J. Biochem. 107, 826-833 (1990)

Purification and Characterization of Two Forms of 2,3,4,7,8-Pentachlorodibenzofuran-Inducible Cytochrome P-450 in Hamster Liver[1]

Nobuyuki Koga, Noritaka Ariyoshi, Hiroshi Nakashima, and Hidetoshi Yoshimura[2]

Faculty of Pharmaceutical Sciences, Kyushu University, Higashi-ku, Fukuoka, Fukuoka 812

Received for publication, September 18, 1989

Two forms of cytochrome P-450 (P-450) from liver microsomes of hamsters treated with 2,3,4,7,8-pentachlorodibenzofuran (PenCDF), which possesses the potent acute toxicity and 3-methylcholanthrene (MC)-type inducing ability of liver microsomal monooxygenases in animals, were purified and characterized. These P-450 forms, designated as hamster P-450H and hamster P-450L, had the molecular masses of 52 and 50 kDa, respectively, and showed the absorption maximum of CO-reduced difference spectra at 446 nm. The absolute spectra of their oxidized forms indicated that hamster P-450H was in high-spin state and hamster P-450L was in low-spin state. A part of PenCDF injected into hamster was tightly bound to purified hamster P-450H at a ratio of 0.107 nmol PenCDF/nmol P-450. In a reconstituted system, both hamster P-450H and hamster P-450L showed relatively low catalytic activities for 3-hydroxylation of benzo[*a*]pyrene and *O*-deethylations of both 7-ethoxyresorufin and 7-ethoxycoumarin, while they both catalyzed 7α- and 2α-hydroxylations of testosterone effectively to a similar extent. Addition of cytochrome b_5 to a reconstituted system accelerated the formation of 7α-hydroxytestosterone 5.3-fold with hamster P-450L and 2.2-fold with hamster P-450H. In addition, hamster P-450H catalyzed estradiol 2-hydroxylation at a high rate but hamster P-450L did not. Immunochemical studies using antiserum to each P-450 form revealed that hamster P-450H and hamster P-450L differ from each other and comprise about 61 and 31% of the total P-450 in PenCDF-treated microsomes, respectively, indicating that these are PenCDF-inducible and major forms of P-450 in PenCDF-treated hamsters. Similarly to PenCDF, inducers such as MC, 3,4,5,3′,4′-pentachlorobiphenyl, and isosafrole also preferentially induced hamster P-450H rather than hamster P-450L, but β-naphthoflavone preferentially increased hamster P-450L. Phenobarbital, pregnenolone 16α-carbonitrile and ethanol did not affect the contents of these forms at all. Analyses of NH_2-terminal amino acid sequences demonstrated that hamster P-450H and hamster P-450L correspond to rat P-450d and rat P-450a, respectively.

2,3,4,7,8-Pentachlorodibenzofuran (PenCDF) is one of the most toxic polychlorinated dibenzofurans (PCDFs) and is also known to be an important causal agent of the disease Yusho, which occurred in the southwestern part of Japan in 1968 (*1*). PenCDF causes body weight loss, atrophies of thymus and spleen, and hyperlipidemia, and also possesses a potent 3-methylcholanthrene (MC)-type inducing ability of hepatic drug-metabolizing enzymes in rats (*1*). Animal species vary greatly in their sensitivity to halogenated aromatic hydrocarbons such as PCDFs, polychlorinated biphenyls (PCBs) and polychlorinated dibenzo-*p*-dioxins; hamsters are most insensitive, while guinea pigs are most sensitive (*2*). In spite of extensive studies (*3-6*), the mechanism of this species difference has not been elucidated yet.

Hamster liver has relatively higher basal levels of drug-metabolizing enzyme activities (*7-9*) and activates chemical carcinogens including benzo[*a*]pyrene (BP) (*10, 11*), phenacetin (*12*), and aflatoxin B_1 (*13*) more efficiently than the liver of other experimental animals. On the other hand, some recent studies have indicated that the induction mode of drug-metabolizing enzymes in hamsters differs from that in rats (*9, 14-16*). In particular, BP 3-hydroxylase, a typical enzyme activity induced in rats by MC-type inducers such as MC, β-naphthoflavone (BNF), and coplanar PCBs, is not stimulated in hamster liver by MC treatment (*9, 14, 16, 17*). Moreover, phenobarbital (PB) treatment markedly induced 7-pentoxyresorufin *O*-dealkylase in rats but not so much in hamsters (*14*). Recently, Watanabe *et al.* (*18*) have isolated two forms of P-450, P-450MC_I, and P-450MC_{II}, from liver microsomes of MC-treated hamsters and demonstrated that neither of

[1] This work was supported in part by a research grant from the Ministry of Health and Welfare of Japan.

[2] To whom reprint requests should be addressed.

Abbreviations: PenCDF, 2,3,4,7,8-pentachlorodibenzofuran; PCDFs, polychlorinated dibenzofurans; PCBs, polychlorinated biphenyls; P-450, cytochrome P-450; fp_2, NADPH-cytochrome *c* reductase; b_5, cytochrome b_5; MC, 3-methylcholanthrene; PB, phenobarbital; ISF, isosafrole; BP, benzo[*a*]pyrene; 7-ER, 2-ethoxyresorufin; 2-EC, 7-ethoxycoumarin; BNF, β-naphthoflavone; PCN, pregnenolone 16α-carbonitrile; DTT, dithiothreitol; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; M_r, apparent monomeric molecular weight as determined by SDS-PAGE; GC, gas chromatography.

them catalyzed BP 3-hydroxylation effectively or cross-reacted with antibody to the MC-induced rat hepatic P-450c. Similarly, Mizokami *et al.* and Fukuhara *et al.* have purified a low-spin form (P-450AFB) of P-450 in MC-treated hamsters, which is responsible for metabolic activation of aflatoxin B_1 (*13*) and have shown that this form was present in hamsters but not in rats, mice, or guinea pigs (*19*).

