Biochemical Pharmacology, Vol. 27, pp. 2595–2603.
Pergamon Press Ltd. 1978. Printed in Great Britain.

EFFECT OF TYPICAL INDUCERS OF MICROSOMAL DRUG-METABOLIZING ENZYMES ON PHOSPHOLIPID METABOLISM IN RAT LIVER*

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(*Received* 1 *September* 1977; *accepted* 3 *March* 1978)

Abstract—The effect of administration of phenobarbital (PB), polychlorinated biphenyls (PCBs) or 3-methylcholanthrene (3-MC) on the metabolism of phospholipids in rat liver was studied by the i.p. injection of [^{32}P]orthophosphate or [Me-^{14}C]choline chloride. The inducers were given to animals for 2 successive days. PB had no significant effect on the incorporation rate of ^{32}Pi into liver microsomal phospholipid classes 48 hr after the first administration. The rate of incorporation of [^{14}C]choline into phosphatidylcholine (PC) in both subcellular components of the liver and blood plasma decreased slightly at this experimental period. In addition, the ratio of [^{14}C] sp. act. of phosphorylcholine to that of microsomal phospholipid was considerably higher on PB-treated rats as compared with the ratio in control rats. These and previous findings strongly suggest that proliferation of liver endoplasmic reticulum (ER) membranes induced by PB would be accompanied by the stimulation of phospholipid synthesis at the early process of induction and subsequently followed by the decrease in turnover rate of microsomal phospholipids. The administration of PCBs, on the other hand, caused strong inhibition of both ^{32}Pi and ^{14}C incorporation into PC in liver subcellular fractions. The secretion of PC from liver cells to blood plasma was also strongly depressed. In addition, phospholipid catabolizing activity was found to be depressed in the liver. These results indicate that hypertrophy of ER membranes in the liver after PCBs administration could be due to a depression of both secretion of lipoprotein from liver cells to plasma and catabolic activity toward membranous phospholipids in liver cells. The decrease in ^{32}Pi incorporation into liver microsomal PC was also observed in rats treated with 3-MC. There occurred a considerable accumulation of ^{14}C activity in phosphorylcholine in the liver of rats treated with either PCBs or 3-MC, suggesting strongly that these drugs caused an inhibition of PC synthesis at the site of CDP-choline formation, namely the inhibition of the reaction catalyzed by cholinephosphate cytidylyltransferase.

Many kinds of lipophilic substances, varying in their chemical structure and biological effect, are known to cause a considerable increase *in vivo* in the rate of biotransformation of foreign compounds by the induction of drug-metabolizing enzyme system in liver ER. In this diversity of inducers, two main groups are discernible differing in principle as to their manifestations [1]. One group of inducers, to which PB belongs, enhances the metabolism of a large varicty of substances and at the same time causes the remarkable proliferation of ER membranes in the liver; another group stimulates the metabolism of only a few substrates without any apparent increases in ER membrane components. Carcinogens having plycyclic hydrocarbon structure such as 3-MC and benzo(a)pyrene are comprised in the latter group. In addition, the effect of these two groups of inducers on the terminal oxidase in the drug-metabolizing enzyme system are recognized to differ from each other. Although both PB and 3-MC increase the concentration of this hemoprotein in liver microsomes, 3-MC induces the formation of cytochrome P448, exhibiting spectral and catalytic activities differing from those of cytochrome P450 induced with PB.

It is very interesting that polychlorinated aromatic hydrocarbons such as Aroclor 1254 (a heterogeneous group of chlorinated biphenyls containing an average of 54% chlorine) and hexachlorobenzene have been recently proposed as representatives of a new class of inducers which share the properties of both PB and polycyclic hydrocarbon groups, causing the formation of cytochrome P448, while eliciting the more general enzyme induction response [2–4].

On the other hand, the important role of membrane phospholipid, especially PC, playing in the microsomal drug-metabolizing system has been demonstrated by the finding that the preparation of solubilized components cannot function in a reconstituted system unless phospholipid is added [5, 6]. The fact that treatment of liver microsomes with phospholipases [7, 8] or with organic solvents [9, 10] causes a significant decrease of drug-metabolizing activity also indicates the essential role of phospholipid in this system.

Thus, the possible relations between difference of manifestations by each group of inducers and requirement of membrane phospholipid for the activity of drug biotransformation have led us to study the

* This work was supported in part by a scientific research grant No. 167277 from the Ministry of Education, Japan.

Abbreviations—PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; Sph, sphingomyelin; ER, endoplasmic reticulum; PB, phenobarbital; PCB, polychlorinated biphenyl; 3-MC, 3-methylcholanthrene.

effect of typical inducers on phospholipid metabolism in liver cells.

In the present study, the characteristic effects of the administration of PB, 3-MC or PCBs on the phospholipid metabolism were demonstrated in rat liver.

MATERIALS AND METHODS

Radioactive materials. [Me-^{14}C]choline (sp. act. 10 mCi/m-mole), [1-^{3}H]ethan-1-ol-2-amine (sp. act. 3.80 Ci/m-mole), phosphoryl [Me-^{14}C]choline (sp. act. 52 mCi/m-mole) and cytidine-5'-diphospho[Me-^{14}C]choline (sp. act. 50 mCi/m-mole) were purchased from Radiochemical Centre, Amersham, U.K. Carrier free $H_3[^{32}P]O_4$ was purchased from Japan Atomic Energy Research Institute.

