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1. We have studied the effects of tetrachloroethylene (PCE) on the kinetics of the P450-dependent monooxygenases in rat liver microsomes.

2. 7-Pentoxyresorufin *O*-depentylase (PROD) and 7-benzyloxyresorufin *O*-debenzylase (BROD) activities in phenobarbital (PB)-treated rat liver microsomes were substantially inhibited by PCE. The inhibition profiles were non-competitive for both enzyme activities; K_i's from Eadie–Hofsee plots were 0·16 and 0·29 mM for PROD and BROD respectively. In contrast, the enzyme activities in untreated, β-naphthoflavone (BNF)-, isoniazid (ISN)- and pregnenolone-16α-carbonitrile (PCN)-induced microsomes were not affected by PCE.

3. 7-Ethoxycoumarin *O*-deethylase (ECOD) activity in PB-induced microsomes was competitively inhibited by PCE, with a K_i that was lower than those of other microsomes.

4. PCE inhibited 7-ethoxyresorufin *O*-deethylase (EROD) activities in some microsomes slightly. The K_i for PCE was the lowest in untreated, followed by ISN-treated microsomes.

5. No effect of PCE upon aniline 4-hydroxylase (AN4H) and testosterone 6β-hydroxylase (TS6BH) activities was evident in any microsomal preparation.

6. These results indicate that PCE inhibits PB-inducible, P450-dependent monooxygenases *in vitro* non-competitively or competitively, and that the P450 enzymes of the P4502B subfamily may contribute to PCE toxicity.

Introduction

Tetrachloroethylene (PCE) is a solvent that is often used in dry-cleaning, metal degreasing and cleaning (Fishbein 1976), and because of its lower toxicity, is replacing trichlorethylene (Ikeda and Ohtsuju 1972). Although PCE is regarded as being of low toxicity, liver impairment, central nervous system depression and disturbances of the peripheral nervous system have been reported (Fishbein 1976). Tetrachloroethylene is also weakly carcinogenic in some mammalian species (Fishbein 1976). These effects may be because tetrachloroethylene contains a double bond that renders it prone to oxidation, and the subsequent formation of highly reactive epoxy intermediates (Yllner 1961).

It has been reported that tetrachloroethylene is metabolized to trichloroacetic acid and chloride, with 2,2,2-trichloroethanol as a minor metabolite *in vivo* (Yllner 1961, Daniel *et al.* 1963, Ikeda and Ohtsjui 1972, Moslen *et al.* 1977). The metabolism of trichloroacetic acid from PCE is catalysed by the hepatic P450 system (Powell 1945, Daniel *et al.* 1963). Furthermore, since PCE binds to the active site

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of P450 *in vitro* (Pelkonen and Vainio 1975, Moslen *et al.* 1977) the toxicities of PCE seem to correlate with P450 binding.

The effect of inducing of different forms of P450 on the spectral binding constant and the maximum extent of PCE binding have been reported by Costa and Ivanetich (1980). The reported that the spectral binding constants in phenobarbital (PB)- and β-naphtoflavone (BNF)-treated rat liver microsomes were similar to that of untreated rat, whereas in pregnenelone 16α-carbonitrile (PCN)-induced rat, they were significantly increased. On the other hand, the maximum extent of spectral binding of PCE was increased by both PCN and PB, with the latter having the greater effect. They also observed that PB decreased the Michaelis constant for the metabolism of PCE and increased the maximum rate *in vitro*. Furthermore, the P450 system appears to catalyse the metabolic activation of PCE to reactive species that may mediate the toxic effects of the parent compound (Costa and Ivanetich 1980). This indicates that the metabolism of PCE is catalysed by the PB-induced P450 system, and that these P450 isozymes may contribute to PCE toxicity.

Thus, the examination of interaction of PCE with drug-metabolizing enzymes *in vivo* and *in vitro* is an important aspect of toxicological research. However, very little has been reported about the interaction of PCE and P450 isozymes. In this study, to clarify the mechanisms of toxicity of PCE, the effects of PCE on several P450-dependent monooxygenase activities were determined by kinetic means *in vitro*.

Materials and methods

Materials

PCE (99·7%) was obtained from W. Merck (Darmstadt, Germany). Resorufin, 7-ethoxyresorufin, 7-pentoxyresurufin, 6β-hydroxytestosterone and PCN were obtained from Sigma Chemical Co. (St Louis, MO, USA). 7-Ethoxycoumarin was obtained from Aldrich Chemical Co. (Milwaukee, WI, USA). 7-Benzyloxyresorufin was purchased from Molecular Probes (Eugene, OR, USA). Aniline hydrochloride, testosterone, BNF, sodium PB and isoniazid (ISN) were purchased from Wako Pure Chemical Industries (Osaka, Japan). NADPH was a product of the Oriental Yeast Co. (Tokyo, Japan). The antibody against rat P4501A1 was prepared as described (Hanioka *et al.* 1994), and anti-rat P4502B1 antibody was purchased from Oxygenase (Dallas, TX, USA). Anti-rat P4502E1 and 3A2 antibodies were from Daiichi Pure Chemical Co. (Tokyo, Japan). Peroxidase anti-rabbit IgG, peroxidase anti-goat IgG and peroxidase substrate kit (4-chloro-1-naphthol) were obtained from Vector Laboratories (Burlingame, CA, USA). All other materials were of the highest quality commercially available.

Animals and treatment

Male Wistar rats (160–190 g) obtained from Nippon Bio-Supply Center (Tokyo, Japan), were fed with laboratory chow (CRF-1) and water *ad libitum*. They were housed in plastic cages at a constant temperature (22–26°C) and humidity (50–60%) under a 12-h light/dark cycle (light: 07:00 to 19:00). Rats were administered four daily intraperitoneal (i.p.) doses of BNF (25 mg/kg), PB (80 mg/kg), ISN (80 mg/kg) or PCN (60 mg/kg) and killed 24 h after the last injection. Prior to killing, all animals were starved for 12 h.

