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ION-EXCHANGE FAST PROTEIN LIQUID CHROMATOGRAPHY; OPTIMIZATION OF THE PURIFICATION OF CYTOCHROME P-450 FROM MARMOSET MONKEYS

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SUMMARY

Fast protein liquid chromatography (FPLC) on Mono Q and Mono S ion-exchange columns was employed to purify marmoset monkey hepatic cytochrome P-450. Cholate-solubilized liver microsomes from untreated animals as well as from animals induced with phenobarbital, β-naphthoflavone, 3-methylcholanthrene, and ethanol were used as starting materials. Since established purification schemes for extensively studied species, such as the rat, were found to be not directly applicable, a purification method was developed and optimized by studying the effects of varying detergent types, detergent concentrations, elution buffers, pH values, elution salts, and flow-rates on the resolution and recovery obtained in analytical chromatograms.

INTRODUCTION

The purification of cytochrome P-450 (P-450)[1] is generally difficult, because its isozymes or forms, are in the molecular weight range of 45 000 to 60 000 daltons, which contains a disproportionately large number of other microsomal proteins. Recent improvements in high-performance liquid chromatographic (HPLC) separations have been reported for rat liver P-450[2–6], but not on primate material. More specific difficulties are encountered when primate material — almost invariably from humans — is investigated[7,8], since here inter-individual differences play a role. Moreover, the concentrations and metabolic activities of P-450 are often low, and different lipid constituents may affect microsome sedimentation and solubilization. Specimens from non-human primates have the advantages that they are more easily accessible and that animals can be induced in a controlled manner. The purification of P-450 from such primates has been published[9], but to our knowledge data on the purification of P-450 from the marmoset monkey have not yet been reported.

It was the aim of the present work to study this species systematically, and to improve the purification by ion-exchange fast protein liquid chromatography (FPLC) by investigating the effects of several chromatographic parameters on resolution and recovery. We consider this to be the first report on the application of Mono Q (instead of DEAE) and Mono S (instead of CM or SP) columns to this problem.

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EXPERIMENTAL

Sample preparation

Male marmoset monkeys (8–14 months old) (*Callithrix jacchus*) were used, which were either untreated or induced with phenobarbital [0.05% (w/v) in drinking water for 10 days], β-naphthoflavone or 3-methylcholanthrene (40 mg/kg subcutaneously each day for 4 days), or ethanol [10% (v/v) in drinking water for 40 days]. The animals were killed by decapitation, and the microsomes were prepared[10]. After solubilization with sodium cholate (6 mg protein and 18 mg cholate/ml)[10] the microsomal material was filtered through Sephadex G-75 at 1 *g*, either as described in ref. 11 or by using disposable plastic 2-ml syringes. For the purpose of comparing the relative effectiveness of Sephadex G-75, Sephadex G-25, Phenyl-Sepharose, and hydroxyapatite for removing detergents and salt, 50 × 5 mm columns were packed with each material. The columns were installed in the chromatographic system (see below) and operated at a flow-rate of 0.2 ml/min. A pre-packed column of Superose 12 was tested in a similar way, except that the dimensions were 300 × 10 mm and the flow-rate was 0.4 ml/min.

Gels and detergents

Mono Q (a strong anion exchanger) and Mono S (a strong cation exchanger), each consisting of 10-μm polymer particles and pre-packed in 50 × 5 mm columns (1 ml volume), as well as Sephadex G-75, Sephadex G-25, Phenyl-Sepharose and Superose 12 were purchased from Pharmacia (Uppsala, Sweden). Hydroxyapatite "Ultrogel HA" was from LKB (Bromma, Sweden).

Octyl glucoside (octyl-β-D-glucopyranoside), octyl thioglucoside (octyl-β-D-thioglucopyranoside), Mega 8 (N-D-gluco-N-methyloctanamide) and Mega 10 (N-D-gluco-N-methyldecanamide)[12] were purchased from Calbiochem (Frankfurt, F.R.G.), sodium cholate from Serva (Munich, F.R.G.), sodium deoxycholate from Merck (Darmstadt, F.R.G.), CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate}[13] and Lubrol PX (polyoxyethylene dodecylalcohol ether) from Sigma (St. Louis, MO, U.S.A.). Renex 690 and Emulgens 911, 913 (polyoxyethylene nonyl phenol ethers) were gifts from Atlas Chemie (Essen, F.R.G.) and Kao Corp. (Tokyo, Japan), respectively.

