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INDUCTION OF LIVER CYTOCHROME P-450 IN MICE BY WARFARIN

COMPARISON OF WARFARIN-, PHENOBARBITONE-, AND COBALT-INDUCED HEPATIC MICROSOMAL PROTEIN PATTERNS BY PAGE AFTER PARTIAL PURIFICATION ON OCTYL-SEPHAROSE CL-4B

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Abstract—A rapid method is presented to separate mouse liver cytochrome P-450 from other components of the microsomal monooxygenase system and to increase specific activity by hydrophobic interaction chromatography on Octyl-Sepharose CL-4B by a factor of between 3.8 and 5.3. In addition it is shown that varieties of cytochrome P-450 can be separated from each other by Octyl-Sepharose CL-4B.

After oral applications of 120 mg/kg warfarin once daily for three days SDS–PAGE analysis of the partially purified cytochrome P-450 fraction revealed a protein pattern in the 50 Kd region that is practically indistinguishable from that after conventional phenobarbitone pretreatment. On the other hand, cobalt pretreatment results in a different pattern that is distinguished from that of normals as well as from that of phenobarbitone- and warfarin-pretreated mice. From these results in conjunction with the previous finding of increased drug metabolic activity after warfarin pretreatment it is concluded that warfarin elicits phenobarbitone-like induction of the hepatic monooxygenases in mice.

The 4-hydroxycoumarin derivative warfarin is known as a drug with anticoagulant activity and is therefore widely used in clinical therapy and in many rodenticides. The principal mode of action is the inhibition of the reduction of vitamin K epoxide (KO) to its hydroquinone form (KH_2) [1]. The oxidation of KH_2 to KO is linked to the conversion of descarboxyprothrombin to prothrombin.

Many drugs are able to alter the anticoagulant action of warfarin by different interaction mechanisms [2]. Since warfarin is metabolized by the microsomal mixed-function oxygenase system (MMFO) drugs inducing this enzyme system decrease the anticoagulant effect of warfarin [3–6].

On the other side warfarin itself exerts active influence on the MMFO. It was demonstrated that several dealkylation and hydroxylation reactions mediated by MMFO were moderately inhibited by warfarin *in vitro* [7, 8] as well as by other naturally occurring coumarins [9, 10]. In contrast, Feuer [11, 12] showed an inducing effect of coumarins like 4-methylcoumarin and some 4-methylcoumarin derivatives *in vivo* on the coumarin 3-hydroxylase activity in rats.

We previously reported the 4-hydroxycoumarin derivative warfarin also to cause induction of the MMFO as characterized by increased microsomal protein and cytochrome P-450 content as well as proliferation of endoplasmic reticulum and some enzyme kinetic data obtained from *in vitro* and *in vivo* tests [8, 13, 14].

This finding prompted us to investigate the effect of warfarin pretreatment on the microsomal protein pattern in male C57 BL/6J Han mice in comparison to conventional phenobarbitone induction and cobaltous chloride pretreatment previously shown to lead to an atypical induction [15–17].

MATERIALS AND METHODS

Animals. All experiments were carried out with male C57BL/6J Han mice (age: 6 + 1 weeks; body weight: 20–25 g) obtained from Zentralinstitut für Versuchstierzucht, Hannover, F.R.G. They were kept on a standard laboratory pellet diet and water *ad libitum* at a fixed 12-hr light/dark cycle beginning at 6.00 a.m.

Pretreatment of animals. The mice were held in our laboratory for 1 week before pretreatment. Racemic sodium warfarin (W) (a gift from Richardson-Merell, Groß-Gerau, F.R.G.) was administered by three daily oral applications of 120 mg/kg in 0.9% sodium chloride solution. Pretreatment with sodium phenobarbitone (PB) was carried out by three daily i.p. injections of 80 mg/kg in 0.9% sodium chloride. 3-Methylcholanthrene (3-MC) was applied by two daily i.p. injections of 30 mg/kg in arachis oil. The animals of these three groups were sacrificed 72 hr after the first application. A fourth group of mice was pretreated with isotonic solution of cobaltous chloride (Co) by two daily s.c. injections each equivalent to 40 mg/kg. These animals were killed 48 hr after the first injection. The group of normals (N) did not receive any injections.