Drug pretreatment of animals. Male Wister rats weighing 90–110 g were used for all experiments. Rats were housed two per cage, fed Clea CF-2 Laboratory Chow, and allowed free access to water. PCBs (Polychloro Biphenyl, Tetra, composed mainly of tetrachlorobiphenyl isomers, obtained from Wako Pure Chem. Ind. Co. Ltd., Japan) and PB were orally given to experimental animals at dosages of 100 mg/kg/day and 80 mg/kg/day, respectively, as previously described [11]. 3-MC dissolved in arachis oil, was i.p. administered at a dosage of 50 mg/kg/day. All inducers were given once a day in the morning for 2 days and control animals were given equivalent volumes of the resolvents. Other details were described in the previous paper [11].

Injection of labeled precursor and preparation of subcellular components. In the morning of day 3, radioactive precursor (200–250 μCi $H_3[^{32}P]O_4$ in 0.5 ml of 0.1 M potassium phosphate, pH 7.4 or 3.8 μCi [Me-^{14}C]choline chloride in 0.5 ml of saline) was i.p. injected into the control and drug-treated rats. At 1 hr after the injection of the precursor, the rats were killed by decapitation. Blood was collected into the test tube containing heparin sodium (100 units). The livers were removed, washed twice with ice-cold saline, and homogenzied in 4 vol. of isotonic KCl with a Potter homogenizer fitted with a Teflon pestle. The homogenate was then subjected to differential centrifugation in a Hitachi model 18PR-3 refrigerated centrifuge and Hitachi model 65P preparative ultracentrifuge. After eliminating cell debris and nuclei by centrifugation at 1,500 *g* for 10 min, mitochondrial fraction was sedimented from the supernatant by centrifugation twice at 15,000 *g* for 20 min, and the resultant supernatant was again centrifuged at 105,000 *g* for 60 min to yield microsomal and cytoplasmic fractions. The firmly packed pellet of microsomes was resuspended in isotonic KCl with the Potter homogenizer and again centrifuged as above to remove soluble cytoplasmic components.

Extraction and fractionation of subcellular phospholipids. Both mitochondrial and microsomal fractions were resuspended in small amount of isotonic KCl, followed by extraction of lipids with 17 vol. of chloroform–methanol (2:1, v/v) according to the method of Folch *et al.* [12]. Microsomal phospholipids were fractionated mainly by two-dimensional thin layer chromatography (t.l.c.) as previously described [11]. To the cytoplasmic fraction, trichloroacetic acid (TCA) was added to a final concentration of 5% and the acid-insoluble materials were then sedimented by centrifugation, followed by extraction of lipids with chloroform methanol as described above.

Analysis of water-soluble radioactivity. Water-soluble radioactive materials were analyzed by a slight modification of the method of Bjørnstad and Bremer [13]. Acid-soluble fraction of 105,000 *g* supernatant was washed three times with 15 ml of diethyl ether to remove most amount of TCA and brought to pH 8–9 with several drops of 1 M KOH, and was then passed through a column (1.5 cm × 30 cm) of Dowex 1 × 4 (200–400 mesh, formate form). Non-radioactive CDP-choline (1 μmole) was added to the fraction before chromatography as an indicator for absorption at 280 nm. The column was washed with water and then eluted with a linear gradient of 0–0.04 N formic acid in a total volume of 300 ml. Three peaks were obtained, containing 85–95 per cent of the applied radioactivity. Peak 1, not retained, contained free choline, betaine and other oxidative metabolites of choline, Peak 2 contained phosphorylcholine and Peak 3 CDP-choline (Fig. 1).

Peak 1 was evaporated to dryness and further chromatographed on Whatman No. 51 paper developed with 95% ethanol–concentrated NH_4OH (95:5, v/v). Four major radioactive bands were obtained. Choline, betaine, sarcosine were identified by reference compounds; a fourth band near the original spot could not be identified.

Peak 2 was chromatographed on paper developed with *n*-propanol–formic acid–water (60:24:16, v/v/v). More than 95 per cent of the radioactivity was chromatographed with authentic phosphorylcholine, which was clearly separated from phosphorylethanolamine, choline, betaine, sarcosine and CDP-choline.

CDP-Choline was located in the fraction (Peak 3) eluted from the column. As the fraction was contaminated with other nucleotides, it was further chromatographed on paper developed with the same solvent system as for Peak 2. Approximately 30–50 per cent of the radioactivity was chromatographed with authentic CDP-choline and the remainder was detected running ahead of CDP-choline, but it was not identified.

Assay of drug-metabolizing activity. Aniline hydroxylase was measured with a slight modification of the method by Imai *et al.* [14]. The reaction mixture contained 10 mM aniline, 100 mM Tris–HCl buffer (pH 7.9), 10 mM nicotinamide, 5 mM $MgCl_2$, 5 mM glucose-6-phosphate (G-6-P), 0.5 mM NADP, 1 unit of G-6-P dehydrogenase (EC 1.1.1.49, from Baker's yeast, Sigma) and a suitable amount (0.2–0.4 mg of protein) of microsomes in a final volume of 1.0 ml. The reaction was carried out for 20 min at 37° aerobically with moderate shaking and stopped by the addition of 0.5 ml of 20% TCA.

