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RESOLUTION OF MOUSE HEPATIC CYTOCHROME P-450 ISOZYMES BY CHROMATOFOCUSING

H. J. MARRIAGE and D. J. HARVEY*
University Department of Pharmacology, South Parks Road, Oxford, 0X1 3QT (U.K.)
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SUMMARY

Constituent cytochrome P-450 isozymes from mouse hepatic microsomes were fractionated by chromatofocusing on Polybuffer Exchanger 94 over the pH range 5.3–8.3 and characterized by polyacrylamide gel electrophoresis. Eight isozymes were detected in fractions from unpretreated mice and seven from phenobarbitone-pretreated animals. Isozyme-containing fractions were reconstituted and shown to produce specific monohydroxy metabolites from both testosterone and Δ^9-tetrahydrocannabinol.

INTRODUCTION

Cytochrome P-450, the major enzyme system mediating biotransformation of lipophilic xenobiotics and certain endogenous compounds, exists as a group of selectively inducible isozymes having different, although somewhat overlapping, substrate specificities[1]. The isolation and purification of these isozymes has posed some difficult biochemical problems, recently reviewed by Guengerich[2]. A large number of chromatographic techniques have been used including such methods as high-performance liquid chromatography (HPLC)[3], *n*-octylamino-Sepharose chromatography[4], DEAE-cellulose chromatography[5] and affinity chromatography[6]. In our work on toxic metabolites formed from drugs and other xenobiotics we required a method for separation of cytochrome P-450 isozymes in an active form for reconstitution and metabolism studies with a variety of substrates. Chromatofocusing appeared to offer the potential for resolution of isozymes as it has greater resolving power than straightforward ion-exchange chromatography but, until recently[7], the technique does not appear to have been exploited for cytochrome P-450 separation. In this paper we describe a chromatofocusing system capable of resolving several cytochrome P-450 isozymes from phenobarbitone-treated and untreated mice and the reconstitution of the enzymes for the study of their catalytic activity.

EXPERIMENTAL

Pretreatment of mice

Mice (Charles River, male, 20–24 g) were treated with phenobarbitone (pH

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adjusted to 7.4 with 1 *M* hydrochloric acid) by including it in their drinking water (0.1%, w/v) for six days. The phenobarbitone was withdrawn 24 h before the animals were killed by cervical dislocation. A second group of mice was supplied with water without phenobarbitone.

Preparation of cytochrome P-450 fractions for chromatofocusing

This was based on the method described by Van der Hoeven and Coon[8]. The livers were removed from the treated and non-treated mice after removal of the gall bladder and placed in ice-cold buffer [0.1 *M* Tris–acetate (pH 7.4), containing 0.1 *M* potassium chloride, 0.1 m*M* EDTA and $2.3 \cdot 10^{-5}$ *M* butylated hydroxytoluene (BHT)]. All subsequent procedures were carried out at 0–4°C. The tissue was cut into approximately 5-mm cubes and homogenized in a Waring blender in four volumes of buffer. The homogenate was centrifuged at 10 000 *g* for 30 min, the supernatant was filtered through cheese cloth and centrifuged further at 105 000 *g* for 90 min. The resulting pellet was suspended in 0.1 *M* potassium pyrophosphate buffer (pH 7.4), containing 0.1 m*M* EDTA and $2.3 \cdot 10^{-5}$ *M* BHT to the same volume as the original homogenate and centrifuged at 105 000 *g* for 60 min. The microsomes were suspended in 10 m*M* Tris–acetate buffer (pH 7.4), containing 20% (v/v) glycerol and 0.1 m*M* EDTA (buffer A) to a protein concentration of about 24 mg/ml for storage at −20°C. The microsomes were then diluted with three volumes of 0.1 *M* Tris–acetate buffer (pH 7.4), containing 0.1 *M* potassium chloride, 20% (v/v) glycerol, 1.0 m*M* EDTA, 1.0 m*M* dithiothreitol and $2.3 \cdot 10^{-5}$ *M* BHT recently flushed with nitrogen. This suspension was solubilized with 10% (w/v) sodium cholate which was added dropwise with stirring to give a cholate-to-protein ratio of 3:1 and was stirred for a further 30 min. Fractional precipitation from this solution was achieved by adding 50% (w/v) polyethylene glycol 8000 solution to give 4%, 6%, 8%, 10% and 13% fractions. The precipitate was obtained by centrifugation at 10 000 *g* for 10 min between each addition of polyethylene glycol. The 8–10% and the 10–13% precipitates, which contained the cytochrome P-450, were suspended in buffer A and dialysed overnight against 50 volumes of buffer A. After dialysis, the fractions were combined and stored at −20°C until required. Before chromatofocusing, the samples were dialysed against 25 m*M* Tris–acetate buffer, (pH 7.4) (three times 1-l), containing 1.0 m*M* dithiothreitol and 0.5% (v/v) Tergitol NP-10. Protein was measured by the method of Lowry *et al.*[9] and cytochrome P-450 was measured by the method of Omura and Sato[10].

