a high incidence of menstrual cycle irregularities, dysmenorrhea and altered serum levels of ketosteroids occurred (*4*). A second mechanism for the action of PenCB and PenCDF is the indirect toxic effect through the enchanced metabolic activation of certain xenobiotics, which is catalyzed by particular forms of cytochrome P-450. We recently reported that

*Faculty of Pharmaceutical Sciences, Kyushu University, Fukuoka 812, Japan.
†Present address: Institute of Pharmaceutical Sciences, Hiroshima University School of Medicine, Hiroshima 734, Japan.

PenCB produced a marked increase in both cytochrome P-450 (448) content and N^4-hydroxylation of sulfanilamide in the kidneys of rats which should stimulate toxic effects of sulfanilamide to the kidneys (*5*). It should be noted in this connection that DT-diaphorase, an enzyme inducible with MC-type PCBs (*2*), is known to catalyze not only the reduction of various quinones (*6*), but also the reduction of the famous precarcinogen, 4-nitroquinoline *N*-oxide (4-NQO) to a proximate carcinogen, 4-hydroxylaminoquinoline *N*-oxide (4-HAQO) (*7*).

The present paper describes the marked induction of liver microsomal cytochrome P-450 (448) and cytosolic DT-diaphorase to cause, respectively, possible disturbance of steroid homeostasis and promotion of carcinogenicity of 4-NQO in rats pretreated with PenCB and PenCDF. For studies on steroid metabolism, progesterone and testosterone were selected as representative substrates.

Materials and Methods

Chemicals

Kanechlor (KC)-400, a Japanese PCB preparation with 48% chlorine content, was supplied by the Ministry of Health and Welfare of Japan. PenCB was synthesized as reported previously (*8*). 2,4,5,2′,4′,5′-Hexachlorobiphenyl (HCB) was purchased from Analab, Inc. (North Haven, CT). The PCDF congeners were generously donated by Dr. Y Masuda, Daiichi College of Pharmaceutical Sciences (Fukuoka). 4-^{14}C-Progesterone and -testosterone (specific radioactivity, 59 mCi/mmole) were purchased from New England Nuclear Corp. (Boston, MA). Unlabeled steroids and metabolites were obtained from Nakarai Chemicals, Ltd. (Tokyo) and Steraloids, Inc. (Wilton, NH), except for 2α-, 6β-, 7α- and 16α-hydroxytestosterone, which were kindly donated by Dr. Y. Nakamura, Shionogi & Co., Ltd. (Osaka). 4-NQO, 4-HAQO and 4-aminoquinoline *N*-oxide (4-AQO) were kindly given by Dr. S. Saeki, Faculty of Pharmaceutical Sciences, Kyushu University (Fukuoka). All other chemicals used were of the highest quality commercially available.

Animal Treatment

All PCBs and PCDFs were dissolved in corn oil and injected IP into male Wistar rats weighing about 100 g. PB in saline and MC in corn oil were injected IP once a day for three consecutive days. Control animals were injected with vehicle alone (2 mL/kg). Following fasting for 18 hr, the animals were killed by decapitation 5 days after the injection of PCBs and PCDFs and 24 hr after the last injection of PB and MC. Day 5 is the time in which optimal induction of the MFO has been observed after pretreatment with PCBs (*9*).

Enzyme Preparation

The homogenate prepared with three volumes of 0.25 M sucrose containing 0.1 mM EDTA and 10 mM Tris-HCl (pH 7.4) was centrifuged at 9000*g* for 20 min. The resultant 9000*g* supernatant was centrifuged at 105,000*g* for 60 min. The pellet washed once with isotonic KCl was resuspended in Tris–sucrose buffer and used for steroid metabolism studies. For 4-NQO reduction, 9000*g* supernatants of liver, lung and skin which were prepared similarly as above from the corresponding 20% homogenate were used as the enzyme sources. The skin sample was 2 cm^2 of shaved skin from the abdomen.