Chromatography

Ion-exchange FPLC was carried out with two pumps, a gradient controller, and other equipment from Pharmacia. Gradients were formed from a buffer solution, (buffer A) containing 10 m*M* Tris or potassium phosphate (pH see below), 20% glycerin, 0.1 m*M* EDTA and a detergent (see above), as described in the legends to Figs. 2–8, and a solution of salt in buffer A (buffer B; indicated in the legend to Fig. 5). The loads of P-450, in mg of protein, are given in the figure legends. The back pressures for these 50 × 5 mm columns were between 3.4 and 3.9 MPa at a flow-rate of 1 ml/min. Absorbances were measured at 405 nm. Fractions of 1 ml were collected and when neccessary combined to re-determine P-450 contents. The temperature for all chromatographic separations was 18°C.

Determination of P-450 and proteins

P-450 contents were measured from CO-oxidized-reduced difference spectra[14]. The protein content was determined by the biuret method with an automatic bichromatic analyzer, ABA 100, Abbott (Wiesbaden, F.R.G.).

RESULTS AND DISCUSSION

As described in the Experimental section and in the legend to Fig. 1, different gel materials were considered for their ability to remove detergent and salt. In these experiments, Renex 690 was replaced by Lubrol PX (which does not absorb at 280 nm). Fig. 1 shows the separations of cytochrome (detection at 405 nm) from Renex 690 (detection at 280 nm) and from salts including sodium cholate (detection by conductivity). It can be seen that Sephadex G-75 produced a 50–70% recovery of P-450 and a 90–95% removal of salt and cholate. G-25 gave a slightly poorer separation. Of these gels Superose 12 yielded the best cholate separation, but it was not used further, because the sample volume on this column is limited to 200 μl and because the elution times are long (up to 1 h). Gel filtration chromatography is, in general, not suitable for exchanging or removing non-ionic detergents when the combined weight of the molecules in a micelle approaches the molecular weight of the protein[15]. This is illustrated in Fig. 1, where the molecular weight of P-450 and the weight of the micelles of either Renex 690 or Lubrol PX are all between 50 000 and 60 000 daltons, and the weight of the cholate micelles is *ca.* 2000 daltons (Lubrol PX does not absorb at 280 nm, whereas Renex 690 does). The usefulness of Phenyl-Sepharose, as described by Robinson *et al.*[16], for exchanging or removing any of the

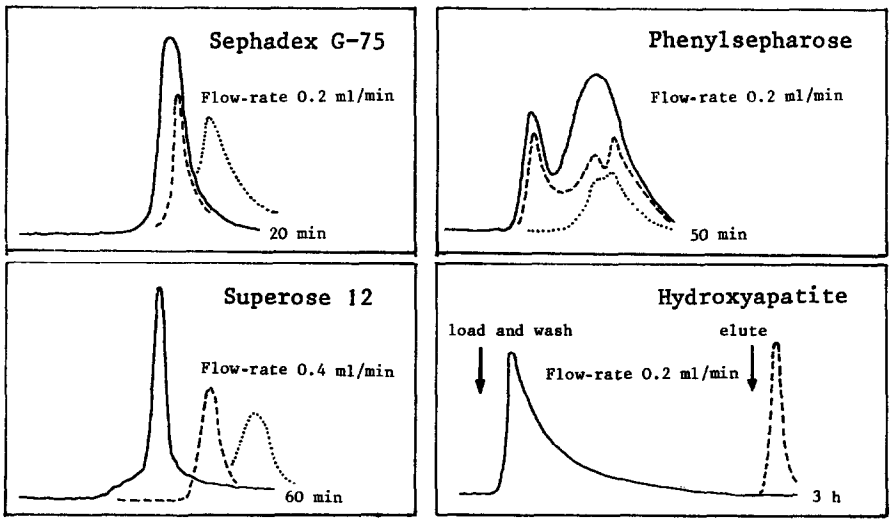

Fig. 1. Comparison of detergent exchange chromatography on different columns. Sample, phenobarbital-induced marmoset liver microsomes in 100 m*M* Tris, solubilized with sodium cholate and Renex 690. Sample size, 200 μl = 780 pmol P-450, 1.2 mg protein, 3.6 mg cholate and 0.4 mg Renex; column size, 50 × 5 mm, except Superose 300 × 10 mm; buffer, 10 m*M* Tris–HCl (pH 7.7)–20% glycerin–0.1 m*M* EDTA–0.3% Lubrol PX; detection, 280 nm at 0.6 a.u.f.s. (solid curve), 405 nm at 0.04 a.u.f.s. (dashed curve), and conductivity with 5.5 mS full scale (dotted curve).