Aminopyrine demethylase was measured by some modified method of Kato and Gillette [15] and or Cochin and Axelrod [16] as follows: the reaction mixture contained 5 mM aminopyrine, 100 mM potassium phosphate buffer (pH 7.4), 10 mM nicotinamide, 5 mM $MgCl_2$, 10 mM neutralized semicarbazide, 5 mM G-6-P, 0.5 mM NADP, 1 unit of G-6-P dehydrogenase and a suitable amount (0.1–0.2 mg of protein) of microsomes in a final volume of 1.0 ml. The reaction

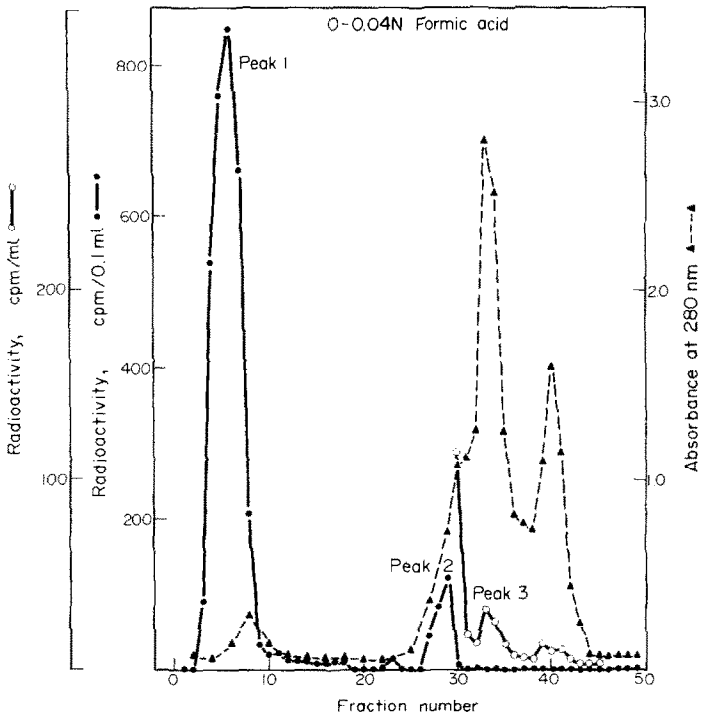

Fig. 1. Fractionation of water-soluble choline compounds through a column of Dowex 1 × 4 (formate form)

Acid soluble fraction of 105,000 *g* supernatant was washed three times with 15 ml of diethyl ether and brought to pH 8–9 with several drops of 1 M KOH, and was then passed through a colmun (1.5 cm × 30 cm) of Dowex 1 × 4 (200–400 mesh, formate). The column was washed with 40 ml of water and then eluted with a linear gradient of 0–0.04 N formic acid in a total volume of 300 ml. Fractions of either 10 ml (fraction No. 1–15) or 5 ml (No. 16–50) were collected and assayed for radioactivity and light absorption at 280 nm as indicated. Non-radioactive CDP-choline (1 μmole) was added to the fraction before chromatography as an indicator for absorption at 280 nm.

was carried out for 30 min at 37° with shaking and stopped by the addition of 0.5 ml 20% (w/v) $ZnSO_4$, and then 0.5 ml of a saturated solution of $Ba(OH)_2$ was added. After centrifugation, a 1.0 ml aliquot was treated with 0.5 ml of double-strength Nash reagent [17], and heated in a water bath at 60° for 30 min and then color intensity was determined. Known amount of formaldehyde (quantitatively derived from hexamethylenetetramine) carried through the incubation and assay procedure was served as standard.

Cytochrome P450 and b_5 content, and ethylisocyanide difference spectra of microsomes were determined by the method of Omura and Sato [18] using Hitachi model 323 spectrophotometer.

Both NADPH-cytochrome *c* and NADH-cytochrome b_5 reductase activities were measured by the method of Omura and Takesue [19] and Takesue and Omura [20], respectively, using a Gilford 240 spectrophotometer.

Phospholipid hydrolyzing activity. Phospholipid hydrolyzing activity in liver homogenate was measured using endogenous substrate labeled by either [Me-^{14}C]choline or [1-^{3}H]ethan-1-ol-2-amine.

Male rats weighing 110–120 g were fasted for 12 hr and i.p. injected with either 15 μCi of [^{14}C]choline or 50 μCi of [^{3}H]ethanolamine, dissolved in 0.5 ml of saline. The rats were killed by decapitation 2 hr after the injection. Liver microsomes were then prepared as described above except that the liver was homogenized with 9 vol. of 0.25 M sucrose containing 10 mM Tris–HCl (pH 7.4). Well washed microsomes, resuspended in small amount of sucrose-buffer, contained approximately 13,000 cpm/mg protein, 850 cpm/μg lipid-P for ^{14}C or 18,000 cpm/mg protein, 1000 cpm/μg lipid-P for ^{3}H, respectively. More than 93 per cent of ^{14}C-activity and 87 per cent of ^{3}H-activity in lipids were detected in PC and PE, respectively, by t.l.c.

The assay system for phospholipid hydrolyzing activity contained 50 mM Tris–HCl (pH 7.4), 20 mM $CaCl_2$, either ^{14}C- or ^{3}H-labeled microsomes (20,000–23,000 cpm) and 0.3 ml of 20 per cent liver homogenate (10–15 mg protein) in a final volume of 1.0 ml. The reaction was carried out 1 or 2 hr at 37° with moderate shaking and stopped by the addition of chloroform–methanol (2:1, v/v) followed by extraction of lipids. The radioactivity found in water–methanol layer was directly counted and ^{14}C- or ^{3}H-labeled hydrolysis products in the chloroform layer were separated by t.l.c. developed with either chloroform–methanol–water (65:25:4, v/v/v) or first chloroform–hexane–acetic acid (65:35:2, v/v/v) followed by chloroform–methanol–water (65:35:4, v/v/v), respectively. In the latter system, lyso-PE could be separated from other phospholipids [21].