Preparation of liver microsomes

The liver was removed from each rat and perfused with ice-cold physiological saline. Livers were finely minced in 3 vols 25 mM Tris–HCl buffer (pH 7·6) containing 0·25 M sucrose, 25 mM KCl and 5 mM $MgCl_2$, and homogenized in a glass–Teflon homogenizer. The homogenates were centrifuged at 9000 *g* for 20 min, and the supernatant fractions centrifuged at 105 000 *g* for 60 min. The microsomal pellets were suspended in 25 mM Tris–HCl buffer (pH 7·6) containing 0·15 M KCl, then centrifuged at 105 000 *g* for 60 min. The microsomes were resuspended in a volume equivalent to the original liver weight of 0·1 M Tris–HCl buffer (pH 7·6) containing 20% (v/v) glycerol. The microsomal fractions were separated into small quantities and stored at − 80°C until use. The protein levels of the microsomes were measured by the method of Lowry *et al.* (1951), using bovine serum albumin as a standard.

Enzyme assays

Total microsomal P450 contents were quantified according to Omura and Sato (1964). The activities of 7-ethoxyresorufin *O*-deethylase (EROD), 7-pentoxyresorufin *O*-depentylase (PROD) and 7-benzyloxyresorufin *O*-debenzylase (BROD) were determined fluorometrically from the amount of resorufin produced (Pohl and Fouts 1980). 7-Ethoxycoumarin *O*-deethylase (ECOD) activity was measured by the fluorometric determination of 7-hydroxycoumarin (Aitio 1978). Aniline 4-hydroxylase (AN4H) and testosterone 6β-hydroxylase (TS6BH) activities were measured by the formation of 4-aminophenol and 6β-hydroxytestosterone (Imai *et al.* 1966, Yoshihara *et al.* 1982). The substrate concentrations, and reaction times were: EROD, 50–800 nM, for 0–5 min; PROD, 250–4000 nM, for 0–15 min; BROD, 25–400 nM, for 0–5 min; ECOD, 50–800 μM, for 0–20 min; AN4H, 50–800 μM, for 0–20 min; and TS6BH, 12·5–200 μM, for 0–20 min.

Western immunoblotting

Rat liver microsomal proteins were separated electrophoretically on a sodium dodecyl sulphate-polyacrylamide gel using 7·5% polyacrylamide (Laemmli 1970). After electrophoresis, the proteins were transferred from gels to nitrocellulose membranes according to Towbin *et al.* (1979). After transfer, non-specific binding sites were blocked by incubating the membranes in phosphate-buffered saline containing 3% (w/v) bovine serum albumin for 16 h at room temperature. The nitrocellulose membranes were incubated with anti-rat polyclonal antibodies for P4501 1A1, 2B1, 2E1 and 3A2 in 3% (w/v) bovine serum albumin for 2 h at room temperature. The membranes were then washed in three successive changes of phosphate-buffered saline containing 0·5% (v/v) Tween 20 for 20 min each. P450 antibodies bound to microsomal proteins were detected by incubating the membranes with anti-rabbit or anti-goat IgG-horseradish peroxidase conjugate for 1 h, followed by a colorimetric determination with 4-chloro-1-naphthol.

Calculations

Apparent K_i was estimated by graphical analyses of Eadie–Hofstee plots using ENZYME KINETICS (Trinity Software, NH, USA). All reported values are mean ± SD.

Results

The total P450 content and the activities of P450-dependent monooxygenase (EROD, ECOD, PROD, AN4H and TS6BH) in microsomes prepared from the livers of rat treated with a variety of xenobiotics are shown in table 1. As expected, EROD activity was highly induced in the livers of rat treated with BNF (142-fold). ECOD activity was increased by BNF (7·4-fold) and that in PB-treated microsomes was also increased 2·4-fold. However, the ECOD activities were not affected by ISN and PCN treatment. PROD and BROD activities in rat liver microsomes were substantially induced by PB (54- and 42-fold respectively). The AN4H activities induced in PB- and ISN-treated microsomes were higher than those in untreated, BNF- and PCN-treated microsomes. TS6BH activity in PCN-treated rat liver microsomes was the highest in the liver microsomes of rat treated with xenobiotics.

Table 1. Total P450 content and P450-dependent monooxygenase activities in rat liver microsomes.

	UT	BNF	PB	ISN	PCN
P450 content[a]	0·77 ± 0·03	1·33 ± 0·04	1·77 ± 0·04	0·81 ± 0·02	1·62 ± 0·04
EROD[b]	0·06 ± 0·00	8·54 ± 0·34	0·07 ± 0·00	0·11 ± 0·00	0·04 ± 0·00
ECOD[b]	0·71 ± 0·01	5·23 ± 0·18	1·67 ± 0·03	0·80 ± 0·03	0·74 ± 0·02
PROD[b]	0·02 ± 0·00	0·04 ± 0·00	1·07 ± 0·05	0·02 ± 0·00	0·03 ± 0·00
BROD[b]	0·06 ± 0·00	0·75 ± 0·03	2·54 ± 0·19	0·09 ± 0·01	0·11 ± 0·01
AN4H[b]	0·45 ± 0·02	0·58 ± 0·01	0·73 ± 0·02	0·97 ± 0·02	0·46 ± 0·02
TS6BH[b]	0·80 ± 0·03	0·67 ± 0·02	0·86 ± 0·02	0·63 ± 0·02	2·11 ± 0·07

Each value represents the mean ± SD of three determinations.

UT, untreatment; BNF, β-naphthoflavone; PB, phenobarbital; ISN, isoniazid; and PCN, pregnenolone 16α-carbonitrile.

[a] nmol/mg protein.

[b] nmol/min/mg protein.