Recently, we have also observed that in hamsters, PenCDF treatment induced liver microsomal enzymes including P-450, BP 3-hydroxylase, and 7-ethoxyresorufin (7-ER) *O*-deethylase much less than in rats, and their relative ratios to the control ranged only from about 200 to 500% (*20*). In the present study, we have purified and characterized two forms of P-450 from liver microsomes of PenCDF-treated hamsters. The results indicate that they are similar to rat P-450a and P-450d, in terms of their catalytic activities and NH_2-terminal amino acid sequences.

MATERIALS AND METHODS

Chemicals—PenCDF was kindly donated by Dr. Masuda (Daiichi College of Pharmaceutical Sciences, Fukuoka). 3,4,5,3′,4′-Pentachlorobiphenyl (PenCB) was synthesized by the method of Saeki *et al.* (*21*). PB (Na salt) and isosafrole (ISF) were purchased from Tokyo Chemical Industry, Tokyo. BNF and MC were obtained from Aldrich Chemical, Milwaukee, Wis. and from Wako Pure Chemical Industries, Osaka, respectively. Pregnenolone-16α-carbonitrile (PCN) and Emulgen 913 were generous gifts from Upjohn Pharmaceutical, Tsukuba, and from Kao-Atlas, Tokyo, respectively. All other chemicals used were of the highest purity commercially available. For purification of P-450, ω-aminooctyl-Sepharose 4B was prepared according to the method of Nishikawa and Bailon (*22*) with some modifications. DE-52, hydroxylapatite, and 2′,5′-ADP-Sepharose 4B were purchased from Whatman, London, from Bio-Rad Lab., Richmond, Calif., and from Pharmacia, Uppsala, respectively.

Treatment of Animals and Preparation of Liver Microsomes—Male Golden Syrian hamsters (70-80 g, 5 wk old) were obtained from Kyudo, Ltd., Tosu, and given chow and water *ad libitum* during the experiment. PenCDF was injected i.p. into 22 hamsters at a single dose of 0.5 mg/2 ml corn oil/kg. Animals were killed 5 days after the injection. The liver microsomes were prepared as described elsewhere (*23*) and used for the purification of P-450 and cytochrome b_5 (b_5). The inducers MC, BNF, PB, ISF, and PCN were injected i.p. daily for 4 days at a dose of 25, 80, 80, 150, and 25 mg/kg, respectively. They were dissolved in corn oil (2 ml/kg) except that PB (Na salt) was dissolved in saline. Ethanol-treated hamsters were given 10% (v/v) ethanol in drinking water for 10 days. All animals were killed after starvation for 12 h and liver microsomes were prepared as described above.

Purification of Hamster P-450H and Hamster P-450L—As shown in Fig. 1, microsomes were solubilized with 1.0% sodium cholate in 0.1 M potassium phosphate buffer (pH 7.25) containing 20% glycerol, 1 mM EDTA, and 0.1 mM dithiothreitol (DTT) (buffer A). The solubilized microsomes were applied to an ω-aminooctyl-Sepharose 4B column (4×18 cm) equilibrated with buffer A containing 0.5% sodium cholate (buffer B). After washing of the column with buffer B, P-450 was eluted as two peaks (peak I and II) with buffer B containing 0.5% Emulgen 913, by monitoring the absorbance at 395 and 416 nm. These two fractions were separately pooled, concentrated, and dialyzed against 10 mM potassium phosphate buffer (pH 7.7) containing 20% glycerol, 1 mM EDTA, 0.1 mM DTT, 0.5% sodium cholate, and 0.2% Emulgen 913 (buffer C). Each fraction was then applied to a DE-52 column equilibrated

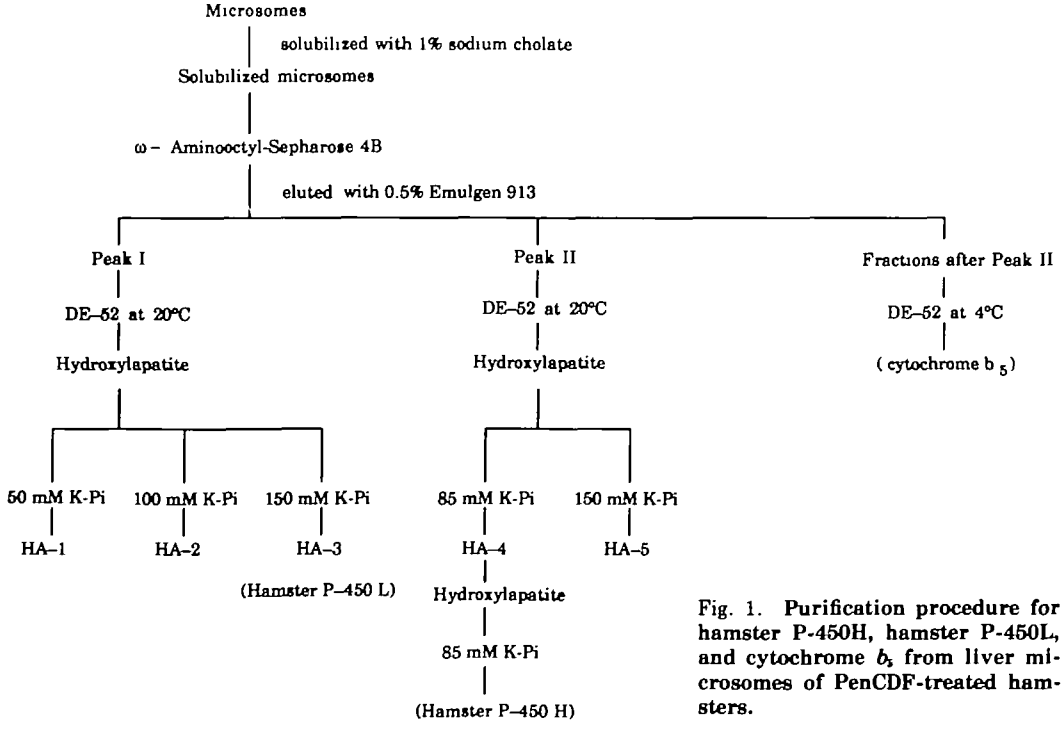

Fig. 1. **Purification procedure for hamster P-450H, hamster P-450L, and cytochrome b_5 from liver microsomes of PenCDF-treated hamsters.**