Other analyses. Organic phosphorus was determined by the molybdate-ascorbic acid method after digesting with 70% (w/v) perchloric acid [22].

Protein was determined by the method of Lowry *et al.* [23] with bovine serum albumin as a standard. Radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer model 2002 using 10 ml of toluene–ethanol (7:3, v/v) with 0.4% (w/v) 2,5-diphenyloxazole and 0.01% (w/v) 2,2′-*p*-phenylene-bis(5-phenyloxazole) for lipid-soluble materials, 1 ml of water and 10 ml of Instagel (Packard) for water-soluble materials. In the case of determining the radioactivity in whole homogenate or blood plasma, 0.2 ml of the sample was solubilized by 1 ml of NCS (Amersham/Searle) overnight, and then counted using toluene–ethanol system described above. Counting efficiency was corrected by the external standard channel ratio method.

RESULTS

Effect of PB, PCBs and 3-MC on microsomal drug-metabolizing components. As shown in Table 1, the administration of either PB or PCBs for 2 days caused significant increases of microsomal protein content, cytochrome P450 content, and sp. act. of both aminopyrine demethylase and NADPH-cytochrome *c* reductase. Aniline hydroxylation activity was also significantly induced by PCBs but not by PB.

No apparent increase, on the other hand, of microsomal protein content, NADPH-cytochrome *c* reductase and aminopyrine demethylase activities was detected by 3-MC, but both cytochrome P450 content and aniline hydroxylation activity were enhanced, though with a lesser magnitude than those by PCBs.

Cytochrome b_5 content and NADH-cytochrome *c* reductase activity, however, neither of which is known to be essentially involved in microsomal drug-metabolizing system, were not significantly changed by the treatment with any of these inducers.

These findings, almost in agreement with those obtained by others [1, 24–26], indicate that the animals in experimental groups made response to given inducers.

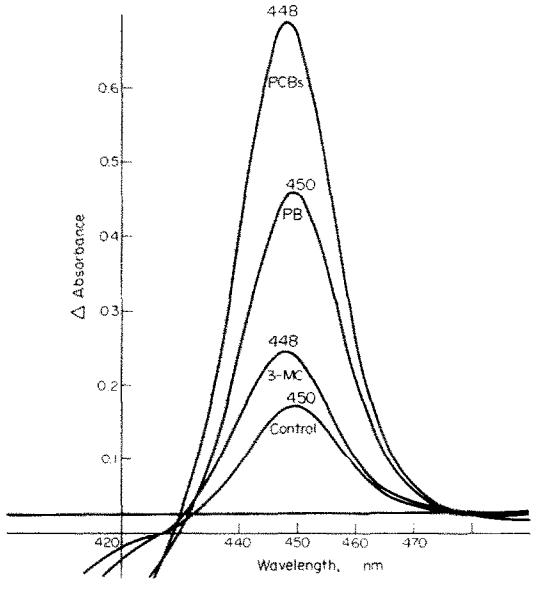

Fig. 2. Carbon monoxide-difference spectra of microsomes from untreated rats and rats treated with PB (80 mg/kg/day), PCBS (100 mg/kg/day) or 3-MC (50 mg/kg/day) for 2 days

CO-difference spectra with a peak at 450 nm were observed with microsomes from untreated or PB-treated rats as expected [27]. When rats were treated with PCBs, a peak shift occurred and CO-binding pigment exhibited an absorption maximum at 448 nm as was seen with microsomes from rats treated with 3-MC (Fig. 2).

When ethylisocyanide, instead of CO, was used as the ligand for the reduced microsomal hemoprotein, spectral peaks at 427 and 451–454 nm were observed (Fig. 3). As can be seen on the inset in Fig. 3, treatment with PCBs resulted in a marked increase in the ratio of 451:427 nm peak, similarly to that with 3-MC. In contrast, with PB, the ratio of 454:427 nm peak was about the same as that observed in untreated rats. The data obtained with 3-MC and PB are similar to those previously reported [27–30] and with PCBs

Table 1. Effect of PB, PCBs or 3-MC on microsomal drug-metabolizing components

	Body wt (g)	Liver (g)	microsomal protein/g liver (mg)	AN hyd.*	AM dem.‡	cytochrome P450†	cytochrome b_5†	NADPH-cytochrome *c*§ reductase	NADH-cytochrome b_5¶
Experiment 1									
Control	104 ± 5	4.3 ± 0.1	17.9 ± 1.2	27.6 ± 3.1	117.7 ± 5.6	0.81 ± 0.09	0.34 ± 0.02	73.4 ± 9.4	3.23 ± 0.14
PB	94 ± 4	4.8 ± 0.6	27.8 ± 0.8	25.5 ± 2.2	215.7 ± 9.3	1.98 ± 0.10	0.26 ± 0.05	114.3 ± 4.4	3.41 ± 0.16
(% of control)			(155)		(183)	(244)		(156)	
Experiment 2									
Control	100 ± 5	4.2 ± 0.3	19.4 ± 2.1	24.1 ± 2.7	108.2 ± 9.0	0.77 ± 0.04	0.33 ± 0.01	59.6 ± 2.9	3.05 ± 0.20
PCBs	100 ± 3	5.9 ± 0.2	33.2 ± 1.8	36.4 ± 3.2	213.2 ± 4.1	3.46 ± 0.40	0.25 ± 0.02	85.7 ± 5.1	2.64 ± 0.41
(% of control)			(171)	(151)	(197)	(449)		(144)	
Experiment 3									
Control	99 ± 4	4.1 ± 0.2	17.0 ± 0.2	24.2 ± 2.9	145.7 ± 8.9	0.78 ± 0.09	0.32 ± 0.02	68.7 ± 4.5	3.69 ± 0.41
3-MC	93 ± 3	4.3 ± 0.1	19.1 ± 0.6	29.7 ± 2.7	139.0 ± 9.6	1.35 ± 0.09	0.26 ± 0.02	64.8 ± 8.0	3.55 ± 0.30
(% of control)				(123)		(173)			

PB (80 mg/kg/day), PCBs (100 mg/kg/day) or 3-MC (50 mg/kg/day) was administered for 2 days. On day 3, the animals were killed by decapitation and liver microsomes were prepared as described in the text. The values are means ± S.E.M. from four animals.