Enzyme Assays

Steroid Metabolism. The incubation mixture consisted of microsomes (about 3 mg of protein), 200 μg of ^{14}C-steroid (0.4 μCi), 3.6 mM $MgCl_2$, 0.5 mM NADP, 6 mM glucose-6-phosphate (G-6-P), 5 units of G-6-P dehydrogenase and 50 mM Tris-HCl (pH 7.4) to make a final volume of 5 mL. Incubation was usually carried out at 37°C for 10 min following a preincubation of 1 min without an NADPH-generating system. The metabolites extracted with ethyl acetate were redissolved in small amounts of methanol and filtered through a membrane filter of 0.45 μm pore size. An aliquot (50–100 μL) of the filtrate (0.2 mL) was applied to a HPLC (Waters Assoc., Inc., Milford, MA, Model ALC 202/401) equipped with 6000 A pump, a solvent programmer type 660 (Waters Assoc.) and a radioactivity monitor, BL 503 (Berthold, Wildbad, Germany). Samples were chromatographed on a Radial-PAK A column (Waters Assoc., reversed phase) by stepwise elution with 30% and 45%, or 25% and 35% tetrahydrofuran in water (v/v), respectively, for progesterone or testosterone metabolites at a flow rate of 3 mL/min. The metabolites were identified by their retention volumes on HPLC relative to reference standards or by comparison of their R_f values on TLC and GC/MS fragmentation patterns with the reported data. A quantitative determination of the metabolites was carried out by measuring absorbance at 254 nm or radioactivity (Packard, Model 3375 liquid scintillation counter) of the corresponding peaks on HPLC.

4NQO Reduction. The DT-diaphorase activity was determined according to the method of Ernster et al. (*6*) by measuring the initial rate of decrease of absorbance at 600 nm in the incubation of 0.08 mM DCPIP, 0.15 mM NADPH, 0.33 mM KCN and 50 mM Tris-HCl buffer (pH 7.4) with 2 to 10 μL of 9000*g* supernatant of the tissue homogenate at 25°C. Activity of 4-NQO reduction was also determined according to the methold of Sugimura et al. (*10*) by measuring the initial rate of decrease of absorbance at 340 nm in the incubation of 0.8 mM 4-NQO, 0.15 mM NADPH and 50 mM phosphate buffer (pH 7.0) with 2 to 10 μL of 9000*g*