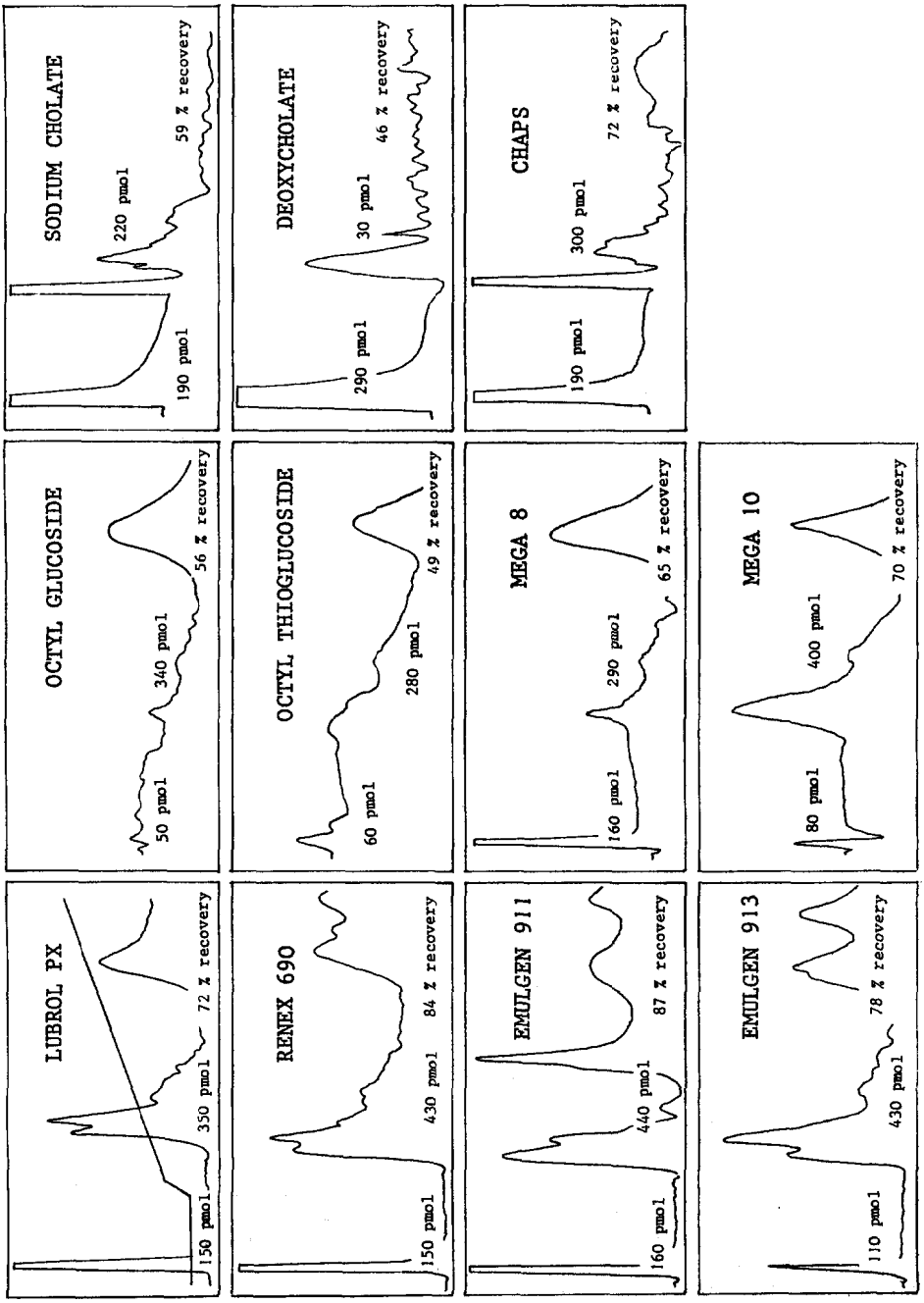

Fig. 2. Effect of various detergents in the eluent buffer on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, filtered though Sephadex G-75. Sample size, 690 pmol P-450 = 1.1 mg protein; column, Mono Q, 50 × 5 mm; flow-rate, 1 ml/min. Buffer A, 10 m*M* Tris–HCl (pH 7.7)–0.1 m*M* EDTA–20% glycerin–0.5% of detergent; buffer B, 1 *M* sodium chloride in buffer A; gradient, 0–40–200 m*M* over 28 ml (see upper left profile); detection, 405 nm at 0.01 a.u.f.s. P-450 elutes throughout the chromatograms. Recovery data are a summation of the recoveries in several parts of the chromatograms.

