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TWO-STEP PURIFICATION OF CYTOCHROME P-450 FROM RAT LIVER MICROSOMES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

Cytochrome P-450 from rat liver microsomes treated with phenobarbital (PB) was separated into six fractions, as was cytochrome P-450 treated with 3-methylcholanthrene (MC), by high-performance liquid chromatography (HPLC) with an anion-exchange column. PB and MC induced three forms and one form of cytochrome P-450, respectively. The major forms induced by PB and by MC were further purified to apparent homogeneity based on sodium dodecyl(lauryl)sulphate—polyacrylamide gel electrophoresis by HPLC using a hydroxyapatite column. These new HPLC techniques are simple, rapid and useful for the purification of major forms of cytochrome P-450 from solubilized microsomes.

INTRODUCTION

Comparison of the many cytochrome P-450 preparations isolated in

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different laboratories is complicated because of the different methods of purification. Ion-exchange chromatography is useful for separating and purifying cytochrome P-450 isozymes [1, 2]. Cytochrome P-450, solubilized from untreated rat liver, was separated into four fractions using DEAE—cellulose chromatography [3]; however, other techniques gave more forms [4, 5]. Conventional ion-exchange chromatography is time-consuming and the resolution and reproducibility of the chromatograms leave much to be desired. Therefore, our aim was to develop a method by which many different forms of cytochrome P-450 may be simultaneously obtained and resolved. We found that high-performance liquid chromatography (HPLC) using an anion-exchange resin facilitates the separation of multiple forms of microsomal cytochrome P-450 [6–8].

We have now isolated six fractions of cytochrome P-450 from rat liver microsomes treated with phenobarbital (PB), and another six with 3-methylcholanthrene (MC) by HPLC using an ion-exchange column; further purification was by HPLC using a hydroxyapatite column.

EXPERIMENTAL

Preparation and solubilization of microsomes

PB (80 mg/kg, dissolved in saline) was given intraperitoneally, daily, for four days to male Sprague—Dawley rats weighing 200–250 g. MC (40 mg/kg, dissolved in corn oil) was injected intraperitoneally daily for three days before the rats were decapitated. Livers were homogenized with a three-fold volume of 1.15% potassium chloride using a Potter glass homogenizer. The homogenate was centrifuged at 10 000 *g* for 30 min and the precipitate was discarded. The supernatant was centrifuged at 100 000 *g* for 1 h and the microsomal fraction obtained was resuspended in ice-cold 1.15% potassium chloride and recentrifuged at 100 000 *g* for 1 h. The microsomal pellet was suspended with 0.1 *M* potassium phosphate buffer (pH 7.4) containing 1 m*M* EDTA, 1 m*M* dithiothreitol and 30% glycerol, and stored at −80°C. Microsomes were solubilized with 10% Emulgen 913 (Kao Chemicals, Tokyo, Japan) and 10% sodium cholate (final concentration of both 1%), with stirring for 30 min at room temperature. If the sample was not clear, it was centrifuged at 100 000 *g* for 30 min before injection into the HPLC apparatus. The protein concentration of the cytochrome P-450 of solubilized microsomes prepared from rats given PB was 31.8 mg/ml and that from rats given MC was 9.1 mg/ml. The cytochrome P-450 level in rats treated with PB was 47.4 nmol/ml and that in rats treated with MC was 15.3 nmol/ml.

Anion-exchange HPLC of solubilized microsomes

Two Altex pumps (Model 100, Berkeley, CA, U.S.A.), equipped with an Altex Model 420 solvent programmer and a spectrophotometer (UV-8, Toyo Soda, Tokyo, Japan) with an 8-μl flow cell, were used. The separation was done using a stainless-steel column (250 × 4.6 mm I.D.) equipped with a guard column (30 × 4.6 mm I.D.) and packed with SynChropak AX-300 (SynChrom, Linden, IN, U.S.A.). The sample was injected using a 500-μl sample loop and elution of haemoprotein was monitored at 417 nm. Ion-exchange chromato-

graphy was done as described elsewhere [6] at a flow-rate of 1.0 ml/min. A linear salt gradient was made using buffer A (0.02 *M* Tris—acetate, pH 7.5, containing 20% glycerol and 0.4% Emulgen 911) and buffer B (1.0 *M* sodium acetate added to buffer A, pH 7.5), so that 20% buffer B was incorporated into buffer A linearly for 20 min and then 20–100% for 30 min, using a solvent programmer at room temperature.