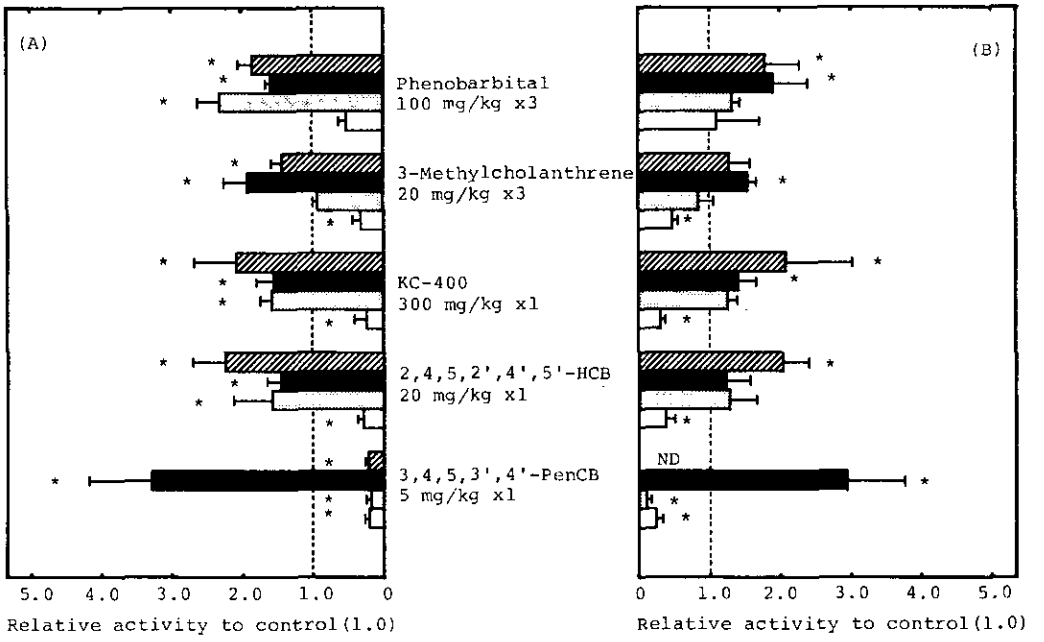

FIGURE 1. Effects of pretreatment with phenobarbital, 3-methylcholanthrene and PCBs on metabolism of (*A*) progesterone and (*B*) testosterone with rat liver microsomes. From Yoshihara et al. (*16*). Experimental conditions as described in Materials and Methods. The activity is expressed relative to that of the control group indicated below (the mean ± SD of four rats). ND = not detectable; the asterisk (*) denotes a value significantly different from the control, $p < 0.05$. Control activity of progesterone metabolism (A): (▨) 16α-OH, 6.93 ± 1.52 nmole/mg protein/ min; (■) 7α-OH, 3.34 ± 1.16 nmole/mg protein/min; (▩) 6β, 14.81 ± 1.03 nmole/mg protein/min. (□) 5α-, 21.13 ± 9.91 nmole/mg protein/min. Control activity of testosterone metabolism (B): (▨) 16α-OH, 8.06 ± 1.79 nmole/mg protein/ min; (■) 7α-OH, 6.70 ± 0.86 nmole/mg protein/min; (▩) 6β-OH, 19.12 ± 1.19 nmole/mg protein/min; (□) 5α, 17.77 ± 8.25 nmole/mg protein/min.

supernatant at 30°C. Dicumarol (0.1 mM) was used for the inhibition experiment. Protein was determined according to the method of Lowry et al., bovine serum albumin being used as a standard (*11*).

Purification of Cytochrome P-450 Isozymes

Liver microsomes of rats pretreated with PenCB at a single IP dose of 5 mg/kg were solubilized with cholic acid and partially purified by aminooctyl Sepharose 4B according to the method of Imai and Sato (*12*). The resultant fractions of cytochrome P-450 were further chromatographed through a DE-52 cellulose column by the method of Warner et al.(*13*) with some modifications, followed by hydroxyapatite and DEAE-Sephacel columns.

Extraction and Determination of PenCB in Rat Liver

Liver lipids extracted from homogenate with a solvent mixture of chloroform and methanol (2:1,v/v) according to the method of Folch et al. (*14*), were dissolved in *n*-hexane, and PenCB in this solution was determined by ECD-gas chromatography (column: 3 mm × 1.7 m, 1.5% OV-17 on Chromosorb W; carrier gas: N_2; column temperature: 190°C).

Results

Effects of PCBs and PCDFs on Steroid Metabolism by Liver Microsomes

In vitro metabolism of ^{14}C-progesterone and ^{14}C-testosterone was first conducted with liver microsomes of rats pretreated with various inducers such as PB, MC and PCBs (KC-400 as mixed type, HCB as PB-type and PenCB as MC-type), analyzing the metabolites by HPLC. The results as relative activities are shown in Figure 1.

Pretreatment with PB, hexachlorobiphenyl (HCB) and KC-400 increased the 16α-, 7α- and 6β-hydroxylations of progesterone 1.5- to 2-fold compared with the control. MC pretreatment also enhanced the 16α- and 7α- hydroxylations but did not affect the 6β-hydroxylation. A similar tendency was observed in the metabolism of testosterone. The most unique change of the metabolic profile of both steroids was demonstrated by pretreatment with the highly toxic PenCB. This PCB enhanced the 7α-hydroxylation more than 3-fold , but, unlike other inducers, the hydroxylations at 16α- and 6β-positions of both steroids were strongly depressed to less than 20% of the control. The 2α-hydroxylation of progesterone and formation of androstenedione from testosterone were also decreased only by PenCB pretreatment, but not by any other pretreatments (data not shown in this figure). In addition, apart from these

Table 1. Effects of pretreatment with 2,3,4,7,8-pentachlorodibenzofuran (PenCDF) on metabolism of progesterone with rat liver microsomes.[a]