detergents from the proteins under investigation here could not be confirmed. Hydroxyapatite, as described elsewhere for the removal of non-ionic detergents[20], was found to give the most complete removal of Renex 690 from the cytochrome, but the times required are much longer (Fig. 1). However, since the aim of the present work was only to remove both the salt and the detergent semi-quantitatively, and to accomplish this in the shortest possible time for numerous subsequent ion-exchange runs, Sephadex G-75 was chosen for further use.

Detergent types

Detergents were compared for their ability to maintain resolution and recovery during chromatography subsequent to solubilization. P-450 was invariably found in most peaks of one and the same chromatogram (Fig. 2). The choice of detergents was based on the facts that (a) Lubrol, Renex, Emulgens, cholate, and deoxycholate have been traditionally used to purify P-450, and that (b) the remaining detergents, while not having been used to this end, are fairly recent in origin and enjoy certain advantages, such as transparency at 280 nm and ease of dialysis[12]. A standard concentration of 0.5% (w/v) was chosen, which is about as high as, or higher than, the micelle concentration of any of the detergents.

The anionic detergents cholate and deoxycholate, and the zwitterionic CHAPS (Fig. 2) gave good recoveries and resolution when used in the elution buffer for the Mono Q column. However, they were excluded from further use, because they invariably showed long equilibration times, baseline drift, a low sample capacity, and a high back pressure. From the other detergents tested Emulgen 911 was chosen on the basis of recovery, resolution, and column hygiene. This detergent has also been employed by other researchers[2,17].

After chromatography with Emulgen 911 under the conditions of Fig. 2, cytochrome P-420 (inactive P-450) was detected in the first (unretained) peak as well as above 350 m*M* sodium chloride and the total amount roughly equalled that originally present in the sample, thus indicating that the FPLC separation itself did not convert a significant amount of P-450 to P-420. Cytochrome b_5 and NADPH-cytochrome C (cytochrome P-450) reductase were found to elute at about 230 and 340 m*M* sodium chloride, respectively, and these peaks were not found to contain any cytochrome P-450[10]. P-450 and reductase, together with phospholipid (but without b_5) could be reconstituted into substrate-metabolizing systems, showing that these proteins retained enzymatic activity after chromatography[10].

Detergent concentration

The concentration of Emulgen 911 was varied to find the lowest concentration that yielded consistently good resolution. This was found to be 0.5 or 0.6% (w/v); 0.4% or lower gave lower peak selectivity; 0.7% or higher had no apparent influence on the chromatographic profile (see Fig. 3).

Buffers

As suggested by the manufacturers of Mono Q and Mono S, only cationic and anionic buffers, respectively, should be used. For the present work, Tris and potassium phosphate were used, which were found to be interchangeable to a large extent (for pH see below). With both columns, Tris gave up to 10% higher yields. When

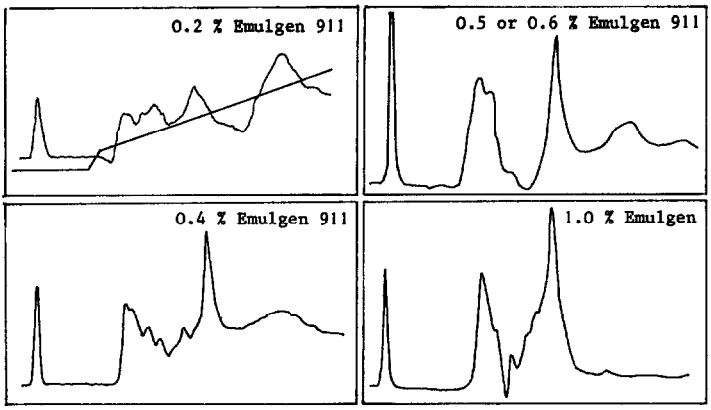

Fig. 3. Effect of detergent concentration in eluent buffer on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, exchanged for various concentrations of Emulgen 911 on Sephadex G-75. Sample size, 580 pmol P-450 = 0.89 mg protein; column, Mono Q, 50 × 5 mm; flow-rate, 1 ml/min. Buffer A, 10 m*M* Tris–HCl (pH 7.7)–20% glycerin–0.1 m*M* EDTA–various concentrations of Emulgen 911; buffer B, 1 *M* sodium chloride in buffer A; gradient, 0–40–200 m*M* over 28 ml (see top left); detection, 405 nm at 0.01 a.u.f.s.