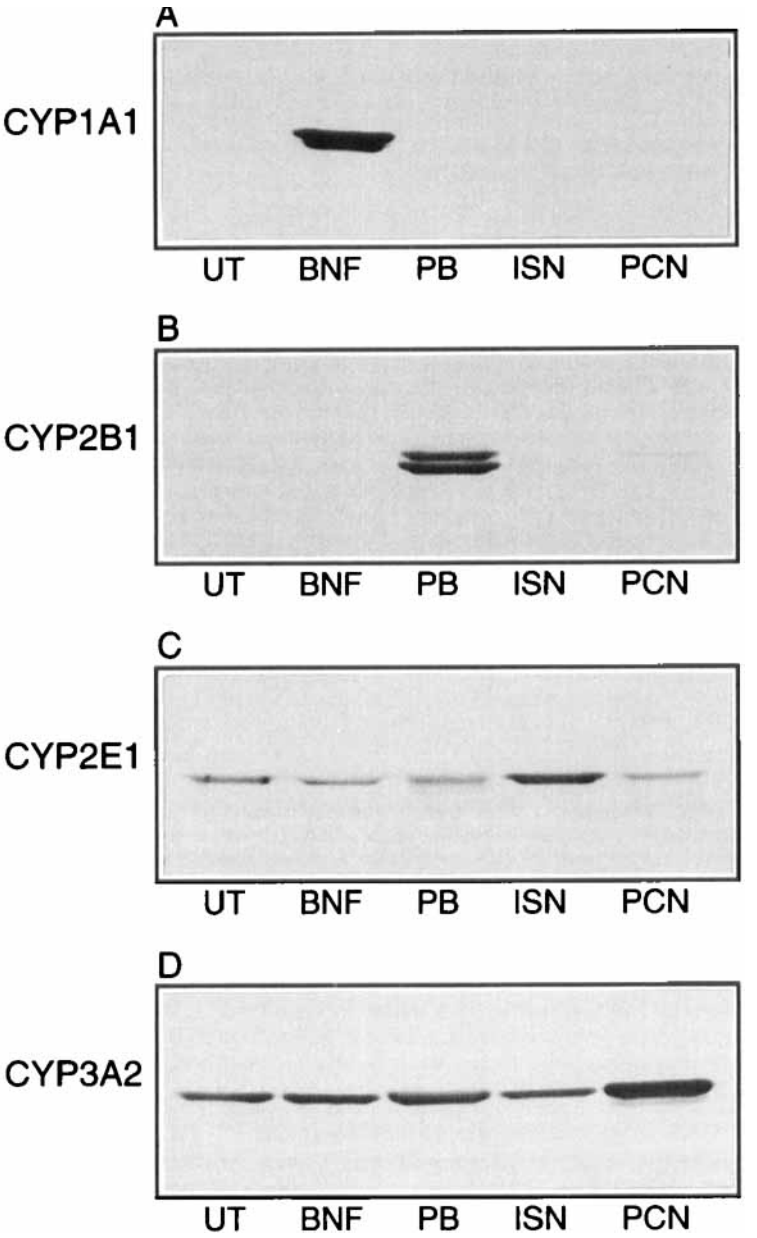

Figure 1. Western immunoblotting of liver microsomes from untreated, BNF-, PB-, ISN- and PCN-treated rat with anti-rat P4501A1, 2B1, 2E1 and 3A2. Liver microsomes (loaded at 10·0, 10·0, 8·0 and 8·0 μg for 1A1, 2B1, 2E1 and 3A2) were resolved by dodecyl sulphate-polyacrylamide gel electrophoresis (7·5% gel) and immunoblotted with anti-rat 1A1 (A), rat 2B1 (B), rat 2E1 (C) and 3A2 (D) respectively. Lanes 1, untreated (UT) microsomes; 2, BNF-induced microsomes; 3, PB-induced microsomes; 4, ISN-induced microsomes and 5, PCN-induced microsomes.

These induction profiles were confirmed by Western immunoblotting using anti-rat P4501A1, 2B1, 2E1, and 3A2 antibodies. Figure 1(A) shows anti-rat P4501A1 immunostained rat liver microsomes. With this antibody, two bands were detected, against which only BNF was an effective inducing agent. When anti-rat P4502B1 was used as the probe, two bands were observed in samples from PB-treated rat (figure 1 (B)). Also, this antibody weakly immunoreacted with the one protein from PCN-treated rat liver microsomes. The band had a mobility similar to that of the upper band observed in the PB-treated rat. Anti-rat P4502E1 and 3A2 antibodies immunoreacted with all rat liver microsomes (figures 1 (C) and (D)). The bands in liver microsomes from ISN- and PCN-treated rat were detected with anti-rat P4502E1 and 3A2 respectively were very intense.

The effects of various concentrations of PCE on P450-dependent monooxygenase activities are shown in figure 2. PCE was dissolved in methanol (final concentration in the reaction medium, 1·0% (v/v)), with methanol being used as the solvent control. PROD and BROD activities in PB-pretreated rat liver microsomes were inhibited at rates of 80 and 81% respectively at a concentration of 2·0 mM PCE. However, there was no inhibitory effect of PCE on the activities of P450-dependent monooxygenase such as EROD, ECOD, AN4H and TS6BH in any rat liver microsomal preparation.

To obtain further information about the type of enzyme inhibition by PCE, Eadie–Hofstee plots were constructed. The inhibition kinetics of P450-dependent monooxygenases in each rat liver microsomal preparation by PCE are shown in figures 3–8. The inhibition curves of PROD and BROD obtained from PB-treated rat liver microsomes were consistent with non-competitive inhibition (figures 5 and 6). ECOD activity in PB-treated rat was inhibited by PCE at low substrate concentration and the inhibition profile was competitive (figure 4).On the other hand, the effect of PCE on EROD, AN4H and TS6BH activities in all microsomes were not striking (figures 3, 7 and 8). The summarized data from all inhibition kinetics are given in table 2. In PB-induced rat liver microsomes, the value of PROD and BROD for PCE were lower that those of untreated, BNF-, ISN- and PCN-treated rat.

Discussion

The results presented here indicated that PCE non-competitively inhibits the PROD and BROD activities in PB-treated rat liver microsomes *in vitro*. It has been reported that PCE is metabolized by the hepatic P450 system *in vivo* and *in vitro* (Pelkonen and Vainio 1975, Moslen *et al.* 1977, Costa and Ivanetich 1980). It is currently accepted that the production of trichloroacetic acid from PCE proceeds via epoxides *in vivo* (Powell 1945, Daniel 1963). Epoxides have been implicated in various toxic reactions such as carcinogenesis and mutagenesis (Jerina and Daly 1974). Furthermore, it has been reported that PCE binds to the active site of the enzyme system, as evidenced by the production of a Type I difference spectrum (Pelkonen and Vainio 1975). Therefore, it is important to clarify the interaction of PCE with hepatic P450 to elucidate PCE toxicity.