with buffer C. The size of the DE-52 column used was 3× 30 cm for peak I and 3×15 cm for peak II. In each case, most of the P-450 was recovered in the excluded fraction and subsequently applied to a hydroxylapatite column (2× 10 cm for peak I, and 2×6 cm for peak II) equilibrated with 10 mM potassium phosphate buffer (pH 7.25) containing 20% glycerol, 0.1 mM DTT, 0.5% sodium cholate, and 0.2% Emulgen 913 (buffer D). The enzymes from peak I were eluted stepwise with potassium phosphate in buffer D to give three peaks (Fig. 2A). P-450 designated as hamster P-450L was obtained in the 150 mM potassium phosphate fraction. On the other hand, the other P-450 form designated as hamster P-450H was eluted in the 85 mM potassium phosphate fraction (Fig. 2B) and applied to a second hydroxylapatite column for further purification. Each purified enzyme migrated as a single band on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), which was performed according to the method of Lugtenberg *et al.* (*24*). The concentration of acrylamide of the lower gel was 11%. The contents of P-450 were determined by the method of Omura and Sato (*25*).

Purification of NADPH-Cytochrome c Reductase (fp_2) and Cytochrome b_5 (b_5)—fp_2 for reconstitution of monooxygenase was purified from liver microsomes of PB-treated Sprague-Dawley rats according to the method of Shephard *et al.* (*26*). The specific activity of the enzyme was 22.2 units/mg protein; one unit was defined as the activity reducing 1 μmol of cytochrome *c* per min at 25°C in 0.1 M phosphate buffer (pH 7.5) by the method of Omura and Takesue (*27*). Hamster b_5 was obtained from a broad fraction following peak II on the ω-aminooctyl-Sepharose 4B column and further purified by the method of Spatz and Strittmatter (*28*) with some modifications. The specific content of the enzyme measured by the method of Takesue and Omura (*29*) was 14.0 nmol/mg protein.

Assays of Monooxygenase Activities—The reconstituted system contained 0.1 nmol of purified P-450, 0.5 unit of purified rat fp_2, 15 μg of dilauroylphosphatidylcholine, NADPH-generating system (0.2 mM NADP, 5 mM glucose 6-phosphate, and 0.1 unit of glucose 6-phosphate dehydrogenase), 3 mM $MgCl_2$, substrate, and 100 mM potassium phosphate buffer (pH 7.5) in a final volume of 1 ml. The concentrations of various substrates used in this study were as described previously (*30*). After sonication for 1 min and preincubation for 1 min at 37°C, the reaction was initiated by addition of the NADPH-generating system.

NH_2-Terminal Amino Acid Sequence Analysis—The NH_2-terminal amino acid sequencing was performed by automated Edman degradation using a Model 470A gas phase sequencer (Applied Biosystems, U.S.A.) equipped with an on-line PTH amino acid analyzer. Prior to analyses, 1.0 nmol of each purified P-450 was dialyzed twice against 2,000 volumes of distilled water for 8 h and washed with 70% ethanol to remove glycerol and free amino acids.

Preparation of Antibodies against Hamster P-450H and Hamster P-450L—About 150 μg of each purified P-450 in Freund's complete adjuvant was injected into the foot pads of rabbits. Every week, the rabbits were boosted by subcutaneous injection of the same amount of antigen. They were bled 4 wk after the first injection, and antisera were collected.

Immunological Analyses—Single radial immunodiffusion analyses for quantitation of hamster P-450H and hamster P-450L in liver microsomes were performed according to the method of Thomas *et al.* (*31*). Immunoblotting of liver microsomes from untreated and inducer-treated hamsters was conducted according to the method of Guengerich *et al.* (*32*) except that 0.05% Triton X-100 instead of 10% (v/v) calf serum was used as the blocking buffer and also that 0.05% Triton X-100 was added to the buffer for washing of the nitrocellulose sheet.

Protein Determination—Protein concentrations of the preparations containing Emulgen 913 and DTT were estimated by the method of Smith *et al.* (*33*), and in other cases the method reported by Lowry *et al.* (*34*) was used. In both methods, bovine serum albumin was used as a standard.

Quantification of PenCDF Content in Hamster P-450H and Hamster P-450L by Gas Chromatography (GC)—PenCDF bound to 100 nmol of the purified enzyme was extracted three times with 2 ml of *n*-hexane, cleaned up on a Wakogel S-I column (5×20 mm) and analyzed by a gas chromatograph (Shimadzu GC-3BE, Kyoto) fitted with an electron capture detector. A glass column (3 mm×1.2 m) packed with Chromosorb W AWCS (60-80 mesh) coated with 1.5% OV-17 was used. The other conditions used were

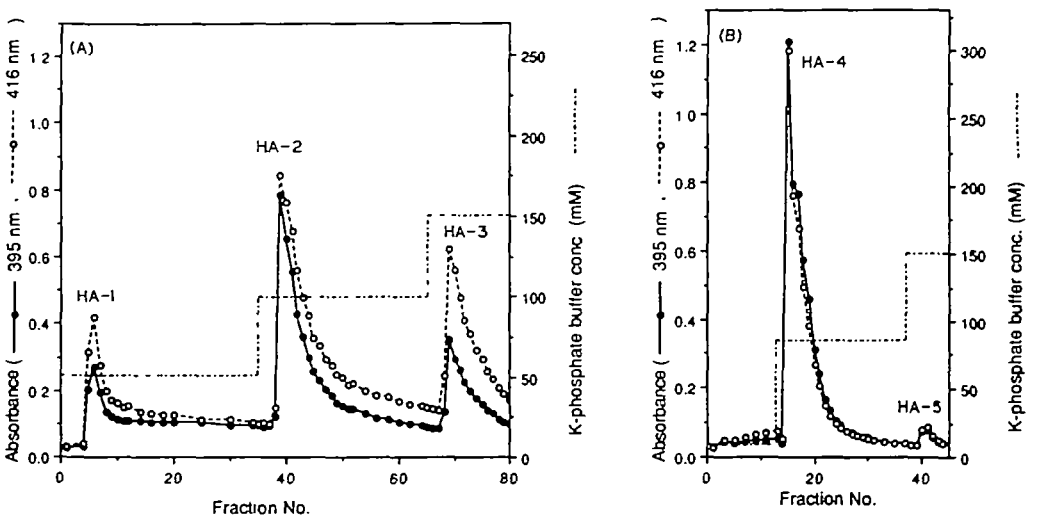

Fig. 2. Elution profile of hamster P-450L (A) and hamster P-450H (B) fractions from the first hydroxylapatite column. Each P-450 eluted in the excluded fraction of DE-52 columns was subjected to hydroxylapatite column chromatography as described in "MATERIALS AND METHODS."

as follows: column temp., 260°C; injection temp., 280°C; carrier gas, N_2; flow rate, 40 ml/min.