Chromatofocusing of cytochrome P-450

This was based on the technique described by Ono *et al.*[11] for squalene epoxidase. A 10-mg/ml sample of 500 mg total protein of the dialysed fraction from the unpretreated mice was applied to a 200-ml column of Polybuffer Exchanger 94 (PBE 94, Pharmacia, Uppsala, Sweden) equilibrated in 25 m*M* Tris–acetate buffer, pH 8.3. For the fraction from the phenobarbitone-pretreated mice, a 5.2-mg/ml sample of 250 mg total protein was applied to a 100-ml column of PBE 94 equilibrated at the same pH. The concentration of material applied to each column was equivalent to 2.5 mg of protein to each ml of column material. Elution of the two columns was performed with 2.5 l and 1.2 l respectively of a mixture of Polybuffer 96 (Pharmacia) (30%) and Polybuffer 74 (70%) adjusted to pH 5.0 with acetic acid at flow-rates of

30 and 20 ml/h respectively. The pH range was chosen following isoelectric focusing of a small sample of the dialysed material on flat bed polyacrylamide gels using the method described by the manufacturer (LKB, Cambridge, U.K.). The gels were stained for protein using Coomassie blue. Fractions of 5.7 and 6.6 ml were collected from the two chromatofocusing columns respectively, and further material was eluted using a salt gradient. Elution profiles were determined either by measuring the absorbance at 405 nm using a flow cell and chart recorder or by measuring at both 270 nm and 405 nm in each fraction. The pH of the fractions was determined within 24 h of elution using a pH meter and electrode calibrated to the pH of the starting buffer. The fractions were combined into samples containing individual cytochrome P-450s as far as could be judged from the elution profiles and frozen at −20°C until required. Under these conditions the cytochrome P-450 was stable for at least 36 weeks. The cytochrome P-450 content was determined as above.

Separation of cytochrome P-450 from polybuffer and detergent

Calcium phosphate gel[12] (approximately 0.5 ml for each 10 ml of cytochrome P-450-containing solution) was added to the fractions and mixed. The gel was centrifuged at 2000 *g* for 15 min and the absorbance of the supernatant was measured at 405 nm to confirm the complete precipitation of the cytochrome P-450. The gel was washed with buffer A until the absorbance at 278 nm was less than 0.1 unit, indicating that the Tergitol had been removed. The cytochrome P-450 was extracted from the gel with 3 ml of 0.3 *M* phosphate buffer containing 20% (v/v) glycerol and 1.0 m*M* EDTA and was centrifuged at 10 000 *g* for 10 min to recover the gel. This procedure was repeated at least three times. The efficiency of the calcium phosphate gel extraction in removal of the Tergitol NP-10 and polybuffers was 97.5% and 100%, respectively, as determined by the absorbances at 273 and 253 nm. The extracts were stored at −20°C until required.

Gel electrophoresis

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with slab gels using the system of Fairbanks *et al.*[13]. Gels were stained for protein with Coomassie brilliant blue R (Sigma, Poole, U.K.), silver stain[14] or, for cytochrome P-450 activity, by peroxidase activity[15]. Molecular weight markers (Sigma) consisting of six proteins with molecular weights between 14 000 and 66 000 daltons were applied to the plates in two positions for development concurrently with the cytochrome P-450. Calibrations were obtained from a regression analysis of the R_F values. Concentration of the samples prior to electrophoresis, if required, was performed by containing the sample in a dialysis bag surrounded by dry Sephadex G 25 to act as a drying agent. This was allowed to stand at 4°C until the required concentration was achieved. Densitometer tracings of the gels stained with Coomassie blue were recorded with a Cecil CE 599 spectrophotometer fitted with a Cecil CE 570 gel scanner. The recording was made at 590 nm at a scan-rate of 5 s/mm.