HPLC using a hydroxyapatite column

Cytochrome P-450 fractions obtained by running two samples through anion-exchange HPLC were pooled and concentrated using an ultra-filtration membrane (UK-50, Toyo Roshi, Tokyo, Japan) and Tris—acetate was replaced with 0.01 *M* sodium phosphate (pH 7.4) containing 20% glycerol and 0.1% Emulgen 911. The prepared cytochrome P-450 was injected into an HPLC system using a hydroxyapatite column (KB-column, 10 × 0.6 cm I.D.) (Koken, Tokyo, Japan) equilibrated with the above 0.01 *M* phosphate buffer at a flow-rate of 1.0 ml/min at room temperature. Cytochrome P-450 was eluted with a linear gradient of 0.01—0.35 *M* phosphate containing 20% glycerol and 0.1% Emulgen 911 in 70 min, and the cytochrome P-450 peaks were collected and concentrated using the ultra-filtration membrane.

Other procedures

The cytochrome P-450 level was measured by the method of Omura and Sato [9] from the CO-reduced difference spectra, using an extinction coefficient of 91 $mM^{-1}\ cm^{-1}$. To measure the protein concentration of the peak fraction containing 0.4% Emulgen, we used the method of Dulley and Grieve [10] with some modification: the final concentration of sodium dodecyl(lauryl)sulphate (SDS) was 1.5%. SDS—polyacrylamide gel electrophoresis (PAGE) was done by the method of Laemmli [11]. To prepare the samples, peak fractions containing 30–50 μg of protein were dialysed against 0.01 *M* sodium phosphate buffer (pH 7.5) containing 0.1 m*M* EDTA for 24 h at 4°C. After being freeze-dried, the preparations were dissolved in a small amount of water and treated with SDS. A slab gel (1.5 mm thick) containing 9% acrylamide separating gel and 3% stacking gel was used. The gel was stained with Coomassie Brilliant Blue R-250. The purity of the bands was calculated from the densitogram of the de-stained gel obtained by a laser densitometer (LKB Model 2202, Bromma, Sweden) and a data processor (Shimadzu C-R1A, Tokyo, Japan).

RESULTS

HPLC profiles of the microsomes from rats treated with PB and MC are shown in Fig. 1. We injected 300 μl (four-fold) of solubilized microsomes from rats treated with PB into the apparatus, or 500 μl (four-fold) of microsomes from rats treated with MC; the peaks were collected and pooled. Differences in the HPLC profile were not found in the four repeats of this step. In the HPLC of microsomes treated with PB, 9.5 mg of protein were loaded on the HPLC column in a single injection, but the peaks did not become rounded. The sample-loading capacity of this column may be < 10 mg of protein,