Preparation and solubilization of liver microsomes. All operations were carried out in the cold (0–4°). Liver microsomes were prepared as described by Netter [18] except that the *in situ* perfusion and the

homogenization of the livers as well as the resuspension of the microsomal pellets were performed with a 20 mM Tris–HCl buffer, pH 7.4, containing 250 mM sucrose, 1 mM EDTA, 1 mM phenylmethanesulfonylfluoride. In addition the microsomal pellets were washed once with 0.15 M KCl to remove glycogen and other adsorbed proteins.

The components of the microsomal electron transfer system were solubilized according to the method of Gibson and Schenkman [19]: The microsomal suspension (about 20 mg of protein/ml) was mixed with an equal volume of a 10 mM sodium phosphate buffer, pH 7.2, containing 1 mM EDTA, 1 M NaCl, 40% glycerol (w/v) and 0.4% (w/v) of the nonionic detergent Emulgen 913 (Kao-Atlas Co., Tokyo, Japan). The solution was gently stirred under a constant stream of N_2 for 30 min and subsequently centrifuged at 218,000 *g* for 45 min. The supernatant was adjusted to 0.5% (w/v) of sodium cholate (E. Merck, Darmstadt, F.R.G., purity: at least 98.5%) before being applied to the hydrophobic interaction column.

Partial purification of cytochrome P-450 on Octyl-Sepharose CL-4B. In order to separate cytochrome P-450 from the other components of the microsomal monooxygenase system and to increase cytochrome P-450 concentration the method of Beaune *et al.* [20] was performed in a modified manner. We used the following buffers: Buffer A: 10 mM sodium phosphate, 1 mM EDTA, 500 mM NaCl, 20% (w/v) glycerol, 0.5% (w/v) sodium cholate, pH 7.4; Buffer B: 10 mM sodium phosphate, 1 mM EDTA, 400 mM NaCl, 20% (w/v) glycerol, 0.44% (w/v) sodium cholate, 0.1% (w/v) Emulgen 913, pH 7.4; Buffer C: 10 mM sodium phosphate, 1 mM EDTA, 20% (w/v) glycerol, 0.2% (w/v) sodium cholate, 2% (w/v) Emulgen 913, pH 7.4. For the 3-methylcholanthrene-pretreated microsomes buffer C contained 0.5% instead of 2% Emulgen 913.

Solubilized microsomes containing 90 to 100 nmol of cytochrome P-450 were applied to an Octyl-Sepharose CL-4B (Pharmacia Chemicals, Freiburg, F.R.G.) column (i.d. 16 mm, length 20 cm) previously equilibrated with 300 to 400 ml of buffer A. The sample was eluted from the column step by step with buffer A until the end of peak 2 then with buffer B until the end of peak 3 followed by buffer C eluting peak 4 (cf. Fig. 1).

The elution profile was monitored measuring the absorbance of the fractions at the wavelength of 417 nm. The column flow rate was 50 ml/hr and the volume per fraction 5 ml.

Analytical techniques. To measure the contents of cytochrome P-450, of cytochrome b_5 and the activity of NADPH–cytochrome *c*-reductase of each of the four peaks the related fractions were pooled. The pool of peak 4 (cf. Fig. 1) containing the cytochrome P-450 served for further SDS–PAGE analysis.

The following assays were applied to the pools:

Cytochrome P-450 was quantitated by recording the CO-reduced minus reduced difference spectrum according to Omura and Sato [21] using the extinction coefficient 91 $mM^{-1}\,cm^{-1}$.

Cytochrome b_5 was determined by the method of Omura and Takesue [22] measuring the reduced minus oxidized spectrum (extinction coefficient 185 $cm^{-1}\,mM^{-1}$).