* nmoles *p*-aminophenol/20 min/mg protein
‡ nmoles formaldehyde/30 min/mg protein
† nmoles/mg/protein
§ nmoles·min/mg protein
¶ μmoles/mg/protein.

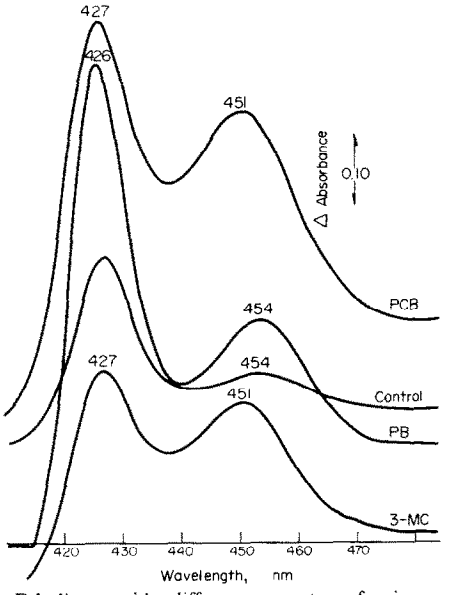

Fig. 3. Ethylisocyanide difference spectra of microsomes from untreated rats and rats treated with PB (80 mg/kg/day). PCBs (100 mg/kg/day) or 3-MC (50 mg/kg/day) for 2 days

Treatment	Ratio of 455 nm to 430 nm peak
Control	0.23
PB	0.31
PCBs	0.71
3-MC	0.82

to those reported by Alvares *et al.* using Aroclor 1254 [2].

Effect of the inducers of drug-metabolizing components on phospholipid biosynthesis in rat liver. It was reported elsewhere that the administration of PCBs caused a significant decrease in the rate of ^{32}Pi incorporation into microsomal phospholipids in rat liver, mainly due to the decrease in incorporation into choline-containing phospholipids such as PC and Sph [11]. PB, on the other hand, had no significant effect on the incorporation rate of ^{32}Pi into liver microsomal phospholipid classes 48 hr after the first administration [11]. Then, additional study was made with respect to the effect of 3-MC on ^{32}Pi incorporation into microsomal phospholipids in rat liver. The results are shown in Table 2 together with the previous data for comparison. No significant increase in microsomal phospholipid as well as protein content (Table 1) was observed in 3-MC-treated rats, suggesting that no apparent proliferation of ER membranes occurred, unlike the case of either PB- or PCBs-treated rats. The rate of ^{32}Pi incorporation into microsomal phospholipids, however, showed significant decrease, which was mainly due to the decrease in incorporation into PC, as in the case of PCBs, although the extent was smaller than that with PCBs.

In order to characterize the inhibitory effects of both PCBs and 3-MC on PC synthesis in rat liver, further investigations were carried out using rats injected labeled choline as to the incorporation into the microsomal phospholipid. The radioactivity of whole liver homogenate, as well as that of blood plasma, was measured 1 hr after the injection. As shown in Table 3, there was no significant difference in ^{14}C-activities in whole liver homogenate between control rats and rats treated with the inducers, suggesting that the process of choline entry into liver cells was not significantly affected by pretreatment with the inducers. In addition, since the activities detected in blood plasma of all groups were far smaller than those in liver homogenate, the influences of any contamination of radioactivity derived from the blood plasma in the liver homogenate could be neglected. On the other hand, the levels of ^{14}C-activities in plasma and also in plasma lipid were lower in the rats pretreated with the inducers as compared with those in the control. Particularly, the activity in plasma lipid in the rats treated with PCBs was remarkably low, showing less than 15 per cent of that in the control rats. It is suggested that either (or both) the secretion of lipoprotein from liver cells or/and the synthesis of PC from free choline in the liver would be strongly depressed in the rats treated with PCBs.

The phospholipid content and ^{14}C sp. act. in both liver subcellular components and blood plasma are shown in Table 4. The phospholipid content in both microsomal and mitochondrial fractions were on the same level in either control rats or rats treated with 3-MC. In contrast, the content in the former was over

Table 2. Effect of PB, PCBs or 3-MC on the incorporation of [^{32}P]orthophosphate into the phospholipid of rat liver microsomes