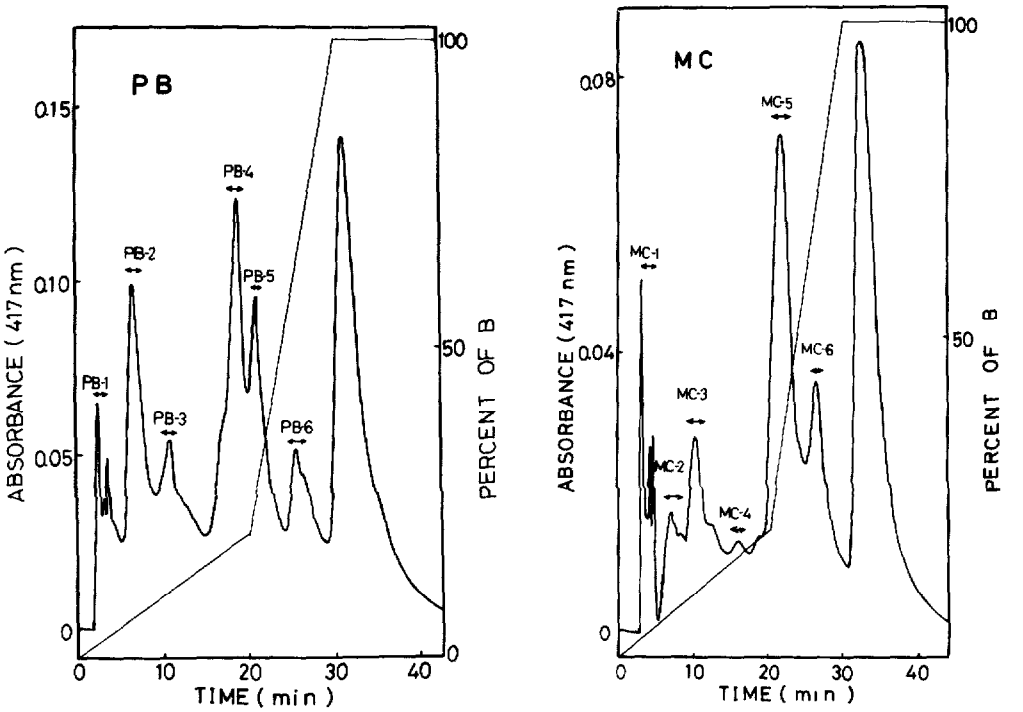

Fig. 1. HPLC profiles of solubilized liver microsomes from rats treated with phenobarbital (PB) or 3-methylcholanthrene (MC). A column (250 × 4.6 mm I.D.) packed with anion-exchange resin (Synchropak AX-300) was used. Fractions indicated by the arrows were collected: PB-1 to PB-6 and MC-1 to MC-6 are fraction names. The percentage of B is the concentration of sodium acetate. The conditions of HPLC are described in Experimental.

because we found elsewhere that resolution worsened when 20 mg of protein were loaded [12]. When the protein sample is of low concentration, a large sample volume of ca. 10 ml can be loaded using a big sample loop. The guard column was renewed after being used five or six times; the main column could be used more than 100 times. The peaks of haemoprotein and the last big peak were identified, spectrophotometrically, to be cytochrome P-450 and b_5, respectively. There were six peak fractions (PB-1 to PB-6) containing cytochrome P-450 isolated from PB-treated microsomes, and also six (MC-1 to MC-6) isolated from MC-treated microsomes. Retention time, the specific content of cytochrome P-450 and recovery for each fraction collected are shown in Table I. PB treatment increased three peaks (PB-2, PB-4, and PB-5) and MC treatment greatly increased one peak (MC-5). We think that these four species of cytochrome P-450 are induced forms. The total recovery of cytochrome P-450 (PB-1 to PB-6) was 42%. The major induced fraction (PB-4) contained 29% of the total cytochrome P-450 recovered as PB fractions. The other two induced fractions (PB-2 and PB-5) contained 24 and 17%, respectively. The specific contents of cytochrome P-450 (nmol/mg of protein) of PB-2, PB-4 and PB-5 were 4.8, 6.1 and 5.7, respectively; this was 3.0-, 3.8- and 3.5-fold that after a single HPLC of the solubilized microsomes. Approximately 37% of the injected cytochrome P-450 after MC treatment was recovered in fractions MC-1 to MC-6. The major MC-induced fraction (MC-5) contained 64%

TABLE I

RETENTION TIME, SPECIFIC CONTENT, SPECTRAL PROPERTIES AND MOLECULAR WEIGHT OF RESOLVED FRACTIONS OF CYTOCHROME P-450 FROM MICROSOMES OF RATS TREATED WITH PHENOBARBITAL (PB) OR 3-METHYLCHOLANTHRENE (MC)

P-450 fraction	Retention time (min)	P-450 (nmol)	Recovery (%)	Specific content (nmol/mg)	CO-reduced maximum (nm)	Molecular weight [$K \cdot 10^3$; purity (%)]
PB microsomes		56.8	100	1.60		
1	2.9	1.62	3.0	1.90	—	—
2	7.1	5.77	10.2	4.78	450	51*K* (62), 46*K* (38)
3	11.6	2.61	4.6	3.32	448	51*K* (73)
4	19.6	6.92	12.2	6.11	450	51*K* (68)
5	21.9	4.04	7.1	5.67	450	52.5*K* (64), 51*K* (28)
6	26.7	2.62	4.6	1.80	449	51*K* (56)
MC microsomes		30.6	100	1.68		
1	3.0	0.70	2.2	0.87	—	—
2	7.1	0.74	2.4	2.46	450	52*K* (23), 50*K* (18)
3	10.1	1.26	4.1	4.94	448	52*K* (34), 50*K* (42)
4	16.1	—	—	—	—	—
5	21.9	7.31	23.8	9.13	447	54*K* (90)
6	26.7	1.48	4.8	3.82	447	49*K* (47)

of the total amount of cytochrome P-450 recovered. The specific content of MC-5 was 9.1 nmol/mg, so the purity was increased 5.4-fold that after a single HPLC procedure. When all eluates of the HPLC system were collected, > 80% of injected cytochrome P-450 was recovered. The yield of cytochrome P-450 in a fraction from PB-treated microsomes was > 1.6 nmol, which is enough to measure the metabolic activity in a reconstituted system and to prepare antibody.