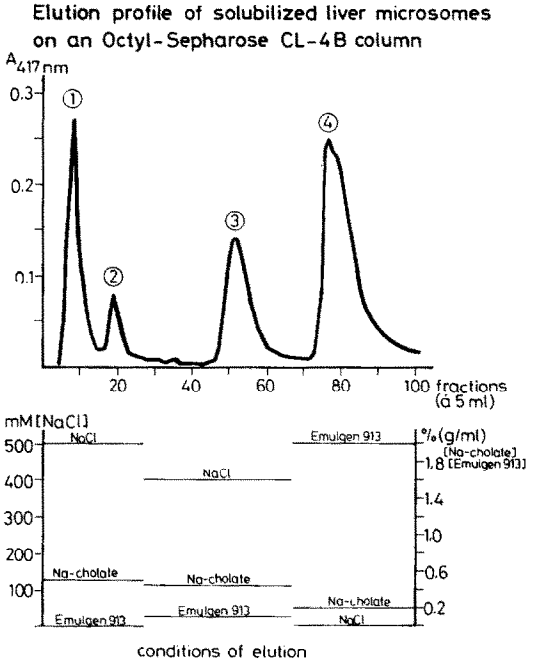

Fig. 1. Elution profile and elution conditions of solubilized mouse liver microsomes (normals) on an Octyl-Sepharose CL-4B column. 91 nmol of Emulgen 913 solubilized cytochrome P-450 were applied to the column. The hydrophobic interaction column was eluted step-wise by changing concentrations of three components in the elution buffers as shown in the diagram. Table 1 relates the fractionated and recovered microsomal components to the different peaks.

NADPH–cytochrome *c*-reductase activity was measured according to the method of Williams and Kamin [23] using cytochrome *c* from horse heart (Boehringer Mannheim, Mannheim, F.R.G.) assuming an extinction coefficient of 19.1 $cm^{-1}\,mM^{-1}$.

Protein was determined with bovine serum albumin as standard by the method of Lowry *et al.* [24] after acetone precipitation in order to avoid Emulgen interaction.

Photometrical measurements were carried out with an Aminco DW2a UV/VIS spectrophotometer except protein concentration which was determined with a Zeiss PMQ II photometer.

SDS–polyacrylamide gel electrophoresis. Aliquots of the peak 4 (Octyl-Sepharose CL-4B column) were diluted with a Tris–HCl buffer, pH 6.8. The samples contained the following final concentrations: 16 mM Tris–HCl (pH 6.8), 0.5% (w/v) SDS, 20% (v/v) glycerol, 1.25% (v/v) 2-mercaptoethanol, and 0.00025% (w/v) bromphenol blue. Samples (200 µl) containing 40 pmol cytochrome P-450 were denaturated at 100° for 2 min and applied to a discontinuous sodium dodecylsulfate–polyacrylamide slab gel [25] constituted by a 8% (w/v) acrylamide, 0.1% (w/v) SDS separating gel and a 3% (w/v) acrylamide, 0.1% (w/v) SDS stacking gel.

Gel electrophoresis was carried out at 11–12°: The samples were run into the stacking gel at 20 mA for about 1 hr and subsequently electrophoresed at 40 mA for about 2 hr. Gels were fixed and stained

for 2 hr in a 45% (v/v) methanol solution containing 10% (v/v) acetic acid and 0.2% Coomassie Brilliant Blue G (Sigma, München, F.R.G.) and then destained in isopropyl alcohol/acetic acid/water (3 + 2 + 15). The following proteins obtained from Bio-Rad Laboratories (Richmond, CA) were used as standards: lysozyme (M_r = 14,400), soybean trypsin inhibitor (M_r = 21,500), carbonic anhydrase (M_r = 31,000), ovalbumin (M_r = 45,000), bovine serum albumin (M_r = 66,200), and phosphorylase B (M_r = 92,500).

3,3',5,5'-Tetramethylbenzidine (TMBZ; Serva, Heidelberg, F.R.G.) –H_2O_2 staining for the peroxidase activity of cytochrome P-450 in SDS-polyacrylamide gels was performed as described by Thomas *et al.* [26]. Samples (120 pmol cytochrome P-450) for subsequent TMBZ staining were prepared under non-reducing conditions, i.e. without 2-mercaptoethanol, and without boiling, and were separated under subdued light at room temperature on gels additionally containing 10% (w/v) glycerol and 3.6 M urea after preelectrophoresis at 20 mA for about 2 hr.