In this study, EROD, ECOD, PROD, BROD, AN4H and TS6BH in untreated, BNF-, PB-, ISN- or PCN-induced rat liver microsomes were used as markers of P450-dependent monooxygenase. It is generally thought that PROD and BROD are mediated by P4502B1 as a PB-inducible isozyme (Burke *et al.* 1985, Lubet *et al.* 1985). PCE non-competitively inhibited PROD and BROD activities in the

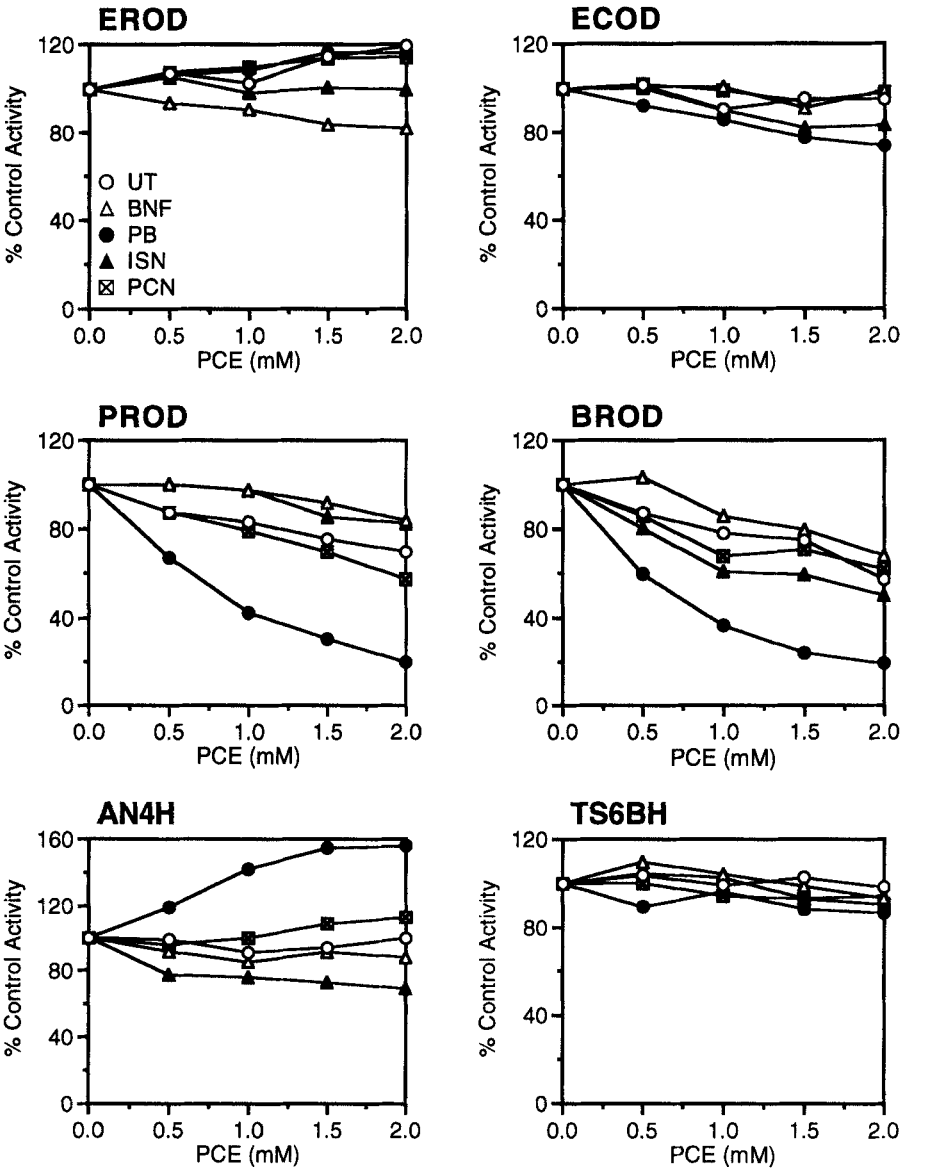

Figure 2. Inhibition of P450-dependent monooxygenase activities by PCE in rat liver microsomes. Substrate concentrations for EROD, ECOD, PROD, BROD, AN4H and TS6BH were 0·8 μM, 0·8 mM, 4·0 μM, 0·4 μM, 0·8 mM and 0·2 mM respectively. Each point represents the mean of two determinations. Control activities (nmol/min/mg protein) in untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were: EROD: UT 0·06; BNF 6·04; PB, 0·07; ISN, 0·11; PB, 0·04; ECOD: UT, 0·82; BNF, 5·08, PB, 2·28; ISN, 0·93; PB, 1·07, PROD: UT,0·02; BNF, 0·04; PB, 0·51; ISN 0·02; PB, 0·02; BROD: UT, 0·04; BNF, 0·63; PB, 2·21; ISN, 0·07; PB, 0·11; AN4H: UT, 0·46; BNF, 0·60; PB, 0·81, ISN, 0·96; PB, 0·43; and TS6BH: UT, 0·88; BNF, 0·65; PB, 0·94; ISN 0·64; PB, 2·36.