	Body wt (g)	Liver (g)	microsomal protein/g liver (mg)	Total PLs	Sp. act. (cpm/μg lipid-P) PC	PE	PI	PS	Sph
Experiment 1									
Control	93 ± 6	4.7 ± 0.8	4.10 ± 0.32	329 ± 36	254 ± 84	559 ± 101	127 ± 44		
PB	97 ± 4	5.3 ± 0.3	9.17 ± 1.51	327 ± 11	244 ± 19	525 ± 53	145 ± 31		
Experiment 2									
Control	98 ± 4	4.0 ± 0.4	5.94 ± 0.53	310 ± 17	297 ± 41	454 ± 42	98 ± 11	21 ± 16	39 ± 8
PCBs	95 ± 1	6.3 ± 0.3	13.48 ± 1.48	134 ± 21	60 ± 20	263 ± 36	83 ± 7	12 ± 12	12 ± 7
Experiment 3									
Control	104 ± 3	4.1 ± 0.1	6.01 ± 0.86	1375 ± 70	1519 ± 91	1687 ± 164	441 ± 58	195 ± 59	210 ± 99
3-MC	109 ± 3	5.0 ± 0.1	5.26 ± 0.48	723 ± 61	619 ± 104	1219 ± 221	521 ± 94	60 ± 10	118 ± 10

PB (80 mg/kg/day), PCBs (100 mg/kg/day) or 3-MC (50 mg/kg/day) was administered for 2 days. On the day 3, [^{32}P]orthophosphate (200–250 μCi) was i.p. injected 1 hr before the animals were killed. Other experimental procedures are described in methods. The values are means ± S.E.M. from four animals.

Table 3. Effect of PB, PCBs or 3-MC on the incorporation of [^{14}C]choline into the liver and blood plasma

Treatment	Body wt (g)	Liver (g)	Radioactivity in liver homogenate (dpm/g liver) $\times 10^4$	Radioactivity in liver homogenate (dpm/whole liver) $\times 10^4$	Radioactivity in blood plasma (dpm/ml plasma)	Radioactivity in blood plasma (dpm in lipid ml plasma)
Control	100 ± 8	4.0 ± 0.5	84.9 ± 13.7	335.3 ± 22.7	19,560 ± 2012	11,138 ± 741
PB	100 ± 6	5.0 ± 0.3	73.2 ± 3.3	366.7 ± 7.3	13,395 ± 186	6453 ± 419
PCBs	100 ± 6	5.7 ± 0.4	65.8 ± 6.9	374.7 ± 28.4	10,154 ± 1689	1526 ± 297
3-MC	96 ± 5	4.4 ± 0.4	81.4 ± 9.3	354.5 ± 9.6	13,206 ± 1867	6738 ± 1543

PB (80 mg/kg/day), PCBs (100 mg/kg/day) or 3-MC (50-mg/kg/day) was administered for 2 days. On the day 3, [Me-^{14}C] choline chloride (3.8 μCi) was i.p. injected 1 hr before the animals were killed. Other experimental procedures were described in the text. The values are means ± S.E.M. from four animals.

Table 4. Effect of PB, PCBs or 3-MC on the incorporation of [^{14}C]choline into the phospholipids of liver subcellular fractions and blood plasma

Treatment	Microsomes PLs/g liver (mg)	Microsomes dpm/μg lipid-P	Mitochondria PLs/g liver (mg)	Mitochondria dpm/μg lipid-P	Cytosol PLs/g liver (μg)	Cytosol dpm/μg lipid-P	Blood plasma PLs/ml plasma (μg)	Blood plasma dpm/μg lipid-P
Control	6.9 ± 0.4	1001 ± 113	6.4 ± 0.2	454 ± 44	822 ± 72	510 ± 58	979 ± 63	286 ± 31
PB (% of control)	14.4 ± 1.7	714 ± 61 (71.3)	5.9 ± 0.3	332 ± 27 (73.1)	659 ± 78	402 ± 18 (78.8)	846 ± 124	195 ± 32 (68.2)
PCBs (% of control)	16.7 ± 1.3	324 ± 63 (32.4)	6.9 ± 0.8	156 ± 33 (34.4)	654 ± 64	171 ± 35 (33.5)	760 ± 68	49 ± 7 (17.1)
3-MC (% of control)	8.0 ± 1.0	839 ± 74 (83.8)	7.5 ± 1.0	399 ± 31 (87.9)	599 ± 46	490 ± 40 (96.1)	987 ± 184	178 ± 59 (62.2)

Experimental conditions were the same as in Table 3. Other details were described in Methods. The values are means ± S.E.M. from four animals.

2-fold higher than in the latter in the rats treated with either PB or PCBs. More than 98 per cent of the ^{14}C-activities incorporated into lipids of each subcellular fraction and plasma lipids were detected in PC, and the remainder, less than 2 per cent were detected in lyso-PC and Sph. The sp. act. of phospholipids were always the highest in microsomal fraction, and declined in the order in cytosol, mitochondrial fraction and the lowest in blood plasma of all experimental groups.

The sp. act. of microsomal, mitochondrial, cytosol fractions and blood plasma in the rats treated with PCBs were 32, 34, 34 and 17 per cent, respectively, of those in the control rats, demonstrating that PCBs caused a considerable inhibition not only of the synthesis of PC in liver cells, but also of the secretion of PC from the liver to the blood plasma in a form of lipoprotein. In the 3-MC-treated rats, on the other hand, there appeared no significant difference in ^{14}C sp. act. of each subcellular component as compared with the control rats, in spite of the fact that ^{32}Pi incorporation into microsomal PC was considerably depressed (Table 2).

Further investigation was carried out with respect to the distribution of ^{14}C-radioactivity in water-soluble percursors of PC. As shown in Table 5, ^{14}C-activity in the water-soluble fraction of the liver from the rats treated with PCBs was relatively higher than that from any of the other three groups. This result is in good agreement with the finding that the rate of ^{14}C-incorporation into the lipids of every subcellular fraction was strongly depressed in the PCBs' group (Table 4).