RESULTS

The solubilization procedure of the membrane-bound cytochrome P-450 in a 0.2% (w/v) Emulgen 913 solution yielded 80% to 90% of the starting amount of the cytochrome P-450 in the microsomes.

The conditions and profile of elution of an Octyl-Sepharose CL-4B column loaded with 90–100 nmol of cytochrome P-450 after Emulgen 913 solubilization are shown in Fig. 1 using microsomes of untreated mice (normals) for illustration. Table 1 summarizes the fractionation, total content and recovery of major components of the microsomal monooxygenase system (e.g. material of non-pretreated mice) applied to the hydrophobic interaction column. Considering the cytochrome P-450 eluted from the column, nearly its total amount is present in the 417 nm-absorbance peak 4, devoid of cytochrome b_5 and NADPH–cytochrome *c*-reductase. Peak 3 mainly consists of cytochrome b_5. NADPH-cytochrome *c*-reductase activity was mainly found (82.3%) in peak 2, whereas the first peak contains about one fifth of the applied cytochrome b_5 as well as some cytochrome P-450 and NADPH-cytochrome *c*-reductase activity (16%). Small amounts of cytochrome P-420 were detected in peak 1 and peak 2. This is true for all pretreatments. Depending on the pretreatment the total recovery of cytochrome P-450 measured in all four elution peaks was 70% to 80% (77.1% for normals, see Table 1) related to the amount of cytochrome P-450 applied to the column.

Table 2 presents a summary of the purification procedure of cytochrome P-450 of variously pretreated mice. Cytochrome P-450 eluted from the Octyl-Sepharose CL-4B column in peak 4 was purified 3.8–5.3-fold with total yields of about 52% to 65%.

Figure 2 shows a magnified section of a SDS–polyacrylamide gel of the pooled fractions of peak 4 (cf. Fig. 1) of the Octyl-Sepharose CL-4B eluate after several pretreatments: cobaltous chloride (track a), normals (track b), sodium warfarin (track c), sodium phenobarbitone (track d). The analysis of the electropherogram reveals that after pretreatment with warfarin as well as with phenobarbitone a new band (approximate molecular weight: 56.4 Kd) appears in contrast to normals and cobalt-pretreated mice. The 50.2 Kd band is predominant after phenobarbitone and warfarin induction, whereas the 49.7 Kd band is less intensive in comparison to normals. Thus warfarin causes the same microsomal protein pattern as phenobarbitone in the 50 Kd region. After cobalt pretreatment the 48.8 Kd band is much more intensive than in normal mice. The cobalt-induced changes do not show any parallelism, either to normals or to warfarin-/phenobarbitone-induced mice.

We made further attempts to characterize the proteins of peak 4 by a hemoprotein sensitive staining procedure. Figure 3 shows a track of a SDS–polyacrylamide gel of the proteins of peak 4 first stained with TMBZ for peroxidase activity and thereafter with Coomassie blue for protein. Non-reducing conditions are necessary for this separation procedure

Table 1. Fractionation and recovery of major components of the microsomal monooxygenase system of nonpretreated mice (normals) applied to an Octyl-Sepharose CL-4B column

	Components of the microsomal monooxygenase system						
	Protein	Cytochrome P-450		Cytochrome b_5		NADPH–cytochrome *c*-reductase activity	
	Total (mg)	Total (nmol)	Yield (%)	Total (nmol)	Yield (%)	Total (U)	Yield (%)
Applied to column	144.0	91.36	100.0	68.44	100.0	21.00	100.0
Peak 1 (fractions 4–13)	87.5	1.30	1.4	14.15	20.7	3.36	16.0
Peak 2 (fractions 14–22)	6.3	0.26	0.3	5.04	7.4	17.29	82.3
Peak 3 (fractions 47–60)	4.4	0.60	0.7	40.22	58.8	n.d.	—
Peak 4 (fractions 74–95)	19.0	68.23	74.7	n.d.	—	n.d.	—
Recovered (in peaks 1–4)	117.2	70.39	77.1	59.41	86.9	20.65	98.3

The mentioned "yields" are related to material applied to the column. See Fig. 1 for condition of elution. n.d. = not detectable.