RESULTS

Purification and Properties of Two Forms of Liver Cytochrome P-450 from PenCDF-Treated Hamsters—The purification of two forms of P-450 was performed as described in "MATERIALS AND METHODS." Figure 2 shows the elution profile of each fraction from the first hydroxylapatite column. Finally, hamster P-450L and hamster P-450H were obtained from fractions HA-3 and HA-4, respectively. As shown in Table I, the specific contents of purified enzymes were 12.8 nmol/mg protein for hamster P-450H and 7.73 nmol/mg protein for hamster P-450L. The yield was 5.1% for hamster P-450H and 8.1% for hamster P-450L. P-450 eluted in fraction HA-2 was found to be hamster P-450H from the absolute spectrum of the oxidized form and the result of SDS-PAGE, but was not subjected to further purification. The other fractions, HA-1 and HA-5, were not characterized because of their low contents of P-450. SDS-PAGE of purified hamster P-450H and hamster P-450L showed that they were homogeneous and had molecular masses of 52 and 50 kDa, respectively (Fig. 3).

Figure 4 shows the absolute spectra of the oxidized and reduced forms, and CO-reduced difference spectra of hamster P-450H and hamster P-450L. Hamster P-450L was so unstable that the absorbance at 416 nm rapidly increased on measuring the CO-reduced difference spectra at room temperature. Therefore, prior to the reduction of hamster P-450L with $Na_2S_2O_4$, the enzyme and the buffer were kept cold in an ice bath. The absorption maximum of the CO-reduced difference spectra was seen at 446 nm for both hamster P-450L and hamster P-450H. The Soret peaks of oxidized forms of hamster P-450L were at 415 nm, indicating that hamster P-450L was in a low-spin state. On the other hand, the absorbance peak at 394 nm and shoulder at 417 nm in the absolute oxidized spectrum indicated that hamster P-450H was composed of both high- and low-spin components. Although the exact proportion of both components is unclear, it appears that a high-spin hemeprotein is predominant.

Catalytic Activities of Hamster P-450H and Hamster

TABLE I. **Purification of two forms of cytochrome P-450 from liver microsomes of PenCDF-treated hamsters.**

Step	Total protein (mg)	Total content (nmol)	Specific content (nmol/mg)	Yield (%)
Microsomes	2,015	2,825	1.402	100
Solubilized microsomes	1,485	2,643	1.780	94
ω-Aminooctyl-Sepharose 4B				
P-450H fraction	139	796	5.74	28
P-450L fraction	825	1,530	1.85	54
DE-52				
P-450H fraction	85.5	537	6.28	19
P-450L fraction	208	878	4.22	31
First hydroxylapatite				
P-450H fraction	29.9	277	9.25	9.8
P-450L fraction	29.7	230	7.73	8.1
Second hydroxylapatite				
P-450H fraction	11.2	144	12.8	5.1

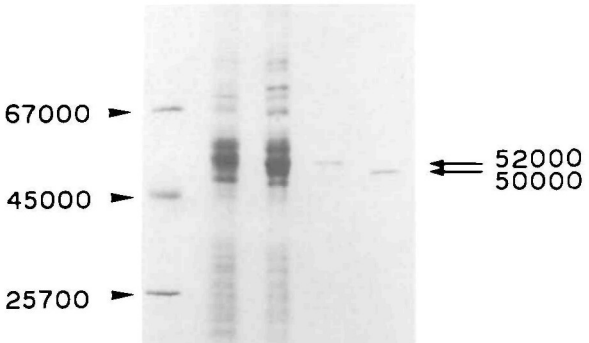

Fig. 3. **SDS-PAGE of liver microsomes of hamsters and two forms of purified P-450.** Lane A, microsomes from untreated hamsters; lane B, microsomes from PenCDF-treated hamsters; lane C, purified hamster P-450H; lane D, purified hamster P-450L. Electrophoresis was carried out according to the method of Lugtenberg *et al.* (*24*) using 11% gel containing 0.1% SDS. Each lane contained 20 μg of microsomes or about 0.5 μg of purified enzyme. The molecular weight standards used were bovine serum albumin (M_r 67,000), ovalbumin (M_r 45,000), and chymotrypsinogen A (M_r 25,700).