Oxidized, reduced and CO-reduced spectra (shown in Table I) of the isolated

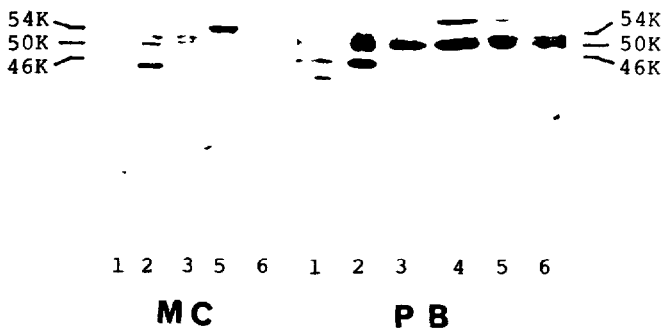

Fig. 2. SDS–polyacrylamide gel electrophoresis of isolated haemoprotein fractions. Liver microsomes from rats treated with phenobarbital (PB) or 3-methylcholanthrene (MC). The well numbers are the same as the fraction numbers in Fig. 1.

cytochrome P-450 were made using buffer A as the reference. Absorption maxima of the CO-reduced form of PB-2, PB-4, PB-5 and MC-5 were 450, 450, 449 and 447 nm, respectively. Only MC-3 contained high-spin haem, as suggested by the absorption maximum at 394 nm for the oxidized cytochromes. SDS—PAGE obtained from each isolated cytochrome P-450 fraction by HPLC is shown in Fig. 2, and the molecular weight of the major protein-staining bands and their purity calculated from laser densitograms are shown in Table I. MC-induced MC-5 was fairly homogeneous (90% pure) and the molecular weight was estimated to be 54 000. PB-4 and PB-5, the major forms of cytochrome P-450 induced by PB, had molecular weights of 51 000 and 52 500, respectively. The purity of PB-4 was 68% and that of PB-5 was 64%. PB-5 contained another band corresponding to PB-4.

Partially purified cytochrome P-450, PB-4, PB-5 and MC-5 were further purified by HPLC with a hydroxyapatite column; their HPLC profiles are shown in Fig. 3. A small peak was observed at the non-retained fraction. A small amount of proteins, which were not cytochrome P-450, eluted before the

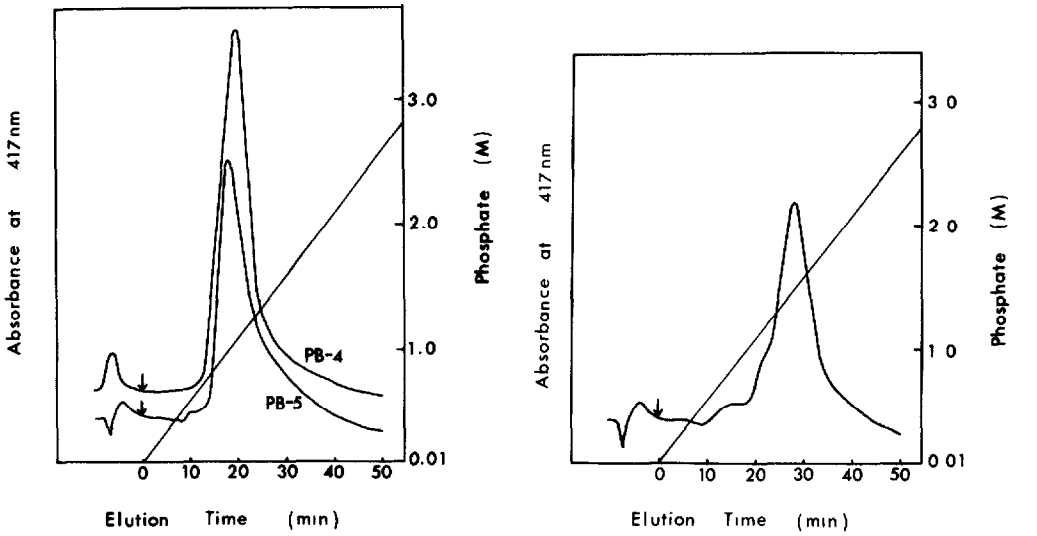

Fig. 3. HPLC profiles of PB-4 and PB-5 (left) and MC-5 (right) using a hydroxyapatite column. The linear gradient of phosphate starts at the point indicated by the arrow.