Reconstitution of cytochrome P-450 for metabolism studies

Reconstitution experiments were performed in 1-ml cuvettes with a light path of 10 mm using a method based on that of Coon[16]. The sample of cytochrome P-450 (0.1 n*M*), cytochrome P-450 reductase (500 units, see below) and dilauroyl-

L-α-phosphatidylcholine (20 μl of a sonicated 0.1% aqueous suspension) were mixed and allowed to stand for 1 min at room temperature. The following components were then added: 0.5 *M* sodium 4-(2-hydroxyethyl)-1-piperazineethanesulphonate (HEPES) buffer at pH 7.4 (100 μl), 0.1 *M* magnesium chloride (50 μl), 0.5% (w/v) sodium deoxycholate (20 μl), 10 m*M* reduced nicotinamide-adenine dinucleotide phosphate (NADPH) (20 μl) and distilled water to 1 ml. The mixture was incubated for 3 min at 37°C before the addition of substrate which was either Δ^9-tetrahydrocannabinol (Δ^9-THC, 40 μg) in ethanol (2 μl) or testosterone (40 μg) in ethanol (4 ml). After further incubation at 37°C for 1 h, the metabolites were extracted with ethyl acetate (three times 1 ml), the combined ethyl acetate fractions were concentrated, transferred to 0.3-ml conical micro-vials, blown to dryness and dissolved in N,O-bis-(trimethylsilyl)trifluoroacetamide (20 μl). This was heated at 60°C for 10 min and aliquots were examined by gas chromatography–mass spectrometry (GC–MS).

GC–MS

GC–MS was performed with a VG Micromass 12B mass spectrometer interfaced to a VG 2050 data system and, via a glass jet separator, to a Varian 2440 gas chromatograph which was fitted with a 2 m × 2 mm glass column packed with 3% SE-30 on 100–120 mesh Gas-Chrom Q. Helium at 30 ml/min was used as the carrier gas and the column was temperature programmed from 170 to 300°C at 2°C/min. The injector and separator temperatures were both 300°C. The mass spectrometer was operated under the following conditions: electron energy, 25 eV; accelerating voltage, 2.4 kV; trap current, 100 μA; ion-source temperature, 260°C; scan speed, 3 s/decade.

Preparation of cytochrome P-450 reductase

This was obtained from the 13% polyethylene glycol supernatant of microsomes from the cytochrome P-450 purification (above) using a method based on that described by Shepard *et al.*[17].

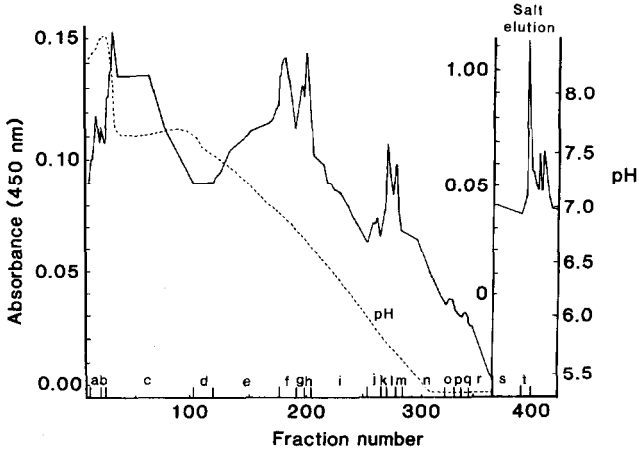


Fig. 1. Elution profile of mouse hepatic cytochrome P-450 (——) and pH (---) obtained by chromatofocusing.

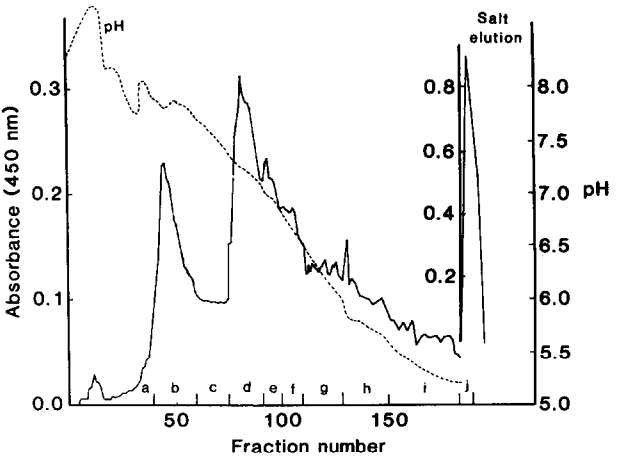


Fig. 2. Elution profile of phenobarbitone-pretreated mouse hepatic cytochrome P-450 (——) and pH (---) obtained by chromatofocusing.