Table 2. Summary of purifications of liver microsomal cytochrome P-450 of mice after several pretreatments

Pretreatment	Purification steps	Cytochrome P-450 Total (nmol)	Yield (%)	Specific activity (nmol × mg^{-1} protein)	Purification (fold)
	Microsomes	104.53	100.0	0.68	1.00
N	Solubilized microsomes	91.36	87.4	0.63	0.93
	Octyl-Sepharose eluate (peak 4)	68.23	65.3	3.59	5.28
	Microsomes	119.50	100.0	1.66	1.00
PB	Solubilized microsomes	96.32	80.6	1.31	0.79
	Octyl-Sepharose eluate (peak 4)	72.04	60.3	6.29	3.79
	Microsomes	105.11	100.0	1.26	1.00
W	Solubilized microsomes	91.76	87.3	1.14	0.90
	Octyl-Sepharose eluate (peak 4)	55.05	52.4	5.37	4.26
	Microsomes	103.63	100.0	0.64	1.00
Co	Solubilized microsomes	94.10	90.8	0.67	1.05
	Octyl-Sepharose eluate (peak 4)	59.22	57.1	3.29	5.14

N = normals, PB = phenobarbitone-, W = warfarin-, Co = cobalt-pretreatment. The estimated "yields" are related to the starting amounts in the integer microsomes.

in order to maintain the integrity of the hemoproteins necessary for TMBZ staining [26, 27]. On the other hand, this method does not result in a satisfactory protein separation into distinct bands within the narrow range of the molecular weights of the observed proteins. Consequently differences in pattern of peak 4 proteins dependent on different pretreatments cannot be visualized in these gels. TMBZ and Coomassie blue stain obviously the same area placed between molecular weights of 46.2 and 57.7 Kd. This range of molecular weights is in accordance with that of Fig. 2. Due to the absence of cytochrome b_5 and