Metabolite	Progesterone metabolized, nmole/mg protein/10 min. Control	PenCDF pretreatment (0.1 mg/kg)
16α-OH	6.93 ± 1.52	3.10 ± 0.83*
7α-OH	3.34 ± 1.16	8.60 ± 4.00*
6β-OH	14.81 ± 1.03	8.81 ± 2.90*
2α-OH	7.31 ± 4.01	0.93 ± 0.38*
5α-Pregnane-3,20-dione	21.13 ± 9.91	13.31 ± 5.15
Total metabolism	68.11 ± 8.23	37.84 ± 11.48*

[a]From Yoshihara et al. (16). Experimental conditions were described in Materials and Methods. The total metabolism was calculated from the amount of substrate disappearing. The value represents the mean ± SD of four rats.
*Significantly different from the control, $p<0.05$.

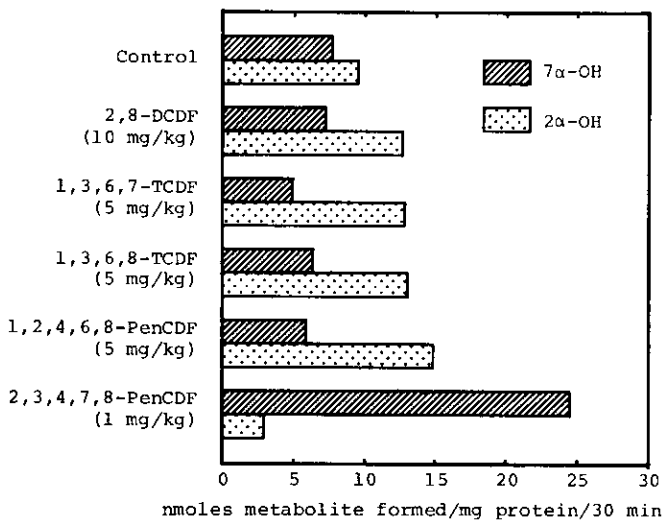

FIGURE 2. Effects of pretreatment with polychlorinated dibenzofuran (PCDF) congeners on 7α- and 2α-hydroxylation of progesterone with rat liver microsomes. From Yoshihara et al. (*16*). The microsomes were obtained from the pooled liver of four rats per group pretreated with respective PCDF congener at a single dose indicated.

hydroxylations, 5α-reduction of both steroids to 5α-pregnane-3,20-dione and to 5α-androstane-17β-ol-3-one, respectively, were decreased more markedly by PenCB than other inducers.

A very similar depressing effect on hydroxylations at the 16α-, 6β- and 2α-positions and 5α-reduction of progesterone were demonstrated by the microsomes obtained from rats pretreated with PenCDF at a single dose of 0.1 mg/kg (Table 1). The 7α-hydroxylation was selectively increased, but the summation of this unique effect of PenCDF resulted in a marked depression of total metabolism, same as the case of PenCB pretreatment.

Contrary to this highly toxic PenCDF, all other PCDFs that are nontoxic and non-MC-type inducers never produced such a dramatic change of steroid metabolism. As indicated in Figure 2, either 7α- or 2α-hydroxylation of progesterone was strongly affected only by pretreatment with PenCDF.

Table 2. Catalytic activity of liver cytochrome P-450 isozymes from rats pretreated with 3,4,5,3′,4′-pentachlorobiphenyl (PenCB).[a]

Hydroxylation	Catalytic activity, nmole product/nmole P-450/min: Low spin P-448	High spin P-448	P-452
Benzo[a]pyrene, 3-OH	2.12	0.08	0.08
Testosterone			
7α-OH	0.09	ND[b]	10.92
16α-OH	Trace	Trace	0.43
6β-OH	0.72	0.28	0.48
Estradiol, 2-OH	1.66	17.05	0.65

[a]The activity is expressed as nmoles product/nmole P-450/min. Benzo[a]pyrene 3-hydroxylase and steroid hydroxylases were assayed as described previously (*2*) and in Materials and Methods, respectively.
[b]ND = not detected.