Mono Q and phosphate, or Mono S and Tris were used, the ratio of the amount of P-450 in the unretained fraction to that eluted during the gradient was occasionally increased up to 4-fold.

pH

Elution of P-450 from a Mono Q column with buffers having a pH between 7.4 and 8.0 clearly revealed an optimum at pH 7.7 (Fig. 4). Other pH values may yield good separations when stepwise gradients are used. For Mono S, a pH of 6.5 was chosen, although here the differences were less striking.

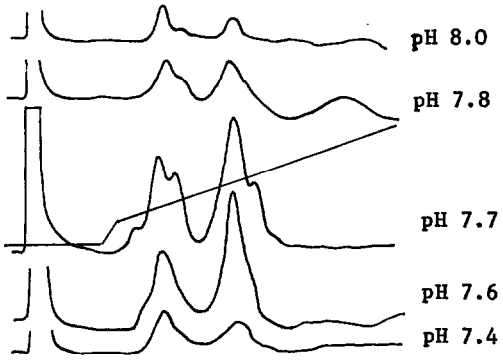

Fig. 4. Effect of pH on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, exchanged for buffer A on Sephadex G-75. Sample size, 3.9 nmol P-450 = 6.0 mg protein; column, Mono Q, 50 × 5 mm; flow-rate, 1 ml/min. Buffer A, 10 m*M* Tris–HCl (varying pH)–20% glycerin–0.1 m*M* EDTA–0.5% Emulgen 911; buffer B, 1 *M* sodium chloride in buffer A; gradient, 0–40–200 m*M* in 28 ml; detection, 405 nm at 0.05 a.u.f.s.

Elution salts

The effects of using six different salts in the elution buffer on the Mono Q column are shown in Fig. 5, where it can again be seen that practically every peak

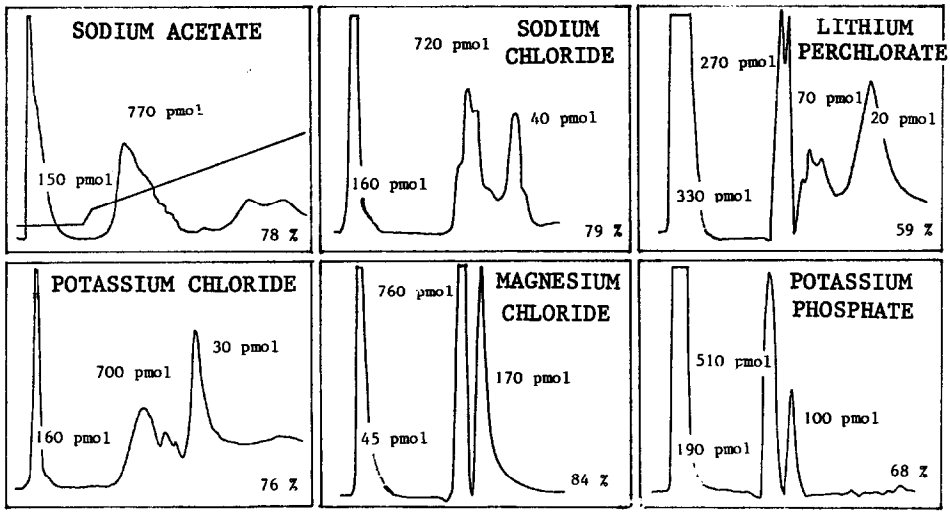

Fig. 5. Effect of various salts in eluent buffer on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, exchanged for buffer A on Sephadex G-75. Sample size, 1.17 nmol P-450 = 1.8 mg protein; column, Mono Q, 50 × 5 mm; flow-rate, 1 ml/min. Buffer A, 10 m*M* Tris (counter-ion of salt in buffer B, pH 7.7)–20% glycerin–0.1 m*M* EDTA–0.5% Emulgen 911; buffer B, 1 *M* of the salt except for potassium phosphate = 400 m*M*; gradient, 0–40–200 m*M* over 28 ml (see top left); detection, 405 nm at 0.02 a.u.f.s.