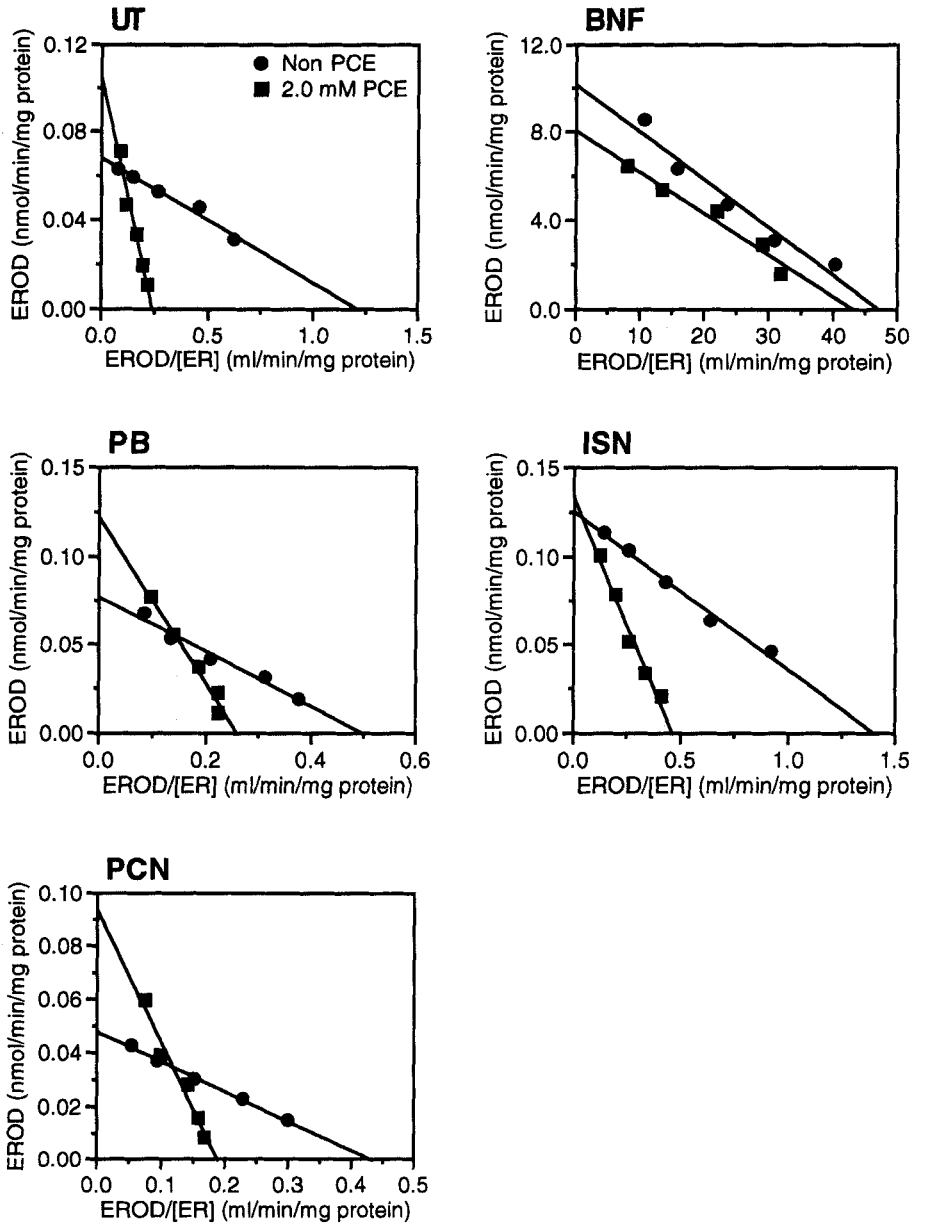

Figure 3. Inhibition of EROD kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for EROD activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

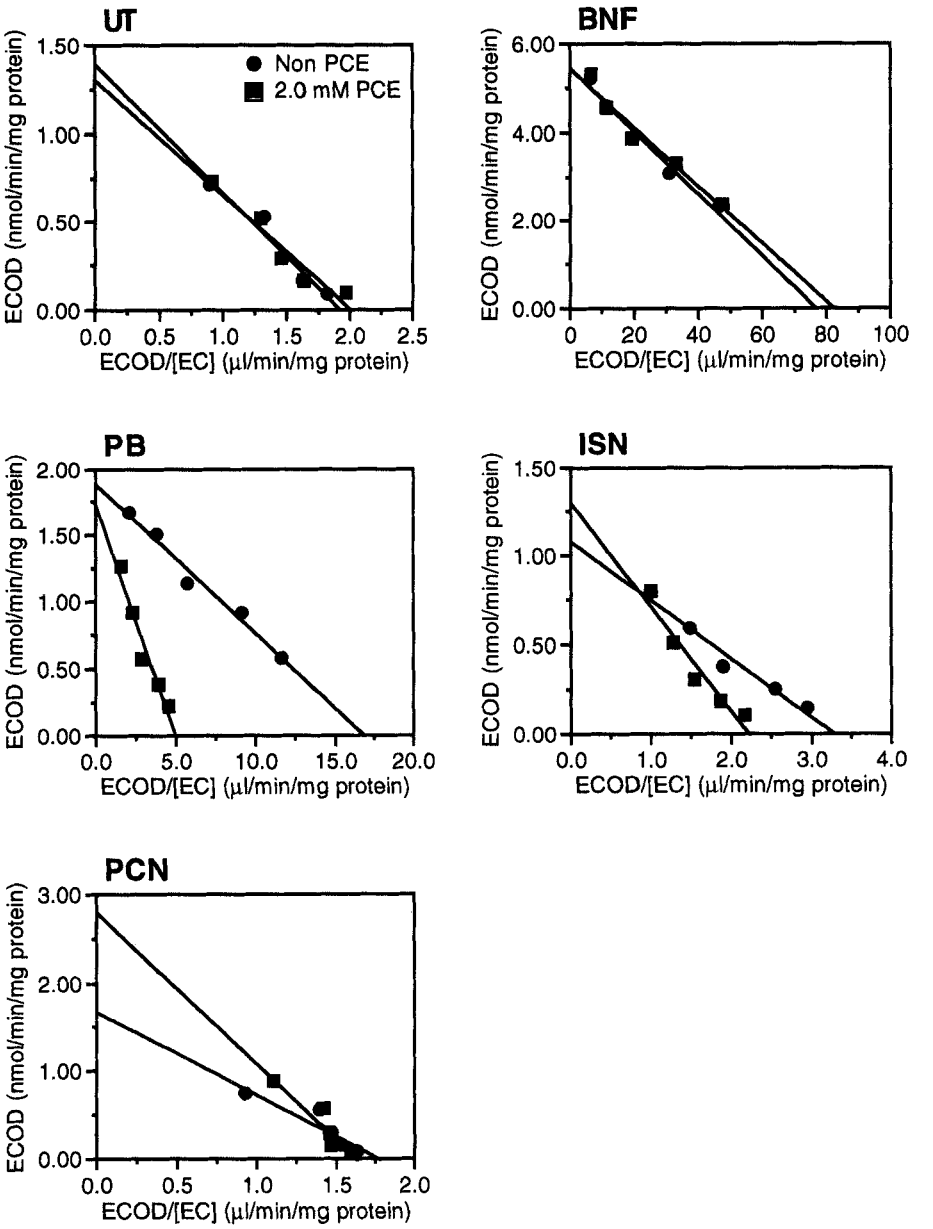

Figure 4. Inhibition of ECOD kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for ECOD activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