It is noteworthy that the amount of radioactivity incorporated into phosphorylcholine was approximately 6-fold and 4-fold higher in the PCBs- and 3-MC-treated rats, respectively, than in the control. The sp. act. of phosphorylcholine in these two groups, on the other hand, was not more than 3-fold and 2-fold, respectively, when compared with that in the control. These findings suggest that there must be a large pool of phosphorylcholine present in the liver of the rats treated with either PCBs or 3-MC. In contrast, the amount of radioactivity incorporated into phosphorylcholine was lower, but sp. act. of phosphorylcholine was higher in the PB-treated rats than in the control, suggesting that there must be a relatively small pool phosphorylcholine in this group.

Peak 1 fraction was further chromatographed on paper into three main components, and per cent activity among them is shown in Table 6. The amount of radioactivity incorporated into betaine plus sarcosine fraction was predominantly higher in both con-

Table 5. Effect of PB, PCBs or 3-MC on the incorporation of [^{14}C]choline into water-soluble components of rat liver

Treatment	Total water-soluble dpm/g liver $\times 10^3$	Peak 1 dpm/g liver $\times 10^3$	Phosphoryl-choline dpm/g liver $\times 10^3$	Peak 3 dpm/g liver	Sp. act. of phosphoryl-choline dpm/μg P
Control	178 ± 54	150 ± 52	12.1 ± 3.9	145 ± 77	1031 ± 245
PB	143 ± 24	115 ± 21	8.1 ± 1.9	222 ± 60	1525 ± 425
PCBs	280 ± 45	164 ± 19	74.5 ± 19.5	332 ± 68	2726 ± 178
3-MC	158 ± 28	107 ± 21	44.7 ± 9.6	165 ± 22	2120 ± 140

Experimental conditions were the same as in Table 3. Other experimental details were described in Methods. The values are means ± S.E.M. from four animals.

Table 6. Distribution of ^{14}C-activity among not-retained compounds through Dowex 1 × 4 column

Treatment	Choline (%)	Betaine plus Sarcosine (%)	Not-identified polar metabolite (%)
Control	15.6 ± 2.4	77.0 ± 1.7	7.1 ± 1.1
PB	9.1 ± 1.0	79.0 ± 6.3	11.9 ± 6.8
PCBs	14.2 ± 2.5	43.0 ± 3.6	42.9 ± 5.8
3-MC	20.3 ± 1.4	52.7 ± 6.8	27.1 ± 7.9

Experimental conditions were the same as in Table 3. Other details were described in the text. The values are means ± S.E.M. from four animals.

Table 7. Effect of administration of PB, PCBs or 3-MC on phospholipid hydrolyzing activity in rat liver

Treatment	^{14}C-activity(%) in water-soluble fraction	lyso-PC	^{3}H-activity(%) in water-soluble fraction	lyso-PE
Control	3.7, 2.8 (0.1, 0.4)	1.9, 3.4 (3.6, 2.7)	4.0, 3.4 (0.2, 0.3)	11.7, 11.1 (8.3, 10.9)
PB	3.2, 3.1 (0.0, 0.6)	1.9, 2.1 (1.9, 3.0)	3.4, 2.9 (0.2, 0.5)	10.9, 12.0 (6.2, 8.2)
PCBs	3.4, 2.5 (0.3, 0.0)	2.8, 2.0 (2.3, 2.5)	3.3, 3.0 (0.2, 0.0)	4.7, 6.9 (2.6, 4.5)
3-MC	4.2, 3.3 (0.4, 0.0)	1.9, 3.2 (2.8, 2.6)	3.5, 3.8 (0.6, 0.4)	9.3, 9.9 (10.1, 6.5)

Rats were administered PB (80 mg/kg/day), PCBs (100 mg/kg/day) or 3-MC (50 mg/kg/day) for 2 days. On day 3, the animals were killed by decapitation. Incubation mixture contained 50 mM Tris–HCl (pH 7.4), 20 mM $CaCl_2$, either ^{14}C- or ^{3}H-labeled microsomes (20,000–23,000 cpm) and 0.3 ml of 20% liver homogenate (10–15 mg of protein) in a final volume of 1.0 ml. The values indicate the results of duplicate determinations and those in parentheses represent the experiments run in the presence of 2% sodium deoxycholate. Other experimental procedures were described in the text.

trol rats and rats treated with PB; 77 and 79 per cent, respectively, of the total activities detected in Peak 1. On the other hand, either PCBs or 3-MC caused considerably high incorporation of radioactivity into unidentified polar component.

Effect of the inducers of drug-metabolizing components on phospholipid catabolism in rat liver. Catabolic activities of endogeneously labeled PC and PE were compared among liver homogenates of four groups of rats. The rate of hydrolysis was shown as the percentages of both water-soluble labeled products released and labeled lyso-derivatives formed, when sodium deoxycholate, which is known to inhibit lysophospholipase activity [21, 31], was either present or not in the incubation mixture.

As seen in Table 7, the percentages of PE hydrolyzed to the lyso-form was considerably less in the PCBs-treated group than those in the other three groups, in either case with or without deoxycholate in the mixture. There was no apparent difference, however, in the activities of lyso-PC formed during the incubation among respective groups.