Separation and Purification of Cytochrome P-450 Isozymes from Liver Microsomes of Rats Pretreated with PenCB

The very interesting phenomenon described above could be explained at least in part by a characteristic change of relative contents of cytochrome P-450 isozymes after pretreatment with the potent MC-type PenCB and PenCDF. In order to confirm this possibility, separation and purification of strongly induced cytochrome P-450 isozymes, some of which may preferentially be concerned with 7α-hydroxylation of the steroids, were undertaken.

SDS–polyacrylamide gel electrophoresis of liver microsomal protein from rats pretreated with PenCB showed a marked change of pattern in comparison with that of the control. Among various isozymes, the three major strongly induced cytochrome P-450 species were then purified by the method of Imai and Sato (*12*) and Warner et al. (*13*) with some modifications. The first one, the most highly induced cytochrome P-450, was a high-spin type showing the absorption maximum of the CO-difference spectrum at 448 nm (a high-spin P-448). Since this cytochrome showed a high activity of 2-hydroxylation of estradiol on reconstitution for MFO (see Table 2), it seemed very likely that this was identical with Levin's $P\text{-}450_d$(*15*), although the molecular weight in the SDS–gel system was different (our sample, 54,000; Levin's, 52,000).

The second cytochrome purified was a low-spin P-448 which had a molecular weight of about 56,000 in the SDS-gel system and was most concerned with 3-hydroxylation of benzo[a]pyrene. These two cytochrome P-448s, however, were found to play only a minor role in 7α-hydroxylation of testosterone (Table 2). The third isozyme that showed an absorption maximum of the CO-difference spectrum at 452 nm (P-452) and had a

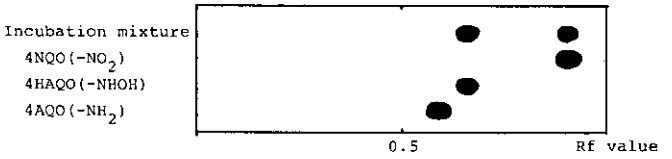

FIGURE 3. Thin-layer chromatogram of reduction product of 4-nitroquinoline *N*-oxide (4-NQO) with rat liver 9000*g* supernatant. From Yoshimura et al. (*17*). Solvent system: isoamyl alcohol–acetone–H_2O (4:2:1, v/v)

molecular weight of 48,000 in the SDS–gel system was found to catalyze 7α-hydroxylation of testosterone with a high activity of about 10 nmole/mmole P-450/min on reconstitution.

Identification of Reduction Product of 4-NQO by Rat Liver 9000*g* Supernatant

In order to confirm the previous result of Sugimura et al. (*7*), 4-NQO was incubated with liver 9000*g* supernatant from untreated rats in the presence of NADPH at 37°C for 1 min. As illustrated in Figure 3, the thin-layer chromatogram of the ethyl acetate extract clearly showed formation of 4-HAQO as sole reduction product. 4-AQO could not be detected at all under the conditions used. A larger spot corresponding to 4-HAQO was observed when microsomes from rats pretreated with PenCB were used as the enzyme source.

Enhancement of Activities of DT-Diaphorase and 4-NQO Reduction in Liver, Lung and Skin of Rats Pretreated with PenCB

Activities of DT-diaphorase (DCPIP reduction) and 4-NQO reduction were determined in the 9000*g* supernatant of liver, lung and skin of rats pretreated with PenCB at a single IP dose of 1.0 mg/kg. As shown in

Table 3. Activities of DT-diaphorase and 4-nitroquinoline *N*-oxide (4-NQO) reductase in 9000g supernatant of the liver, lung and skin of rats pretreated with 3,4,5,3′,4′-pentachlorobiphenyl (PenCB).[a]

Tissue	Treatment	DCPIP activity, μmole[b]	4NQO activity, μmole[c]
Liver	Control	0.652 ± 0.038	0.381 ± 0.026
	PenCB	6.830 ± 0.801	3.281 ± 0.513
Lung	Control	0.311 ± 0.052	0.162 ± 0.029
	PenCB	0.879 ± 0.087	0.457 ± 0.052
Skin	Control	0.060 ± 0.008	0.132 ± 0.016
	PenCB	0.309 ± 0.038	0.285 ± 0.037

[a]From Yoshimura et al. (*17*). The value represents the mean ± SD of four rats.
[b]Dichlorophenolindophenol (DCPIP) reductase μmole/mg protein/min.
[c]NADPH oxidized, μmole/mg protein/min.