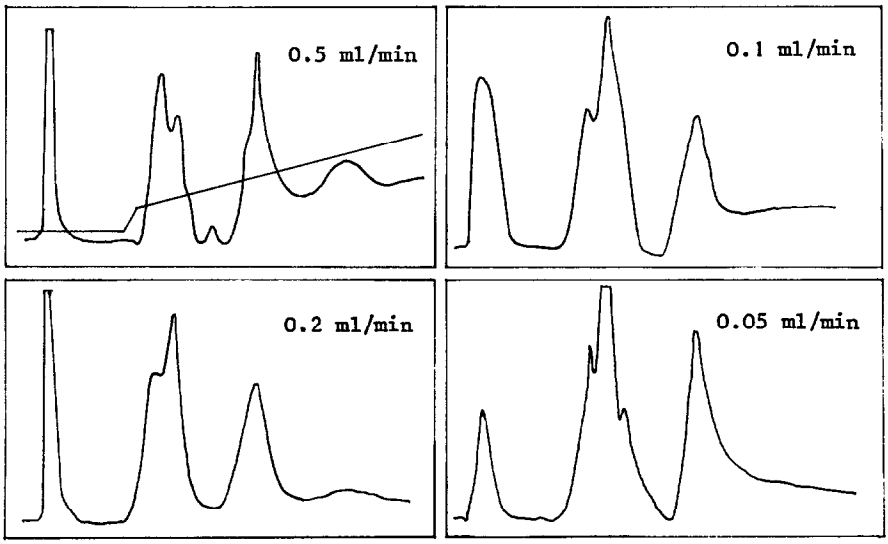

Fig. 6. Effect of flow-rate on resolution. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, dialyzed in buffer A. Sample size, 3.9 nmol P-450 = 6.0 mg protein; column, Mono Q, 50 × 5 mm. Buffer A, 10 m*M* Tris–HCl (pH 7.7)–20% glycerin–0.1 m*M* EDTA–0.5% Emulgen 911; buffer B, 1 *M* sodium chloride in buffer A; gradient, 0–40–200 m*M* over 31 ml (see top left); detection, 405 nm at 0.05 a.u.f.s.