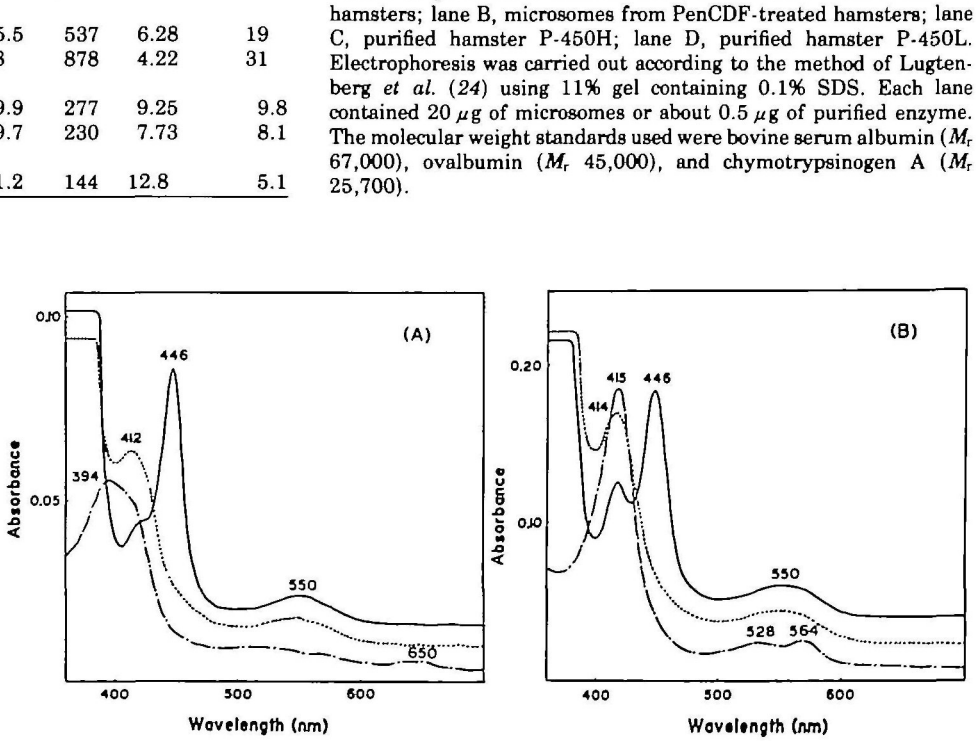

Fig. 4. **Absolute, and CO-reduced difference spectra of hamster P-450H (A) and hamster P-450L (B) from PenCDF-treated hamsters.** Each purified enzyme was dissolved in 100 mM potassium phosphate buffer, (pH 7.25) containing 20% glycerol and 0.2% Emulgen 913. The oxidized (———) and reduced (·····) spectra were recorded with the reference cuvettes containing the same buffer as the sample. The CO-reduced difference spectra (——) were generated according to the method of Omura and Sato (*25*).

TABLE II. **Catalytic activities of hamster P-450H and hamster P-450L purified from liver microsomes of PenCDF-treated hamsters in the absence or presence of cytochrome b_5.** The activities are expressed as nmol product formed/min/nmol P-450. The amount of b_5 added to a reconstituted system was 0.1 nmol. The figures in parentheses are the relative ratio to those in the absence of b_5.

Substrate	Concentration (mM)	$-b_5$		$+b_5$	
		P-450H	P-450L	P-450H	P-450L
Aminopyrine	1	7.1	2.0	6.9 (0.9)	2.0 (1.0)
Benzphetamine	1	2.8	6.5	2.6 (0.9)	5.8 (0.9)
Ethylmorphine	1	2.8	1.6	2.8 (1.0)	1.1 (0.7)
Benzo[*a*]pyrene	80	0.2	0.5	0.3 (1.5)	0.7 (1.4)
7-Ethoxyresorufin	3	0.3	<0.1	0.4 (1.3)	<0.1
7-Ethoxycoumarin	0.125	5.1	4.6	11.1 (2.2)	11.4 (2.5)
p-Nitroanisole	0.4	3.2	2.5	4.6 (1.4)	5.6 (2.2)
Testosterone	0.14				
7α-OH		0.20	0.22	0.44 (2.2)	1.15 (5.3)
16α-OH		0.06	N.D.	0.03 (0.5)	N.D.
6β-OH		0.05	N.D.	0.04 (0.9)	N.D.
2α-OH		0.24	0.29	0.27 (1.1)	0.41 (1.4)
androstene-3,17-dione		0.10	0.07	0.12 (1.2)	0.10 (1.4)
Estradiol-17β	0.15				
2-OH		0.70	N.D.[a]	1.16 (1.7)	N.D.

[a]N.D., not detected.

CYTOCHROME	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
Hamster P-446H	Ala	Leu	Ser	Gln	Tyr	Thr	Ser	Leu	Ser	Thr	Glu	Leu	Val	Leu	Ala	Thr	Ala	Ile
Hamster P-446L	Met	Leu	Val	Ser	Gly	Met	Leu	Leu	Val	Val	Val	Leu	X	X	Leu			
Rat P-450a	Met	Leu	Asp	Thr	Gly	Leu	Leu	Leu	Val	Val	Ile	Leu	Ala	Ser	Leu	Ser	Val	Met
Rat P-450c	Pro	Ser	Val	Tyr	Gly	Phe	Pro	Ala	Phe	Thr	Ser	Ala	Thr	Glu	Leu	Leu	Leu	Ala
Rat P-450d	Ala	Phe	Ser	Gln	Tyr	Ile	Ser	Leu	Ala	Pro	Glu	Leu	Leu	Leu	Ala	Thr	Ala	Ile

Fig. 5. **Comparison of NH_2-terminal amino acid sequence of hamster P-450H and hamster P-450L with those of rat P-450 forms, P-450a, P-450c, and P-450d (*35*, *36*).** The underlined amino acids represent significant homology between hamster P-450 and rat P-450. X indicates an unidentified amino acid.

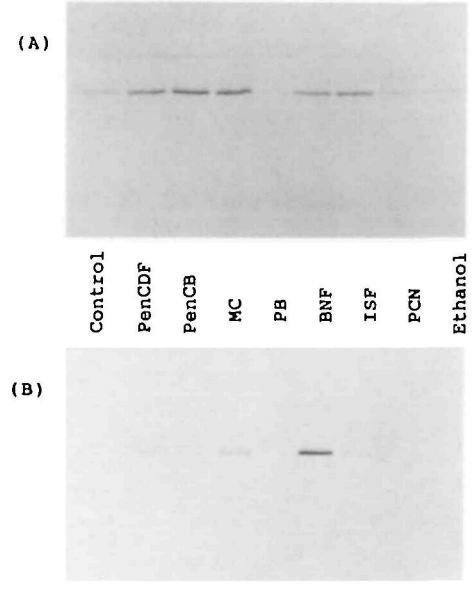

Fig. 6. **Immunoblot analyses of liver microsomes from untreated and inducer-treated hamsters.** Each well contained 5 μg (untreated) or 1 μg (inducer-treated) of microsomal protein. The proteins were transferred to the nitrocellulose sheet, treated with rabbit antisera against hamster P-450H (A) and hamster P-450L (B), and further stained with peroxidase and anti-peroxidase complex using 3,3′-diaminobenzidine as a substrate for peroxidase.