Table 3, DT-diaphorase activity increased about 10- ,3- and 5-fold, respectively, in liver, lung and skin on PenCB pretreatment. Along with this induction, activities of 4-NQO reduction were also increased about 9-, 3- and 2-fold, respectively. It should be noticed that these activities were completely inhibited by addition of 0.1 mM dicumarol, a specific inhibitor of DT-diaphorase in the incubation mixture, indicating that the reduction of 4-NQO in this *in vitro* system was catalyzed solely by DT-diaphorase.

To confirm more precisely the parallel increase of the two activities, individual values of reduction activities of DCPIP and 4-NQO in the control and PenCB-treated rats were compared. As indicated in Figure 4, 4-NQO reduction activities correlated well with DCPIP reduction activities in all the three organ preparations, and the ratios of both reduction activities were almost constant among the three organs. The above findings suggest that the same enzyme (DT-diaphorase) that can be induced by PenCB treatment is concerned with reduction of 4-NQO to a proximate carcinogen, 4-HAQO in rats. These data were not shown in this paper, but a similar effect was also obtained from the experiments with PenCDF.

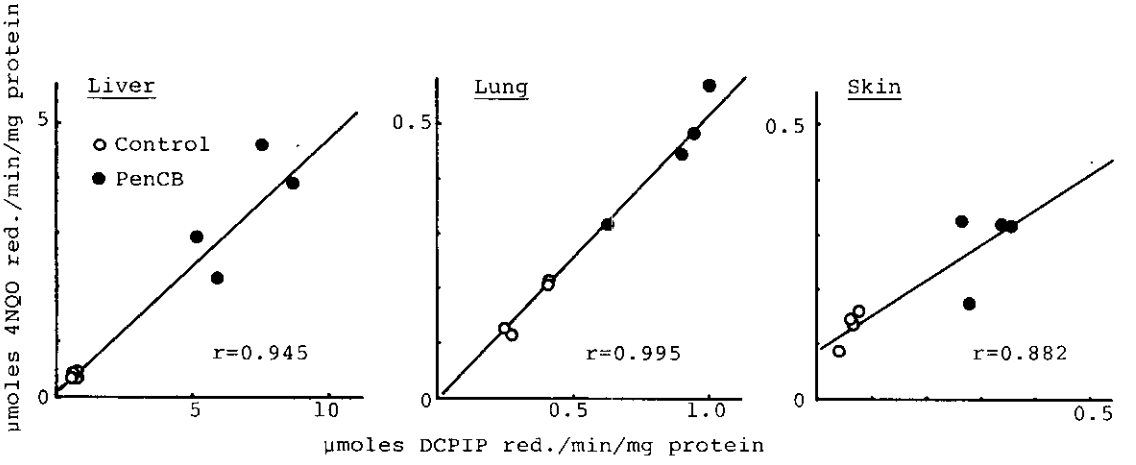

FIGURE 4. Correlation between activities of DT-diaphorase and 4-nitroquinoline *N*-oxide (4-NQO) reductase. From Yoshimura et al. (*17*).