RESULTS

The elution profiles and pH from the two chromatofocusing columns are shown in Figs. 1 and 2 for the untreated and phenobarbitone-treated mice, respectively. The profile from the unpretreated animals was obtained by monitoring the column eluent in a flow cell whereas that from the other column was constructed using the absorbance of each fraction determined in a scanning spectrophotometer. The slope of the absorption towards lower values during the elution is thought to be due to changes in the absorbance of the buffer.

Difference spectroscopy[18] revealed that both cytochrome P-450 and P-420 were eluted from the column but varied between the fractions. For example, fraction 42 from the phenobarbitone-induced P-450 gave a reduced carbon monoxide spectrum with maxima at 449 nm and 550 nm whilst fraction 82 from the same column gave substantial maxima at 420 and 450 nm and no detectable maximum at higher wavelength. Removal of the tergitol NP-10 and Polybuffers altered the spectral characteristics of several fractions to show increased absorbance at 450 nm and reduction of the peak at 420 nm. The reverse situation was not observed. This was most apparent in fraction 82 from the phenobarbitone-pretreated mice where, after removal of the detergent and Polybuffers, the difference spectrum maximized almost entirely at 450 nm with a broad absorbance band at 550 nm. Fractions and their properties are shown in Tables I and II for the unpretreated and phenobarbitone treated mice, respectively.

The quantity of recovered cytochrome P-450 was measured by the method described by Omura and Sato[10]. The total recovery of cytochrome P-450 with chromatofocusing was about 40% from both columns. Values for the individual fractions are listed in Tables I and II.

Individual fractions were examined by SDS-PAGE and the gel scans of the fractions, recorded at 550 nm, are shown in Figs. 3 and 4. Molecular weights and other properties are listed in Tables I and II.

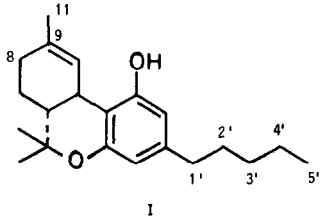
TABLE I

PROPERTIES OF THE FRACTIONS OBTAINED FROM CHROMATOFOCUSING OF MOUSE HEPATIC CYTOCHROME P-450

Fraction	*Elution pH*	*Absorption maximum (nm)*	*Molecular weight (× 1000)*	*Specific activity (nM/g/min)*	*Total P-450 recovery (%)*
b	8.3	449.0	45	–	
c	7.8	451.0	49	5.1	69.3
f	7.0	451.0	51, 56	3.3	5.8
g	6.7	449.0	46, 51, 53	1.7	1.7
h	6.6	451.5	46, 51	1.3	1.2
i	6.0–6.6	451.0	50, 55	1.6	7.8
j	6.0	451.0	50, 55	0.8	11.6
k	5.8	450.0	50, 55, 61	1.1	1.0
l	5.7	448.0	–	0.8	0.3
m	5.6	449.0	55, 57	1.3	1.5

Compounds eluted with salt at the end of the chromatofocusing phase were mainly cytochrome b_5 and cytochrome P-450 reductase.

Reconstitution experiments were successful for every cytochrome P-450-containing fraction and produced metabolites from both Δ^9-THC (I) and testosterone. Fractions not containing P-450 were inactive. All fractions from both untreated and phenobarbitone-pretreated mice produced the two major monohydroxy allylic



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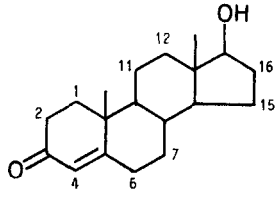

II

TABLE II

PROPERTIES OF THE FRACTIONS OBTAINED FROM CHROMATOFOCUSING OF PHENOBARBITONE-PRETREATED MOUSE HEPATIC CYTOCHROME P-450

Fraction	*Elution pH*	*Absorption maximum (nm)*	*Molecular weight (× 1000)*	*Specific activity (nM/g/min)*	*Total P-450 recovery (%)*
a	8.3	449.5	45, 54	0.3	1.2
b	8.0	451.5	47, 51	1.7	17.3
c	7.3–8.0	451.0	49, 51	2.9	29.6
d	7.3	451.5	49	1.7	21.8
e	7.0	451.0	51	1.0	8.5
f	6.6	451.5	51	1.4	12.5
g	6.2	450.0	51, 59	0.8	3.7
h	5.7	449.0	60	0.4	2.2
i	5.4	449.0	50, 59	0.3	3.3