P-450L in a Reconstituted System—Table II shows the catalytic activities of the two forms of hamster P-450 toward various substrates in a reconstituted system. The effects of addition of hamster b_5 on these catalytic activities are also shown in Table II. Hamster P-450H and hamster P-450L had relatively low activity for BP 3-hydroxylation and *O*-deethylation of both 7-ER and 7-EC, which are typical activities markedly induced by MC-type inducers in rats, although the activity of 7-EC *O*-deethylation was increased about 2-fold by addition of b_5. Each enzyme also showed catalytic activity for *N*-demethylations of aminopyrine, benzphetamine, and ethylmorphine and *O*-demethylation of *p*-nitroanisole but the activities were not characteristic and these activities were not affected by addition of b_5, except that *O*-demethylation of *p*-nitroanisole was stimulated 2.2-fold. On the other hand, hamster P-450H and hamster P-450L were considerably active in the hydroxylation of testosterone at 7α- and 2α-positions. In particular, when b_5 was added to the system, 7α-hydroxylation was accelerated 5.3-fold with hamster P-450L and 2.2-fold with hamster P-450H. Hamster P-450H also catalyzed estradiol 2-hydroxylation at a relatively high rate but hamster P-450L did not. This activity was elevated 1.7-fold by addition of b_5.

NH_2-Terminal Amino Acid Sequences of Hamster P-450H and Hamster P-450L—The NH_2-terminal amino acid sequences of hamster P-450H and hamster P-450L were compared to those of the previously reported rat P-450 forms, P-450a, P-450c, and P-450d (*35*, *36*), which

TABLE III. **Immunochemical quantitation of hamster P-450H and hamster P-450L in liver microsomes of untreated and PenCDF-treated hamsters.** Each value represents the mean ± SD of triplicate determinations, and the figures in parentheses are percent of total P-450 content in microsomes.

Treatment	Total P-450	Hamster P-450H (nmol/mg protein)	Hamster P-450L
Untreated	1.030±0.183 (100)	0.115±0.020 (15)	N.D.[a]
PenCDF-treated	1.355±0.075 (100)	0.821±0.010 (61)	0.420±0.076 (31)

[a]N.D., not detected.

were forms of P-450 markedly induced by treatment of rats with MC-type inducers (*37, 38*) (see Fig. 5). Hamster P-450H began with alanine and had 13/18 identical amino acids at the NH_2 terminal with rat P-450d reported previously by Botelho *et al.* (*35*). The result obtained by the Ouchterlony double diffusion method that hamster P-450H and rat P-450d cross-reacted with antisera against both enzymes supported this structural similarity (data not shown). On the other hand, hamster P-450L began with Met-Leu and the degree of sequence homology with rat P-450a was quite high (60% homology), whereas only 20% sequence homology was observed between hamster P-450L and rat P-450c. Moreover, the sequence data showed that hamster P-450H and hamster P-450L are different molecular species.

Detection of Hamster P-450H and Hamster P-450L in Liver Microsomes from Untreated and Inducer-Treated Hamsters—Hamster P-450H and hamster P-450L in liver microsomes were detected by Western blotting (Fig. 6). In untreated hamsters, hamster P-450L was not detected, whereas hamster P-450H was observed at a relatively high concentration. In inducer-treated hamsters, hamster P-450H and hamster P-450L were markedly increased by induction with MC, PenCB, BNF, or ISF as well as PenCDF. Although BNF treatment induced hamster P-450L much more than hamster P-450H, other inducers showed preferential induction of hamster P-450H. The treatment with PB, PCN, or ethanol did not significantly alter the content of either enzyme. In addition, it is apparent from these immunoblot analyses that hamster P-450H and hamster P-450L are not immunochemically identical.

Immunochemical Quantitation of Hamster P-450H and Hamster P-450L in Hamster Liver Microsomes—SDS-PAGE and immunoblot analyses using antiserum to each enzyme showed remarkable increases of both forms of P-450 by PenCDF. Therefore, the contents of hamster P-450H and hamster P-450L in liver microsomes of untreated and PenCDF-treated hamsters were determined by single radial immunodiffusion analyses as shown in Table III. Hamster P-450H accounted for about 15% of total P-450 in untreated microsomes and was elevated to 61% of total P-450 by PenCDF treatment. Although hamster P-450L was not detected in untreated microsomes, it comprised 31% of total P-450 in PenCDF-treated microsomes. These results confirmed that both hamster P-450H and hamster P-450L are PenCDF-inducible.

Quantitation of PenCDF Bound to Hamster P-450H and Hamster P-450L by GC—Several inducers such as ISF, MC, and PenCDF bind to a high-spin form of P-450 purified from rats and rabbits (*39-42*). Therefore, each purified enzyme preparation was treated with *n*-hexane, and the amount of PenCDF in this extract was quantified by GC. Hamster P-450H contained PenCDF at a much higher level than hamster P-450L. The amounts of PenCDF found were 0.107 nmol per nmol of hamster P-450H and 0.004 nmol per nmol of hamster P-450L.

DISCUSSION

In the present study, we purified two forms of P-450 from liver microsomes of PenCDF-treated hamsters. The molecular masses, spectral properties, immunological properties and NH_2-terminal amino acid sequences of both forms of P-450 showed that they are distinct molecular species. In addition, immunological studies demonstrated that hamster P-450H and hamster P-450L are both PenCDF-inducible and accounted for 61 and 31% of total P-450 in liver microsomes of PenCDF-treated hamsters, respectively. Comparison of NH_2-terminal amino acid sequences of hamster P-450H and hamster P-450L with those of three PenCDF-inducible forms of rat P-450 (P-450a, P-450c, and P-450d) showed that hamster P-450H and hamster P-450L had considerable structural similarity to rat P-450d and rat P-450a, respectively. Although hamster P-450L was thought to correspond to rat P-450c in terms of the spin

TABLE IV. **Comparison of properties of hamster P-450H and hamster P-450L with those of several forms isolated in other laboratories.**

P-450	Inducer	Molecular mass (kDa)	Spin state	Reduced CO-complex λ_{max} (nm)	Reference
P-450H	PenCDF	52	high+low	446	This study
P-450L	PenCDF	50	low	446	This study
P-450MCI	MC	56	high	446.5	(*18*)
P-450MCII	MC	53.5	low	446.5	(*18*)
P-450III	MC	59.5	low	448.0	(*19*)
P-450II	MC	58.5	high	447	(*19*)
P-450AFB	MC	56	low	448.5	(*13, 19*)
P-450IA2	BNF	52.5	high	447	(*50*)
Form 1	MC	48.5	—[a]	—	(*49*)
Form 2	PB	50	—	—	(*49*)
Form 4	MC	53.5	—	—	(*49*)

[a]—, not determined.

state and the absorption maximum of the reduced CO-complex, it was greatly different from rat P-450c in NH_2-terminal amino acid sequence.