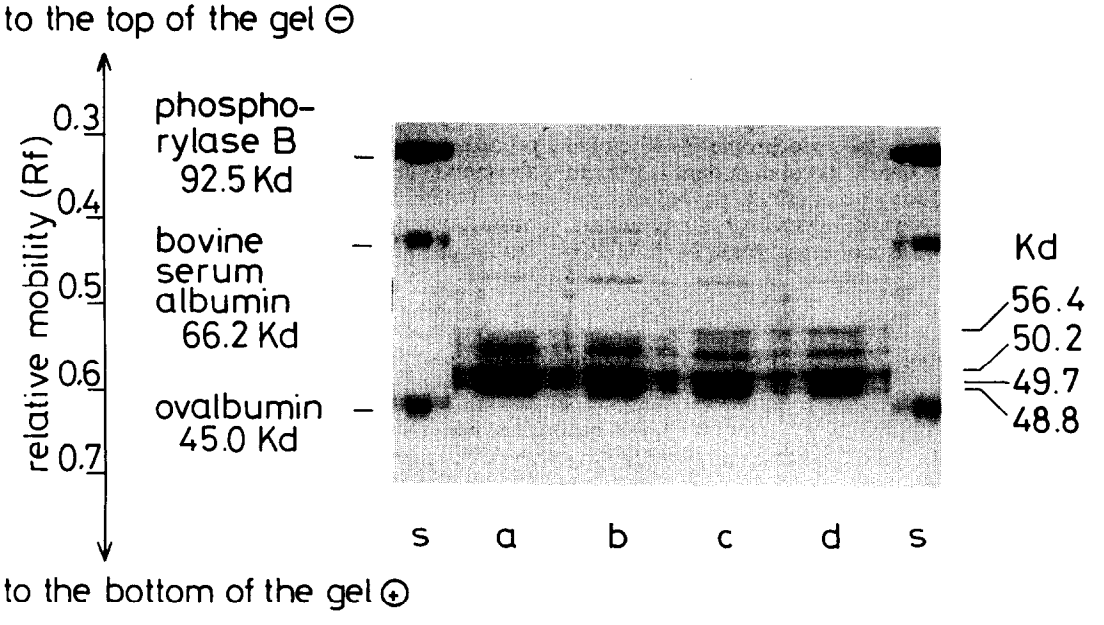

Fig. 2. SDS–polyacrylamide gel electrophoresis of microsomal proteins of peak 4 (cf. Fig. 1) obtained by hydrophobic interaction chromatography on Octyl-Sepharose CL-4B. A photograph (magnified section) is shown with the approximate molecular weights of the standards (s) and those bands altered by the following pretreatments (see also Results): cobaltous chloride (track a), normals (track b), sodium warfarin (track c), and sodium phenobarbitone (track d). Each of these tracks was loaded with samples containing 40 pmol cytochrome P-450 of the pooled peak 4. This amount of protein was chosen with the aim of visualizing the higher molecular weight bands enabling a better differentiation of the critical bands above 50 Kd. The transverse leakage from the sample wells provided a better comparison between the bands of different tracks in the intermediate spaces.

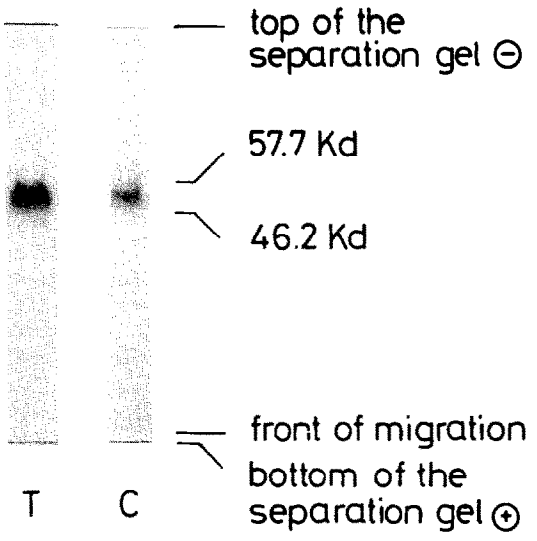

Fig. 3. SDS–polyacrylamide gel of proteins of peak 4 (cf. Fig. 1). One individual track is shown first stained for peroxidase activity with TMBZ (T) and subsequently for protein using Coomassie blue (C). The track was loaded with a sample containing 120 pmol cytochrome P-450 of normals. For details see Materials and Methods.

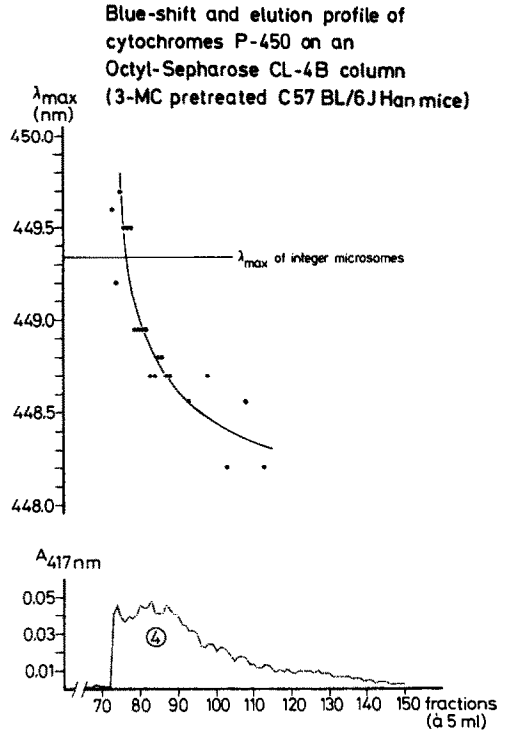

Fig. 4. The conditions of elution up to fraction 65 were the same as demonstrated in Fig. 1. Peak 4 was eluted by a buffer containing a reduced concentration (0.5%) of Emulgen 913. The upper panel shows the decreasing wavelength of the absorbance maximum of the CO-complex of reduced cytochrome P-450 in relation to the fraction number. The lower panel shows peak 4 measured by monitoring optical density at 417 nm.

hemoglobin in peak 4 as estimated by spectral analysis we suggest the hemoproteins to be cytochromes P-450.