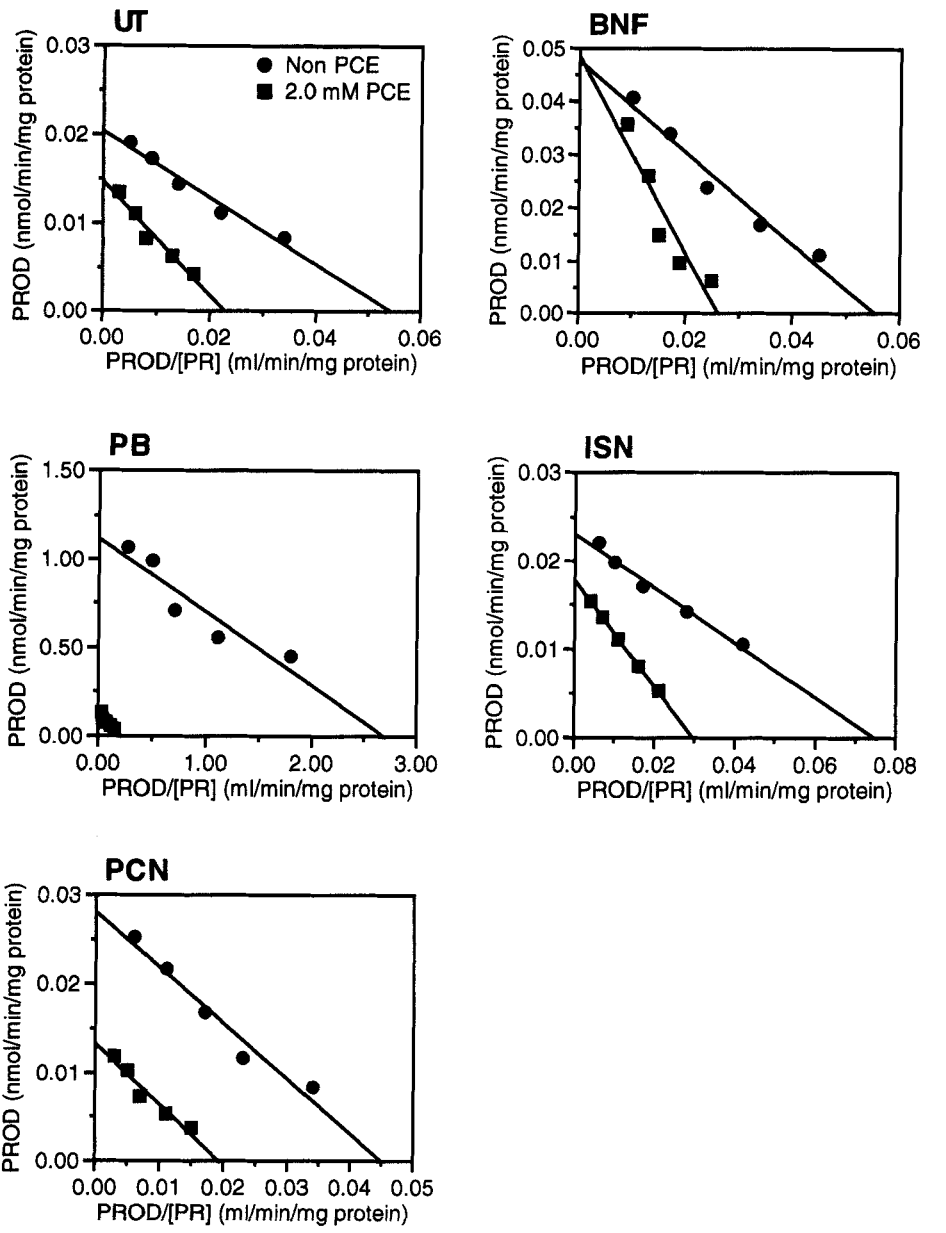

Figure 5. Inhibition of PROD kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for PROD activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

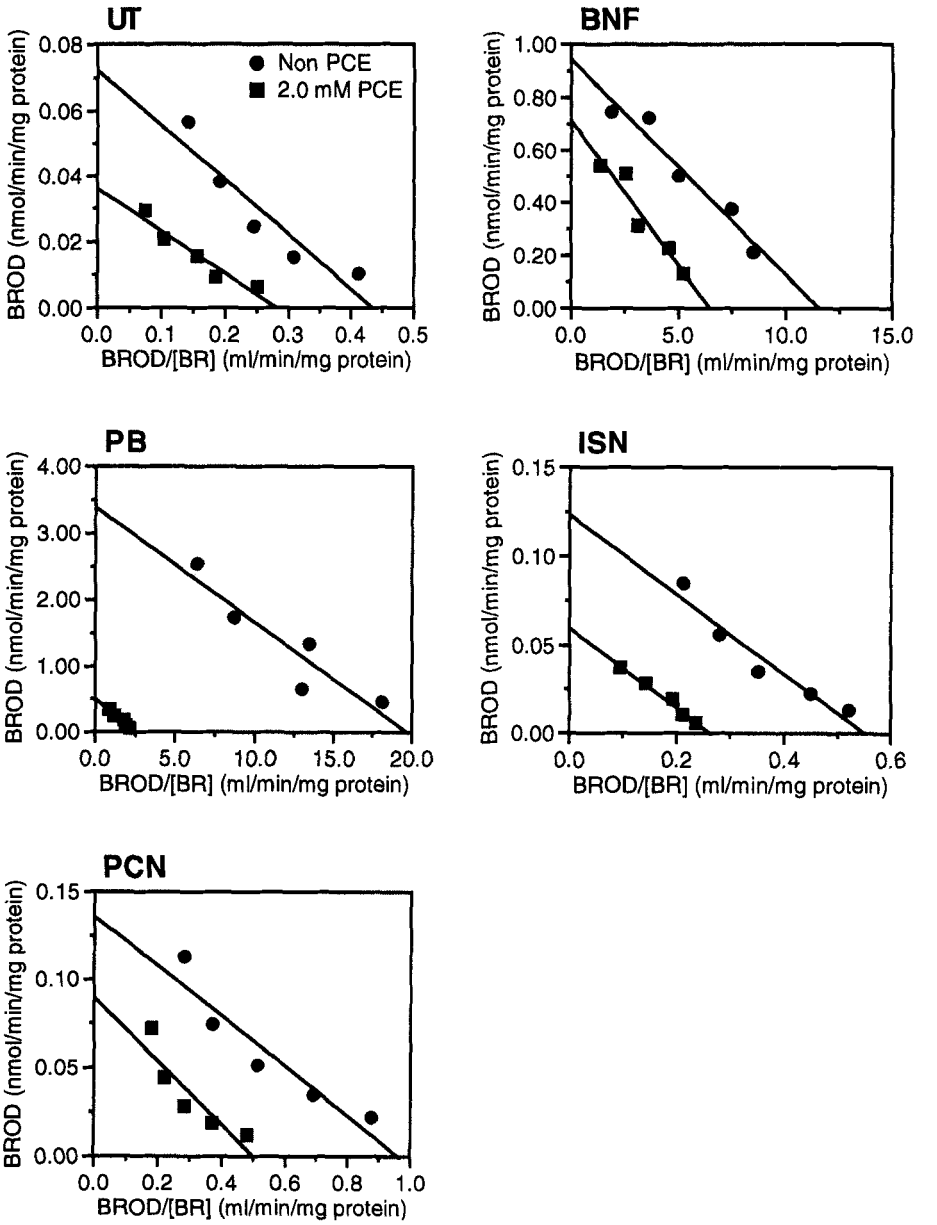

Figure 6. Inhibition of BROD kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for BROD activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