Hamster P-450H and hamster P-450L did not show high catalytic activity for exogeneous substrates including BP, 7-ER, 7-EC, and benzphetamine used in this study. However, both enzymes catalyzed hydroxylations of testosterone at the 7α- and 2α-positions to similar extents. Moreover, hamster P-450H catalyzed estradiol 2-hydroxylation at a rather high rate, supporting the similarity of hamster P-450H to rat P-450d (*23, 43*). It is noteworthy that addition of b_5 to a reconstituted system resulted in a 5.3-fold increase of testosterone 7α-hydroxylation activity catalyzed by hamster P-450L. This result suggested the similarity of hamster P-450L to rat P-450a, which is induced by MC-type inducers and also shows high catalytic activity for 7α-hydroxylation of testosterone (*44, 45*).

A number of investigators have shown that addition of purified b_5 to the reconstituted system brings about stimulation or inhibition of metabolism, depending on the forms of P-450 involved, the substrate used, and the assay conditions (*46-48*). In the present study, we observed that addition of an equimolar quantity of b_5 stimulated several reactions such as testosterone 2α-hydroxylation and 7-EC *O*-deethylation by both enzymes, *p*-nitroanisole *O*-demethylation by hamster P-450L, and also estradiol 2-hydroxylation by hamster P-450H in addition to a potent acceleration of testosterone 7α-hydroxylation by hamster P-450L. On the contrary, an inhibitory effect of b_5 was observed in testosterone 16α-hydroxylation catalyzed by hamster P-450H. These results suggest the significant involvement of b_5 in testosterone metabolism, although the mechanism is not clear at present.

Table IV shows a comparison of the properties of hamster P-450H and hamster P-450L with those of other hamster hepatic P-450 reported so far. Although there were some differences among the hamster P-450 preparations in the molecular masses and the spectral data, the low catalytic activities for BP 3-hydroxylation, 7-EC *O*-deethylation, and benzphetamine *N*-demethylation were common to all P-450 preparations (*13, 18, 19, 49*). Recently, Raucy *et al.* (*50*) have purified a high-spin form of P-450 (hamster P-450IA2) from liver microsomes of BNF-treated hamsters and found that the NH_2-terminal sequence of the first 18 amino acid residues of this enzymes showed 89% homology to rat P-450d. The comparison of our sequence data of hamster P-450H with theirs indicated a difference in 3 amino acid residues. This result might indicate the existence of microheterogeneity between hamster P-450H and P-450IA2. On the other hand, quite recently, Fukuhara *et al.* (*51*) have reported the complete cDNA sequence of P-450AFB, which is MC-inducible and responsible for the activation of aflatoxin B_1. The amino acid sequence deduced from P-450AFB cDNA showed 65% homology with that of rat P-450a. When comparing the amino acid sequence at the NH_2 terminal, the sequence of 13 amino acid residues of hamster P-450L was in complete agreement with their data.

It is of interest that hamster P-450H comprises about 15% of total P-450 in untreated microsomes (Table III). Since in rats or in chickens the amount of the corresponding P-450, rat P-450d and chicken P-448H, accounted for only about 5% and less than 3% of total P-450 in untreated microsomes, respectively (*39, 30*), such a high content of this form is characteristic of hamsters.

The determination of PenCDF content in purified enzymes revealed that hamster P-450H also contained a much higher concentration of PenCDF (0.107 nmol/nmol P-450) than did hamster P-450L. We previously reported in rats that PenCDF is distributed almost exclusively to the liver (*52*) and bound noncovalently but tightly to a high-spin form, P-448H (rat P-450d), among three PenCDF-inducible forms of P-450, at a molar ratio of 0.86 (*41*). Considering these results, the affinity of PenCDF to hamster P-450H appears to be lower than that to rat P-448H. However, since a high level of PenCDF (48% of dose) was detected in hamster liver 5 days after a single i.p. injection (*20*), hamster P-450H might contribute, at least in part, to the retention of PenCDF in the liver.