A pronounced tailing of peak 4 is provoked if the elution procedure is performed with a lower concentration (0.5%) of Emulgen 913 (cf. Fig. 1 and Fig. 4). 3-Methylcholanthrene-induced samples show a blue-shift of the CO-reduced minus reduced absorbance maximum of the cytochromes P-450 dependent on the progredient elution in peak 4. This may indicate the potency of Octyl-Sepharose CL-4B to separate different forms of cytochrome P-450 by selective retention.

DISCUSSION

In general

As a result of optimization of both solubilization and separation procedure on Octyl-Sepharose CL-4B, originally described by Gibson and Schenkman [19] and Beaune *et al.* [20], a rapid method is presented to investigate induction phenomena in mice. The advantages of the method are that at most ten animals are needed (in contrast to Levi and Hodgson [28]) and that the recovery of cytochrome P-450 in peak 4 as well as the purification of cytochrome P-450 in this peak are higher than those reported by Beaune *et al.* [20]. The proteins of peak 4 were subjected to conventional SDS–polyacrylamide gel analysis revealing clear differences among the various inductions investigated. Furthermore, the use of Octyl-Sepharose CL-4B implies the possibility to compare the hydrophobic character of cytochromes P-450 from different organisms.

In detail

After solubilization of mouse liver microsomes with 0.2% (w/v) Emulgen 913 we obtained 80% to 90% of the cytochrome P-450 in the supernatant. The experiments were performed according to Gibson and Schenkman [19]. In contrast, the solubilization procedures according to Beaune *et al.* [20] in a 0.75% Na–cholate solution recovered only smaller percentages.

For purification we chose hydrophobic interaction chromatography using a high Emulgen 913 concentration (2% w/v) to elute a highly concentrated cytochrome P-450 in peak 4 (cf. peak 4 in Fig. 1 and 4). The column recovery of cytochrome P-450 in peak 4 is 60–75% (74.7% for normals, see Table 1) depending on pretreatment. Purification of cytochrome P-450 in peak 4 was 3.8–5.3-fold gaining total yields of 52% to 65% as related to the amount of cytochrome P-450 in microsomes (see Table 2).

Levi and Hodgson [28] reported that, while very much information has been published on the purification and characterization of multiple forms of cytochrome P-450 in rabbits and rats, there exist in untreated male ICR mice at least five forms of cytochrome P-450 with molecular weights between 48 and 56 Kd. As shown in Fig. 2 seven resp. eight protein bands in peak 4 (containing 3.8–5.3-fold purified cytochrome P-450 devoid of cytochrome b_5 and hemoglobin) can be distinguished in the molecular weight range between 47.8 and 56.4 Kd. Since this area is stainable with TMBZ (Fig. 3) for hemo-

proteins we suggest these protein bands to be cytochromes P-450.

After sodium warfarin pretreatment an additional protein band is present at 56.4 Kd, and the 50.2 Kd band becomes predominant in comparison with normals. Thus after warfarin administration the SDS–PAGE protein pattern of peak 4 (containing partially purified cytochrome P-450) in male C57 BL/6J Han mice is phenobarbitone-like (Fig. 2). This fact agrees with previously shown results from this laboratory characterizing enzymatic action of warfarin pretreated microsomes [8, 13]:

Besides the increase in microsomal protein and cytochrome P-450 content (twofold) a proliferation and vesiculation of smooth endoplasmic reticulum has been observed in hepatocytes of warfarin—as well as phenobarbitone-treated mice. On the other hand, 3-methylcholanthrene did not cause a significant proliferation of endoplasmic reticulum [29].

Warfarin also shortened the hexobarbitone sleeping time highly significantly to about one third (phenobarbitone reduces it to about 20%) and zoxazolamine paralysis to one fifth of normals' paralysis time (phenobarbitone to one-eighth).