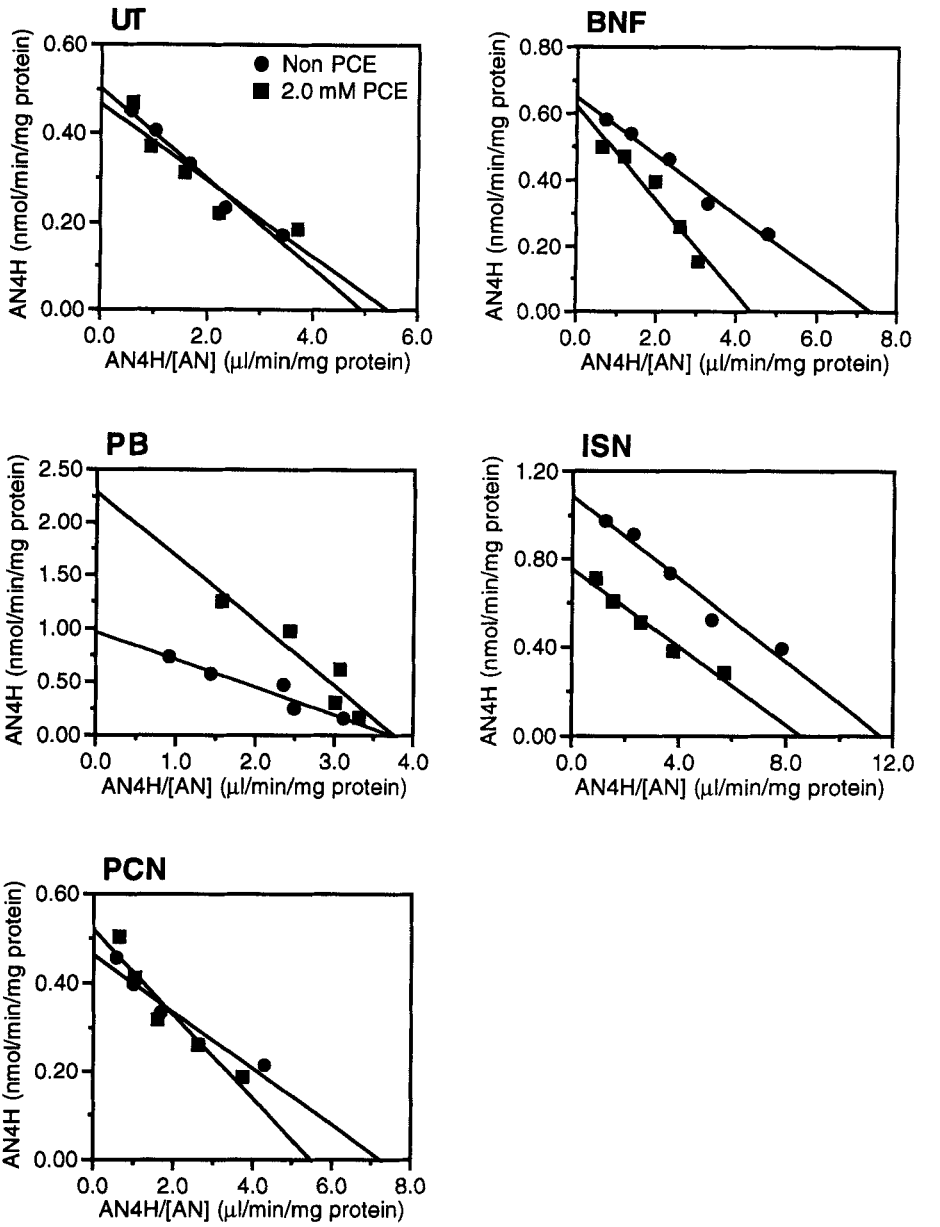

Figure 7. Inhibition of AN4H kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for AN4H activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