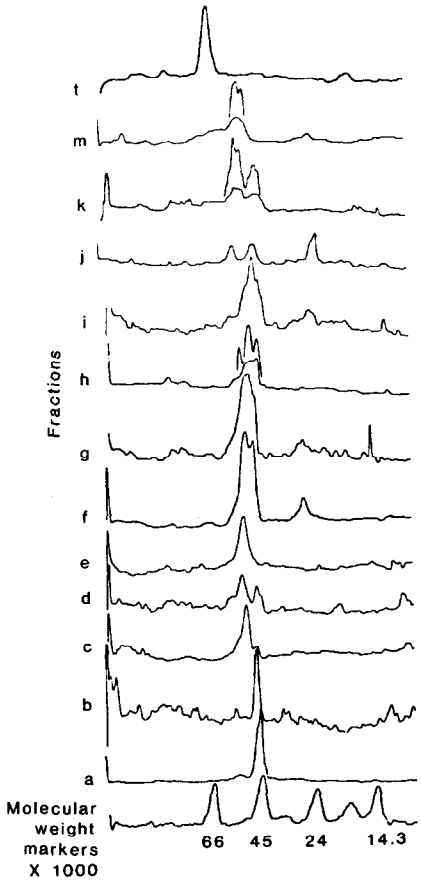

Fig. 3. Scans of SDS-PAGE of fractions from chromatofocusing of mouse hepatic cytochrome P-450.

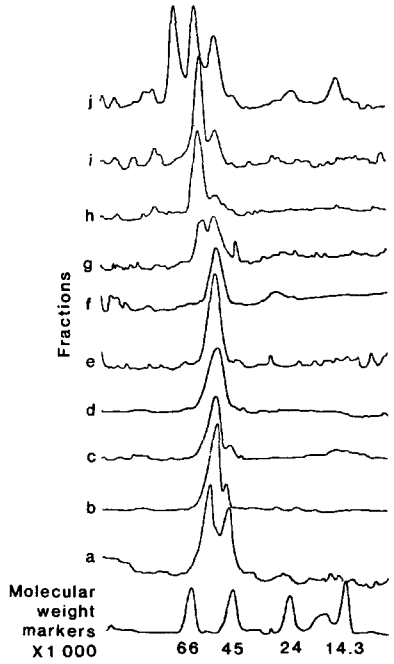

Fig. 4. Scans of SDS-PAGE of fractions from chromatofocusing of phenobarbitone-pretreated mouse hepatic cytochrome P-450.

metabolites of Δ^9-THC, namely 8α- and 11-hydroxy-Δ^9-THC, although in different proportions. Fractions k–m from the untreated mice produced the side-chain monohydroxy metabolites, 3′- and 4′-hydroxy-Δ^9-THC with 3′-hydroxy-Δ^9-THC being the major metabolite in fraction m. This fraction also produced large quantities of a fourth but, as yet, unidentified metabolite. The profiles from the phenobarbitone-pretreated mice were similar in that all fractions, except fraction f, produced more 8α- than 11-hydroxy-Δ^9-THC and, in addition, this fraction produced substantial quantities of another unidentified metabolite. This metabolite was detected in the profile from fraction f from the unpretreated mice but not in any of the other fractions. Identification of the known metabolites was based on a comparison of their GC–MS properties with those of authentic standards and with published spectra[19]. Full details of the metabolites of Δ^9-THC identified in these fractions will be published later.

Monohydroxy metabolites of testosterone (II) were identified by a comparison

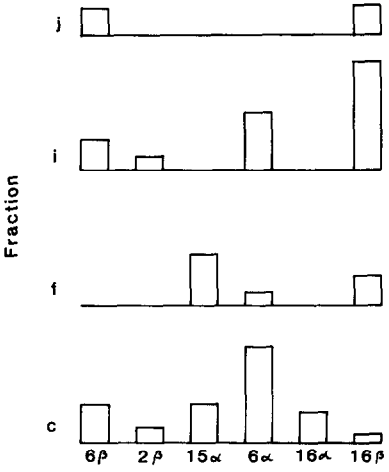

Fig. 5. Testosterone metabolites identified by GC–MS from reconstituted fractions following chromatofocusing of mouse hepatic cytochrome P-450. Compounds are listed in order of elution from the GLC column.

of their GC–MS properties with those of published spectra as follows; 2-β-, 16-α- and 16-β-[20], 6-α-[21], 6-β-[22] and 15-α-hydroxy-testosterone[23]. Their relative proportions produced in four fractions from the unpreteated mice are shown in Fig. 5.