DISCUSSION

Orrenius and co-workers [32–34] first found that PB markedly increased the rate of ^{32}Pi-incorporation into phospholipid of liver microsomal fraction, and they suggested that enhanced phospholipid synthesis was an early step in the proliferation of liver ER induced by PB. Young *et al.* [35] and recently Davison and Wills [36] reported that an increased synthesis of phospholipid in the liver of PB-treated rats resulted from a stimulated synthesis of PC via methylation of PE. In addition, it has been shown that the activities of *S*-adenosylmethionine: PE methyltransferase [36, 37], acyl CoA:*sn*-glycerol-3-phosphate acyltransferase [37, 38] and acetyl CoA carboxylase [39] increase *in vitro* at an early time after the injection of PB. Holtzman and Gillette [40], Stein and Stein [41] and Infante *et al.* [42], however, failed to confirm these results and found that PB increased hepatic microsomal phospholipid by inhibiting the catabolism rather than by stimulating the synthesis. According to our previous paper [11], an increase of microsomal phospholipid content caused by PB was probably attributable, at least in part, to an actual increase in hepatic PC synthesis, since the rate of incorporation of ^{32}Pi into phospholipid, especially into PC, was markedly stimulated 12 hr after a single dose of PB.

In the present study, however, the incorporation experiment of [^{14}C]choline indicates that the synthesis of PC in the liver keeps relatively low 48 hr after the first administration of PB (Table 4). In addition, the ratio of sp. act. of phosphorylcholine

to that of microsomal phospholipid (Tables 4 and 5) was considerably higher in the PB-treated rats (approximately 2.1) as compared with the ratio in the control (1.0), also suggesting a slower conversion of phosphorylcholine to PC in this experimental group.

Thus, our results *in vivo* support a view that proliferation of ER membrane induced by PB would be accompanied by the stimulation of phospholipid synthesis at the early process of induction and subsequently followed by the decrease in turnover rate of microsomal phospholipids. However, the *in vitro* study of phospholipid catabolism using liver homogenate showed no significant difference between control and PB-treated rats (Table 7), unlike the results reported by Stein and Stein [41].

In the previous paper [11], it was suggested that PCBs (or the metabolites) caused a severe inhibition of PC synthesis in the process of CDP-choline formation via phosphorylcholine, namely the inhibition of reactions catalyzed by choline kinase and/or cholinephosphate cytidylyltransferase.

The present study demonstrated that there could be at least 2-fold accumulation of phosphorylcholine occurred in the liver of rats treated with PCBs (Table 5). These findings suggest strongly that the process from phosphorylcholine to CDP-choline is the possible site of PCBs-induced inhibition. In view of this point, it has been proposed that the reaction catalyzed by cholinephosphate cytidylyltransferase is the rate-limiting step of PC synthesis in the liver [43] as well as other tissues [44–46]. It has been also suggested that the activity of the liver enzyme, mainly located in 100,000 *g* supernatant fraction, is possibly controlled by some kinds of membranous phospholipids [47, 48]. Therefore, it would be expected that cholinephosphate cytidylyltransferase activity would be easily affected by the lipophilic environment, especially by the highly lipophilic invader such as PCBs.

Phospholipids synthesized in ER membranes of the liver are considered to be rapidly transferred to other intracellular membranes such as mitochondria by phospholipid exchange proteins [49, 50]. However, since the ratios of sp. act. of microsomal, mitochondrial and cytosol fractions in the rats treated with PCBs to those in the control rats, were on the similar level (Table 4) the transfer activity of PC would not be significantly affected by the PCBs-administration. On the other hand, the secretion process of PC from liver to blood plasma in a form of lipoprotein was considerably inhibited by PCBs or the metabolites (Tables 3 and 4). A decrease of enzyme activity in phospholipid catabolism was also observed in the liver of the rats treated with PCBs (Table 7) when endogenous PE was used as a substrate. However, the hydrolyzing activity to PC detected by liver homogenate was too small to be compared among each group.

In conclusion, the phospholipid accumulation in the liver ER membranes after PCBs-administration is strongly suggested by the data presented here to be due to a depression in the secretion from hepatocytes to plasma. The inhibition of catalytic activity to phospholipid would significantly participate in the hypertrophy of ER membranes in the liver.

Few findings have been reported concerning the alteration of phospholipid metabolism caused by the administration of 3-MC. Recently Davison and Wills [36] reported that 3-MC caused a considerable decrease in the turnover rate of microsomal phospholipid in rat liver, although the precise mechanism remained obscure. In the present study, it was demonstrated that the rate of ^{32}Pi incorporation into liver microsomal PC decreased to less than one half of that in the control rats (Table 2). In addition, the radioactivity derived from [^{14}C]choline was accumulated preferentially in phosphorylcholine in the liver as compared with that in the control (Table 5). These findings suggest that 3-MC, as in the case of PCBs, causes an inhibition of PC synthesis at the site of CDP-choline formation.

On the other hand, the rate of incorporation of [^{14}C]choline into liver subcellular fractions did not significantly decrease by 3-MC (Table 4), differing from the results with ^{32}Pi as the precursor (Table 2). From the present result, however, no definite explanation on these differences can be drawn. [^{14}C]-Choline is incorporated into PC by CDP-choline pathway [51], but two other known ways could be considered; the methyl group could be relocated to methionine during oxidation of choline [52] and utilized in the methylation of PE [53], or [^{14}C]-choline could be incorporated into PC by an exchange reaction similar to that observed in a system *in vitro* [54]. Thus, the stimulation of the activities involved in either or both of the above pathway(s) might be a plausible explanation. In connection with the former possibility, it would be interesting to note that a relatively high amount of ^{14}C-radioactivity was observed in the water-soluble polar component in the liver of rats treated with 3-MC (Table 6).

The precise mechanism of inhibition, caused by both PCBs and 3-MC, in the reaction catalyzed by cholinephosphate cytidylyltransferase is now under investigation in our laboratory.

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