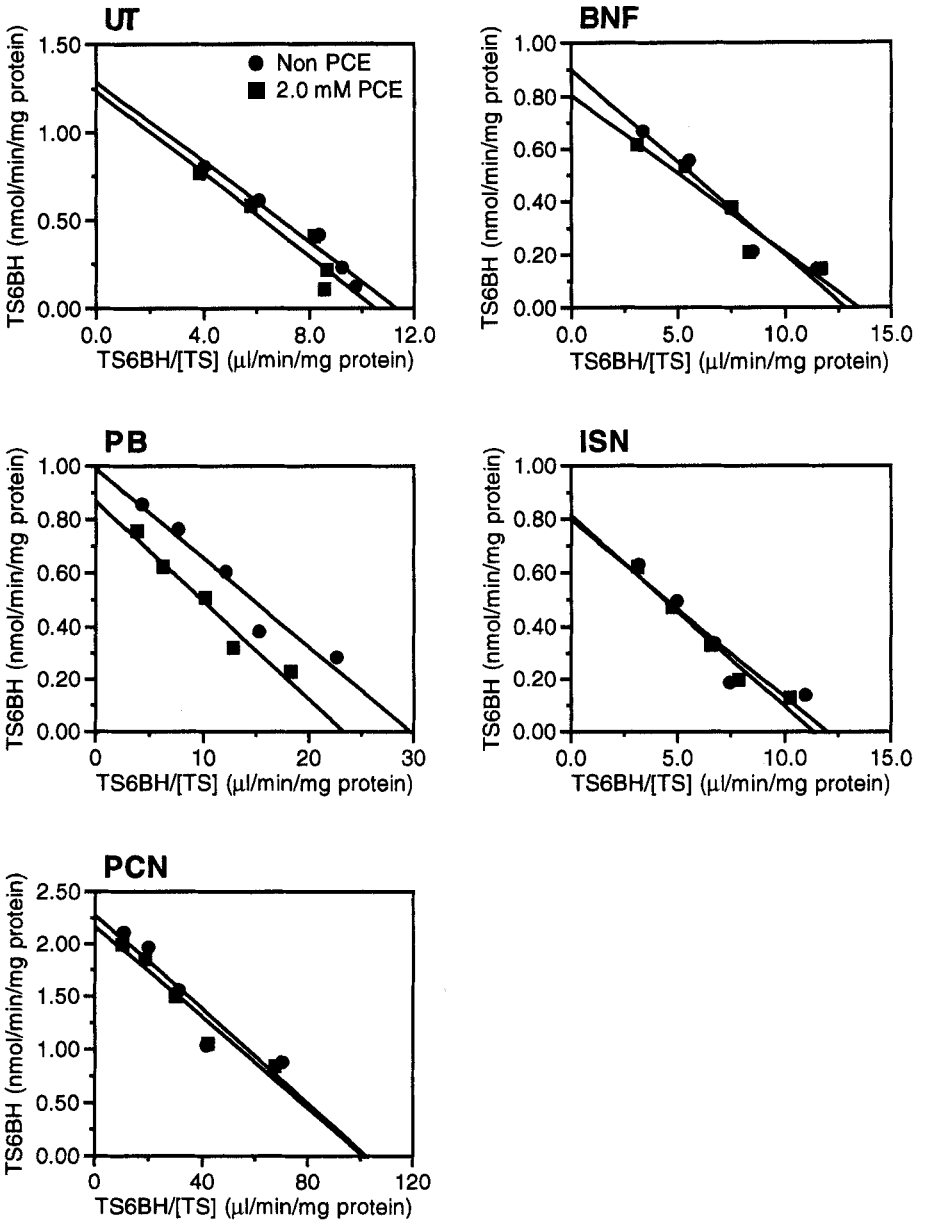

Figure 8. Inhibition of TS6BH kinetics in rat liver microsomes by PCE. Data were analysed by Eadie–Hofstee plots. Liver microsomes from untreated (UT), BNF-, PB-, ISN- and PCN-induced rat were assayed for TS6BH activity in the absence or presence of 2·0 mM PCE. Each point represents the mean of three determinations. The lines are computer-generated, best-fit curves from which the apparent kinetic parameters were derived.

Table 2. Inhibition of P450-dependent monooxygenase activities in rat liver microsomes by PCE.

	K_i (mM)				
	UT	BNF	PB	ISN	PCN
EROD	0·50 ± 0·06	> 5·00	2·20 ± 0·33	0·98 ± 0·02	1·57 ± 0·19
ECOD	ni	ni	0·83 ± 0·02	4·37 ± 1·06	> 5·00
PROD	1·50 ± 0·34	1·84 ± 0·23	0·16 ± 0·03	1·35 ± 0·18	1·48 ± 0·08
BROD	4·00 ± 1·28	2·76 ± 0·93	0·29 ± 0·03	1·87 ± 0·35	2·22 ± 0·41
AN4H	ni	3·00 ± 0·52	ni	> 5·00	> 5·00
TS6BH	ni	ni	> 5·00	ni	ni

K_i's for PCE were calculated from Eadie–Hofstee plots of inhibition kinetics.
Each value represents the mean ± SD of three determinations.
ni, Not inhibited; UT, untreatment; BNF, β-naphthoflavone; PB, phenobarbital; ISN, isoniazid; and PCN, pregnenolone 16α-carbonitrile.

PB-treated rat liver. However, the effect of PCE on these activities in untreated, BNF-, ISF- and PCE-treated rat was not observed. K_i's for the inhibition of PROD and BROD in PB-treated rat liver microsomes by PCE were lower than those of other microsomal preparations. Also, it has been reported that ECOD activity is catalysed by P4501A1 or 2B1 (Ryan *et al.* 1982, Ryan and Levin 1990). As shown in figure 5, the ECOD activity in PB-treated rat was inhibited by PCE and the K_i was lower than those of other microsomal preparations. However, this type of inhibition differed from those of PROD and BROD. This difference may be due to the substrate specificity of each enzyme. On the other hand, the inhibition rates by PCE of EROD associated with the P4501A1 was not striking in any of the rats studied. In this case, the K_i in the untreated rat was the lowest among the tested microsomes of rat livers, followed by the ISN-treated rat. With regard to the other P450-dependent monooxygenase activities assayed, AN4H and TS6BH in rat liver microsomes would indicate the induction of the P4502E1 and 3A2 subfamilies P450s (Waxman *et al.* 1985, Nagata *et al.* 1987, Levin 1990, Ryan and Levin 1990). Although these activities in liver microsomes were induced with ISN and PCN *in vivo*, no effects of PCE *in vitro* were apparent. Thus, PCE inhibits PB-inducible P450-dependent monooxygenase activities, indicating that PCE may have a high affinity for the P450s of 2B subfamily.

It has been suggested that the formation of an intermediate epoxide of PCE and trichloroethylene by P450-dependent monooxygenase is the initial step for their metabolic transformations in mammals (Henshler 1977). This epoxide can react with cellular nucleophiles such as DNA, protein or glutathione by alkylation or it may undergo intramolecular rearrangement, forming acid chlorides or chlorinated aldehydes. PCE has been tested for genotoxic proteins in short-term mutagenicity systems using various strains of *Staphylococcus typhimurium* (Baden *et al.* 1979, Bartsh *et al.* 1979, Oesch *et al.* 1983), *Escherichia coli* K12 (Greim *et al.* 1975) and *Saccharomyces cerevisiae* D7 (Bronzetti *et al.* 1981, 1984). However, trichloroethylene exhibited only weak mutagenicity, whereas PCE had no effect. Furthermore, it has been suggested that PCE is metabolized by the PB-inducible P450 system (Costa and Ivanetich 1980), whereas trichloroethylene is metabolized by P4502E1 in mouse and rat *in vitro* (Nakajima *et al.* 1990, 1993). The current study has indicated that PCE substantially inhibited P4502B1-dependent monooxygenases such as PROD and BROD in PB-induced rat liver microsomes. Therefore, the difference in mutagenicity between PCE and trichloroethylene may be due to

the associated P450 isozyme(s). Carcinogenesis studies in mouse have shown that orally administered PCE induces a high incidence of hepatocellular carcinomas, whereas in rat an increased tumour incidence was not apparent (National Cancer Institute 1977). PCE inhalation studies in rat and mouse showed that there was no increase in tumour frequency (Rampy *et al.* 1977). However, these negative results have to be considered in the light of limitations of the test system (Theiss *et al.* 1977, IARC 1979). Thus, since the carcinogenesis of PCE has not yet been clarified, P4502B1, which is associated with the metabolism of PCE, may be a key enzyme involved in bioactivation and carcinogenesis. Further studies are required to identify the relationships between PCE toxicities and P450.

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