In vitro studies revealed hexobarbitone binding affinity to warfarin-induced microsomes to be increased 2.5-fold and to phenobarbitone pretreated microsomes 5.9-fold.

Inhibition curves of the 7-ethoxycoumarin-deethylation *in vitro* by metyrapone show similar sigmoidal shapes after warfarin and phenobarbitone induction, which are both different from normals. The warfarin induced cytochrome P-450 is 10 times, the phenobarbitone induced cytochrome P-450 90 times more susceptible to metyrapone inhibition than normals, measured by I_{50}-values. In warfarin- as in phenobarbitone-induced microsomes inhibition of 7-ethoxycoumarin-deethylation by warfarin occurred more strongly than in normals.

Warfarin like phenobarbitone induction causes an increase of the molecular activity of cytochrome P-450 on *in vitro* 7-ethoxycoumarin-deethylation of about 60% and on 7-methoxycoumarin-demethylation of about 30%. 7-Methoxycoumarin-demethylation *in vivo* ($^{14}CO_2$-exhalation analysis) revealed a maximum $^{14}CO_2$-exhalation rate per mouse 1.5-fold higher in warfarin-, and 1.8-fold higher in phenobarbitone-pretreated mice compared to normals. Also the ratio of demethylation of scoparone in position 6 and 7 is affected *in vivo* by warfarin pretreatment [14].

Current investigations show that warfarin indeed also induces its own metabolism much more strongly (2.3- to 6.5-fold molecular activity determined by measuring four different metabolites) than the 7-ethoxy- and 7-methoxycoumarin metabolism. 3-Methylcholanthrene slightly decreases the molecular activity of cytochrome P-450 towards warfarin metabolism.

All data obtained by enzyme kinetic analysis and SDS–PAGE qualitatively show phenobarbitone-like induction phenomena after warfarin pretreatment. The observed effects after warfarin induction, however, are quantitatively not as pronounced as those after phenobarbitone pretreatment. This may lead to the conclusion that the quantities of different forms of cytochrome P-450 being responsible for the alteration in metabolic activity is different after phenobarbitone and warfarin pretreatment within the tested dose.

Cobalt is known to decrease microsomal cytochrome P-450 content when administered *in vivo* [15, 30]. On the other hand, Legrum *et al.* [15, 16, 31] demonstrated an induction-like effect after cobaltous chloride treatment characterized by enhanced molecular activity towards metabolism of coumarin and several 7-substituted coumarin derivatives. However, the metabolism of the 4-hydroxycoumarin derivative warfarin is less markedly increased after cobaltous chloride pretreatment [32]. Stuehmeier *et al.* [17] reported phenobarbitone and cobaltous chloride seemingly to have similar inductive effects on biphenyl metabolism, but deeper analysis revealed distinguishable effects on both modifiers. This finding seems to coincide with the present study showing that cobaltous chloride pretreatment produces a different protein pattern in the 50 Kd region in comparison to that of phenobarbitone pretreatment and to that of untreated animals (normals). Cobalt mainly causes an intensification of the 48.8 Kd band (Fig. 2).

Kahl *et al.* (33) reported that variations in absorbance maxima of cytochromes P-450 depend on the amounts of cytochrome P-448 and cytochrome P-450 present in a sample. Even cytochrome P-448 of 3-methylcholanthrene-induced responsive mice exhibits a heterogeneous character, because it is constituted by cytochrome P-448 itself as well as by several forms of cytochrome P-450. In Fig. 4 we demonstrate a selective retention of cytochromes P-450 of 3-methylcholanthrene-pretreated mice on an Octyl-Sepharose CL-4B column showing a blue shift of λ_{max} during the progredient elution of peak 4. Thus we suggest that the proportion of the composition of cytochrome isoenzymes absorbing light in the 450 nm region to those absorbing light in the 448 nm region is altered in such a way that at the beginning of peak 4 mainly cytochromes P-450 and less cytochrome P-448 are eluted while at the end of the elution period the situation is reversed. This finding prompts us to formulate the hypothesis that in mice cytochrome P-448 has to be considered to be more hydrophobic than cytochrome P-450. But further subtile experiments are necessary to strengthen this contention.

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