Table III lists the properties of the cytochrome P-450 reductase present in microsomes and recovered from the affinity column.

DISCUSSION

With this study we have demonstrated the use of chromatofocusing as a technique for preliminary fractionation of cytochrome P-450 isozymes. Most fractions, designated by letters in the figures, obtained by combination of the individual fractions from the column, contained mixtures of enzymes, but this can partly be ascribed to the way in which the individual fractions from the chromatofocusing column were combined. The highest purity from the experiments described, was obtained with

TABLE III

PROPERTIES OF THE CYTOCHROME P-450 REDUCTASE ISOLATED FROM MOUSE LIVER MICROSOMES

Fraction	*Total protein (mg)*	*Total* activity (μM/min)*	*Specific activity (μM min^{-1} mg^{-1})*	*Degree of purification*	*Yield (%)*
Microsomes	585.0	98	0.167	1	100
Solubilized microsomes	516.0	145	0.281	1.68	149
Affinity column	2.1	55	26.2	157	56

* Activity is expressed as *μM* of cytochrome *c* reduced per minute at 30°C.

fraction 42 from the phenobarbitone-pretreated mice. A gel scan of the SDS-PAGE experiment indicated that 97% of the material in the P-450 was concentrated in a single band (mol. wt. 47 000). Better control over the combination of the individual column fractions would undoubtedly allow more of the isozymes to be obtained in a reasonably pure state. This technique, however, appears to be a useful method for preliminary fractionation of the isozymes and provides a number of physical characteristics as listed in Tables I and II. Recovery was generally good and twice that obtained by Huang *et al.*[24] who used DEAE-cellulose fractionation for mouse cytochrome P-450.

The only other report of cytochrome P-450 separation by chromatofocussing appears to be that of Pasanen and Pelkonen[7] who used the technique to separate twenty-one pools of the enzyme over the pH range 5–7.5. Their technique differs from ours in that preliminary purification was accomplished by chromatography on both phenyl-Sepharose and DEAE-cellulose and thus has the capability of achieving a more complete separation. Our method, however, has been demonstrated to work on a less pure preparation of the original enzyme mixture and to achieve substantial separation of the isozymes.

As it has been suggested that the elution pH of proteins from chromatofocusing columns can be considered as approximations of their isoelectric points[25], the elution pH values determined in the present experiments can be compared with the published pI values of cytochrome P-450. These are, for example, in the same range (pH 5.0–8.0) as those observed for cytochrome P-450 from rat liver[26,27]. Results from separation of cytochrome P-450 from the mouse[28] on DEAE-cellulose suggest the presence of two isozymes with p*I* values above pH 7.8 and three below. The elution of phenobarbitone-induced mouse liver isozymes[24] from DEAE-cellulose suggests two isozymes with p*I* values above pH 7.7 and two below. Chromatofocusing has given more accurate estimates of these values and has also suggested the presence of more isozymes; eight from the untreated mice and seven from the phenobarbitone-induced animals. The p*I* values of cytochrome P-450 reductase and cytochrome b_5 from the rat[27] of 5.4 and 4.9, respectively, confirm that these enzymes should be retained on the chromatofocusing column under the conditions used.

Experiments on the suitability of isoelectric focusing to separate microsomal proteins[29] demonstrate that these techniques can produce artefactual heterogeneity. It was suggested that this could be caused by the binding of carrier ampholites and strong protein-protein interactions. In these chromatofocusing experiments, these problems may have been overcome by the use of Polybuffers which were subsequently removed by calcium phosphate gel extraction and by using a sample loading of only 2.5 mg protein per ml of chromatofocusing gel.

Reconstitution experiments demonstrated that the fractions isolated from the chromatofocusing column were active and that they metabolized the two substrates tested with a certain degree of specificity. As most of the fractions contained a mixture of isozymes it is not possible from these experiments to determine the individual catalytic profile of each isozyme but the results are generally in agreement with work of others that some of the isozymes catalyse the production of individual metabolites. It is interesting that for Δ^9-THC, the major 8α- and 11-hydroxy-metabolites appear to be produced by most of the isolated isozymes whereas the production of the minor metabolite in the mouse[30], 3′-hydroxy-Δ^9-THC is much more specific. This undoubt-

edly explains why side-chain hydroxylated metabolites are produced in higher concentration by the lung[31] where the profile of cytochrome P-450 isozymes would be expected to be different. Work is underway to purify these enzymes further and to characterize fully their catalytic activity.

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