REFERENCES

1. Yoshihara, S., Nagata, K., Yoshimura, H., Kuroki, H., & Masuda, Y. (1981) *Toxicol. Appl. Pharmacol.* **59**, 580-588
2. Poland, A. & Knutson, J.C. (1982) *Annu. Rev. Pharmacol. Toxicol.* **22**, 517-554
3. Olson, J.R., Holscher, M.A., & Neal, R.A. (1980) *Toxicol. Appl. Pharmacol.* **55**, 67-78
4. Gasiewicz, T.A. & Rucci, G. (1984) *Mol. Pharmacol.* **26**, 90-98
5. Gasiewicz, T.A., Rucci, G., Henry, E.C., & Baggs, R.B. (1986) *Biochem. Pharmacol.* **35**, 2737-2742
6. Henry, E.C. & Gasiewicz, T.A. (1987) *Toxicol. Appl. Pharmacol.* **89**, 165-174
7. Burke, M.D. & Mayer, R.T. (1974) *Drug Metab. Dispos.* **2**, 583-588
8. Litterst, C.L., Mimnaugh, E.G., Reagan, R.L., & Gram, T.E. (1975) *Drug. Metab. Dispos.* **3**, 259-265
9. Thorgeirsson, S.S., Atlas, S.A., Boobis, A.R., & Felton, J.S. (1979) *Biochem. Pharmacol.* **28**, 217-226
10. Schreiber, H., Martin, D.H., & Pazmino, N. (1975) *Cancer Res.* **35**, 1654-1661
11. Mass, M.J. & Kaufman, D. (1983) *Carcinogenesis* **4**, 297-303
12. Nohmi, T., Yoshikawa, K., Nakadate, M., & Ishidate, M., Jr. (1983) *Biochem. Biophys. Res. Commun.* **110**, 746-752
13. Mizokami, K., Nohmi, T., Fukuhara, M., Takanaka, A., & Omori, Y. (1986) *Biochem. Biophys. Res. Commun.* **139**, 466-472
14. Blaich, G., Gottlicher, M., Cikryt, P., & Metzler, M. (1988) *Chem. Biol. Interact.* **67**, 129-138
15. Wroblewski, V.J. & Olson, J.R. (1988) *Drug Metab. Dispos.* **16**, 43-51
16. Wroblewski, V.J., Gessner, T., & Olson, J.R. (1988) *Biochem. Pharmacol.* **37**, 1509-1517
17. Burke, M.D. & Prough, R.A. (1976) *Biochem. Pharmacol.* **25**, 2187-2195
18. Watanabe, M., Fujii, H., Sagami, I., & Tanno, M. (1987) *Arch. Toxicol.* **60**, 52-58
19. Fukuhara, M., Nohmi, T., Mizokami, K., Sunouchi, M., Ishidate, M., Jr., & Takanaka, A. (1989) *J. Biochem.* **106**, 253-258
20. Koga, N., Nakashima, H., Kamimura, H., Hokama, Y., & Yoshimura, H. (1989) *Fukuoka Acta Med.* (in Japanese) **80**, 227-234
21. Saeki, S., Yoshihara, S., Uchino, Y., & Yoshimura, H. (1979) *Fukuoka Acta Med.* (in Japanese) **70**, 85-87
22. Nishikawa, A.H. & Bailon, P. (1975) *Anal. Biochem.* **64**, 268-275
23. Nagata, K., Buppodom, P., Matsunaga, T., Ishimatsu, M., Yamato, H., Yoshihara, S., & Yoshimura, H. (1985) *J. Biochem.* **97**, 1755-1766
24. Lugtenberg, B., Meijers, J., Peters, R., Hoek, P., & Alphen, L. (1975) *FEBS Lett.* **58**, 254-260
25. Omura, T. & Sato, R. (1964) *J. Biol. Chem.* **239**, 2370-2378
26. Shephard, E.A., Pike, S.F., Rabin, B.R., & Phillips, I.R. (1983) *Anal. Biochem.* **129**, 430-433

27. Omura, T. & Takesue, S. (1970) *J. Biochem.* **67**, 249-257
28. Spatz, L. & Strittmatter, P. (1971) *Proc. Natl. Acad. Sci. U.S.* **68**, 1042-1046
29. Takesue, S. & Omura, T. (1970) *J. Biochem.* **67**, 259-266
30. Hokama, Y., Koga, N., & Yoshimura, H. (1988) *J. Biochem.* **104**, 355-361
31. Thomas, P.E., Korzeniwsky, D., Ryan, D.E., & Levin, W. (1979) *Arch. Biochem. Biophys.* **192**, 524-532
32. Guengerich, F.P., Wang, P., & Davidson, N.K. (1982) *Biochemistry* **21**, 1698-1706
33. Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., & Klenk, D.C. (1985) *Anal. Biochem.* **150**, 76-85
34. Lowry, O.H., Rosebrough, N.J., Farr, A.L., & Randall, R.J. (1951) *J. Biol. Chem.* **193**, 265-275
35. Botelho, L.H., Ryan, D.E., Yuan, P.-M., Kutny, R., Shively, J.E., & Levin, W. (1982) *Biochemistry* **21**, 1152-1155
36. Haniu, M., Ryan, D.E., Lieber, S., Levin, W., & Shively, J.E. (1984) *Arch. Biochem. Biophys.* **235**, 304-311
37. Thomas, P.E., Reik, L.M., Ryan, D.E., & Levin, W. (1983) *J. Biol. Chem.* **258**, 4590-4598
38. Parkinson, A., Safe, S.H., Robertson, L.W., Thomas, P.E., Ryan, D.E., Reik, L.M., & Levin, W. (1983) *J. Biol. Chem.* **258**, 5967-5976
39. Ryan, D.E., Thomas, P.E., & Levin, W. (1980) *J. Biol. Chem.* **255**, 7941-7955
40. Imai, Y., Hashimoto-Yutsudo, C., Satake, H., Girardin, A., & Sato, R. (1980) *J. Biochem.* **88**, 589-503
41. Kuroki, J., Koga, N., & Yoshimura, H. (1986) *Chemosphere* **15**, 731-738
42. Voorman R. & Aust, S.D. (1987) *Toxicol. Appl. Pharmacol.* **90**, 69-78
43. Ryan, D.E., Thomas, P.E., Reik, L.M., & Levin, W. (1982) *Xenobiotica* **12**, 727-744
44. Wood, A.W., Ryan, D.E., Thomas, P.E., & Levin, W. (1983) *J. Biol. Chem.* **258**, 8839-8847
45. Sonderfan, A.J., Arlotto, M.P., Dutton, D.R., McMillen, S.K., & Parkinson, A. (1987) *Arch. Biochem. Biophys.* **255**, 27-41
46. Kuwahara, S. & Omura, T. (1980) *Biochem. Biophys. Res. Commun.* **96**, 1562-1568
47. Chiang, J.Y.L. (1981) *Arch. Biochem. Biophys.* **211**, 662-673
48. Levin, W., Thomas, P.E., Oldfield, N., & Ryan, D.E. (1986) *Arch. Biochem. Biophys.* **248**, 158-165
49. Chiang, J.Y.L. & Steggles, A.W. (1983) *Biochem. Pharmacol.* **32**, 1389-1397
50. Raucy, J.L., Lasker, J.M., Lieber, C.S., & Black, M. (1989) *Arch. Biochem. Biophys.* **271**, 270-283
51. Fukuhara, M., Nagata, K., Mizokami, K., Yamazoe, Y., Takanaka, A., & Kato, R. (1989) *Biochem. Biophys. Res. Commun.* **162**, 265-272
52. Yoshimura, H., Kuroki, J., Koga, N., Kuroki, H., Masuda, Y., Fukasaku, N., & Hasegawa, M. (1984) *J. Pharmacobio-Dyn.* **7**, 414-419