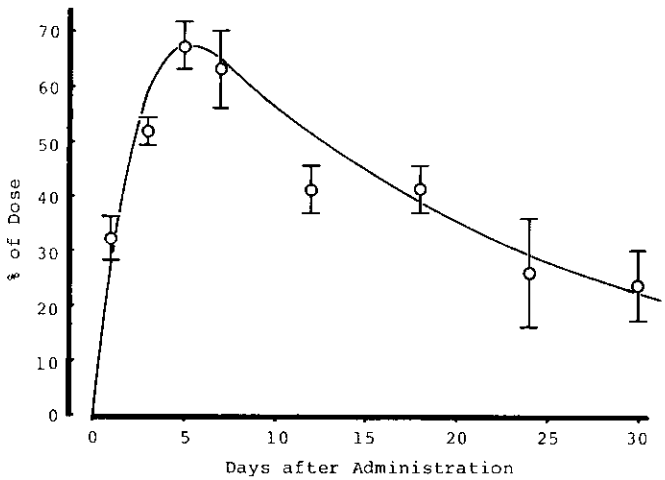

FIGURE 5. Time course of 3,4,5,3′,4′-pentachlorobiphenyl (PenCB) accumulation in the liver of rats given a single dose of 0.5 mg/kg.

Persistency of PenCB and PenCDF in Rat Liver

The induction effects of the highly toxic MC-type PenCB and PenCDF are very strong and long lasting. It is, therefore, important to learn the time course of the concentration of PenCB and PenCDF in the liver. The study was first performed by using the liver of rats orally administered PenCB at a single dose of 0.5 mg/kg. As illustrated in Figure 5, about 68% of the dose accumulated in the liver 5 days after the administration. This high level of accumulation persisted over a long period, and over 20% of the dose was still retained in the liver after 30 days. Such a surprisingly high affinity to the liver is also seen in the case of PenCDF even more markedly (data not shown), but not in any other PCBs including HCB, which is a typical PB-type inducer and does not have a high toxicity.

Discussion

PCBs and PCDFs are closely related families of compounds, similar not only in structure, but also in biological and toxicological nature (*1*). Among these, PenCB and PenCDF are most unique with respect to inductive effect and toxicity. Both compounds reveal a significantly potent MC-type induction and acute toxicity at a single dose as low as 0.1 mg/kg (PenCB) or 0.01 mg/kg (PenCDF) in rats (*3*). The present study has demonstrated further the characteristic properties of PenCB and PenCDF, using effects on steroid metabolism and on 4-NQO reduction as the biological and possible toxic indicators (*16,17*).

The remarkable effect of PenCB and PenCDF on the metabolism of progesterone and testosterone was an increase of 7α-hydroxylation and decrease of 16α-, 6β- and 2α-hydroxylations. Pretreatment with these inducers caused also depression of 5α-reduction which is a rate-limiting step in catabolism of Δ^4-steroids, resulting in a marked decrease of total metabolism of both steroids. Similar effect has been reported by pretreatment with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (*18,19*).

This unique change of steroid metabolism might be due to qualitative and quantitative changes of cytochrome P-450 isozymes. The present paper provided evidence to explain an increase of 7α-hydroxylation by purification of preferentially induced cytochrome P-452. Both PenCB and PenCDF have a very high persistency in the liver, and therefore, may afford a significant effect on steroid metabolism *in vivo* not only with a high potency but also over a long period, if animals are once exposed to appreciable amounts of these compounds. Based on these findings, it is very likely that a disturbance in steroid homeostasis caused by the change of relative contents of cytochrome P-450 isozymes is responsible for the endocrine symptoms seen in Yusho patients.

The marked increase of 4-NQO reduction activity is parallel with a potent induction of DT-diaphorase in several organs of rats by pretreatment with PenCB is also important with respect to possible promotion of carcinogenicity of 4-NQO. These experiments, however, were conducted only in an *in vitro* system. It is, therefore, impossible at present to conclude whether or not exposure to PenCB or PenCDF could really promote carcinogenicity of 4-NQO. *In vivo* studies on this line are progressing in this laboratory, and the results will be reported elsewhere in the near future.

This work was supported in part by a research grant provided by the Ministry of Health and Welfare of Japan to which the authors are greatly indebted. They also thank Drs. Masuda (Daiichi College of Pharmaceutical Sciences), Saeki (Faculty of Pharmaceutical Sciences, Kyushu University) and Nakamura (Shionogi & Co., Ltd.) for their generous gifts of valuable chemical samples.

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