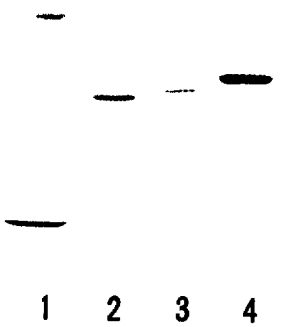

Fig. 4. SDS—polyacrylamide gel electrophoresis of purified P-450 by hydroxyapatite HPLC. Tracks 1—4 are the standard protein, PB-4, PB-5 and MC-5, respectively.

P-450 peak (data not shown). The elution times of PB-4, PB-5 and MC-5 were 19.8, 18.5 and 27.9 min, respectively. This result supports the idea that PB-4, PB-5 and MC-5 are different forms of cytochrome P-450. The recovery of PB-4, PB-5 and MC-5 during HPLC using hydroxyapatite was 81, 77 and 57%, respectively. The specific content of purified PB-4, PB-5 and MC-5 was 11.0, 8.8 and 12.4 nmol/mg of protein, respectively. Denaturation to cytochrome P-420 was not seen in the CO-reduced difference spectra of PB-4, PB-5 and MC-5. SDS—PAGE of purified PB-4, PB-5 and MC-5 is shown in Fig. 4. PB-4 and MC-5 were homogeneous on SDS—PAGE, while PB-5 had a minor band, the purity of which was calculated from the laser densitogram to be 86%. The apparent molecular weight of PB-4, PB-5 and MC-5, calculated from their mobility on SDS—PAGE, was 51 000, 52 500 and 54 000, respectively.

DISCUSSION

Solubilized liver microsomes from rats treated with PB were resolved into six fractions of cytochrome P-450 by HPLC using an anion-exchange column. The same number of fractions were obtained from rats treated with MC. This method separated the multiple forms of cytochrome P-450 better than conventional cellulose ion-exchange chromatography [1—3], because the column support is different in material, particle size, pore size and rigidity [13, 14]. Another advantage of HPLC is that the sample-loading capacity of the column support is much greater, because macroporous spherical microparticles are used for the column support material. By this new HPLC technique, it is possible to prepare, in a single day, many forms of cytochrome P-450 with high recovery and reproducibility. Usually, polyethylene glycol fractionation and chromatography using amino-octyl Sepharose 4B, DEAE—cellulose, CM—cellulose, and hydroxyapatite are all used to obtain homogeneous cytochrome P-450 by SDS—PAGE. This takes time and recovery is low. Bansal et al. [15] obtained three forms of P-450 (a, b and c) at elution positions between those of PB-4 and PB-5. We do not know how these three forms correspond to the six forms that we found. Possibly, the difference arises from differences in the rat microsomes; there is variation in the multiplicity of cytochrome P-450, depending on strain and colony [16, 17]. On the other hand, the HPLC profiles depend on chromatographic conditions, especially on the type and concentration of detergent and on the solubilization of microsomes [7]. Hydroxyapatite chromatography has been used to purify cytochrome P-450, in which species are eluted stepwise with phosphate [3, 18]. It was easy to elute cytochrome P-450 with a linear gradient of phosphate in our method. The elution order of three of the forms of cytochrome P-450 on hydroxyapatite HPLC was PB-5 > PB-4 > MC-5, which agrees with the results of Waxman and Walsh [2] and Ryan et al. [18].

PB-4 is the major form of cytochrome P-450 induced by PB. It has strong monoxygenase activity on the demethylation of benzphetamine [8]. MC-5 is the major form of cytochrome P-450 induced by MC. It strongly catalyses the hydroxylation of benzo[*a*]pyrene and the de-ethylation of 7-ethoxycoumarine [8]. The results concerning monoxygenase activity, spectral properties, monomeric molecular weight and chromatographic behaviour

strongly suggest that PB-4 and PB-5 correspond to the PB-4 and PB-5 purified by Waxman and Walsh [2] and also to the P-450b and P-450e purified by Ryan et al. [18]. MC-5 corresponds to the P-450c purified by Reik et al. [19].

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