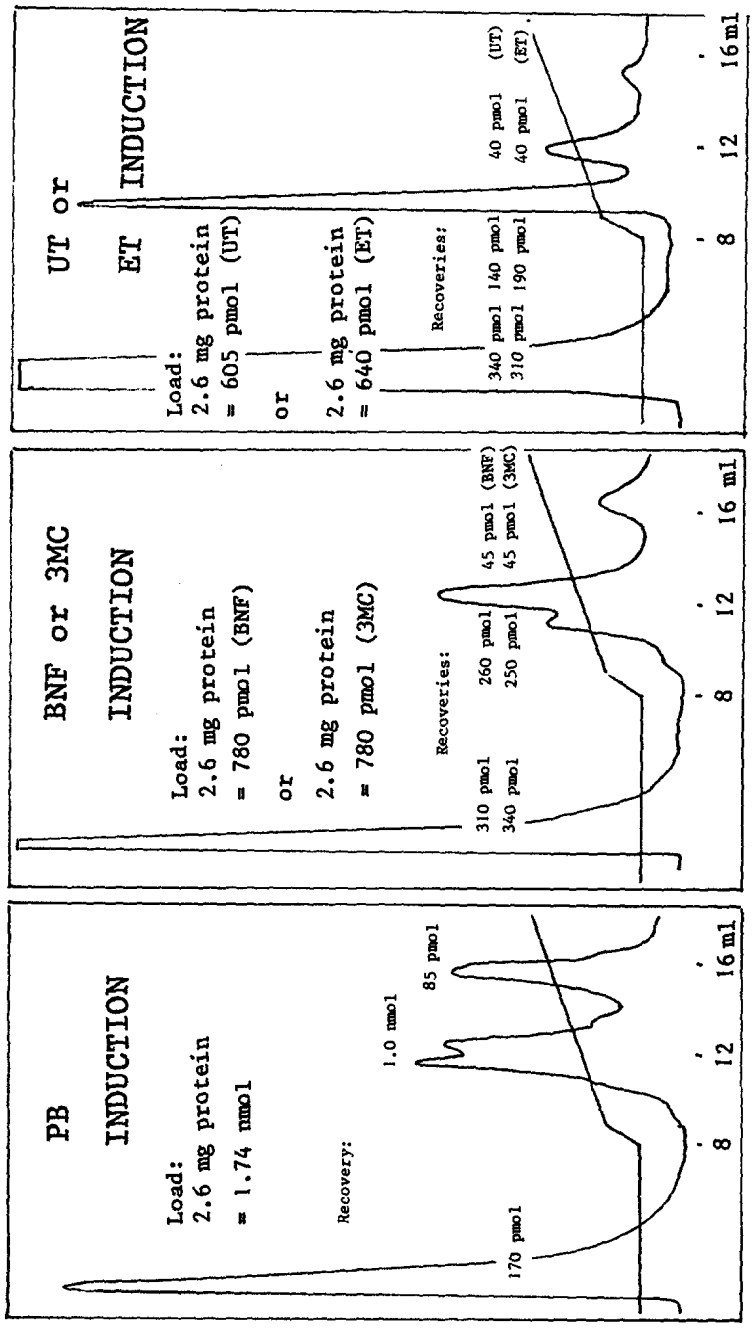

Fig. 7. Anion-exchange chromatograms on Mono Q of cholate-solubilized liver microsomes from untreated (UT) male marmosets and from marmosets induced with phenobarbital (PB), β-naphthoflavone (BNF), 3-methylcholanthrene (3MC), and ethanol (ET). The samples were filtered through Sephadex G-75 and then dialyzed in buffer A. All samples contain 2.6 mg protein (P-450 see above); column, Mono Q, 50 × 5 mm; flow-rate, 0.5 ml/min. Buffer A, 10 m*M* Tris–HCl (pH 7.7)–20% glycerin–0.1 m*M* EDTA–0.5% Emulgen 911; buffer B, 1 *M* sodium chloride in buffer A; gradient, 0–40–120 m*M* over 28 ml; detection, 405 nm at 0.04 a.u.f.s.

contains P-450. If they indeed represent different P-450 isozymes or forms, then the discrimination between them, for example by various substrate assays[10] requires high resolution. Therefore, sodium chloride was prefered to sodium acetate and potassium chloride. Previously it has been reported that for the purification of rat P-450 on DEAE columns sodium acetate gave the best resolution[2].

In the present work, lithium perchlorate apparently gave the best resolution, but not the highest recovery. Moreover, P-450 fractions were found to be unstable in the presence of this salt after a few hours. Magnesium chloride and potassium phosphate, two salts which have much higher elution strengths, yielded excellent selectivities but were eventually discarded because of significant increases in back pressure. This was found also to apply, albeit to a lesser extent, to elution from Mono S column.

Flow-rate

A decrease in the flow-rate for the Mono Q column from 1.0 to 0.05 ml/min (0.2–0.01 ml cm^{-2} min^{-1}) resulted in a slight increase in resolution (Fig. 6), but the positions of the three main peaks remained the same. 0.5 ml/min was chosen because (a) higher flow-rates often resulted in increased back pressure when the sample load and/or the salt concentration was increased, and (b) lower flow-rates increased the amount of material eluted in the third peak at the expense of material in the first (unretained) peak. The first peak was found to be electrophoretically more homogeneous than the second or third peak[10].

Inducing agents

The elution profiles described so far were observed with phenobarbital-induced microsomes as sample. Inspection of the chromatograms obtained from untreated (UT) animals as well as animals induced with β-naphthoflavone (BNF), 3-methylcholanthrene (3MC), or ethanol (ET) reveals several differences (Fig. 7; note that equivalent protein loads gave different total P-450 contents). Such differences have also been reported for rat liver specimens[2,5], where the specific P-450 contents were much higher.

It is interesting that the chromatograms for BNF and 3MC (and UT and ET) are quite similar, because this may reflect that the isozymes or forms of P-450 present are also similar or even identical. This has been suggested for chromatograms of induced rat liver P-450[18]. All of the peaks in Fig. 7 contain cytochrome P-450 and any of them may contain more than one cytochrome (see also Conclusions).

Finally, it is significant that upon induction with PB (or BNF or 3MC) the relative alterations in intensities of the first (unretained) peak and the second peak (at about 10 ml) are quite different. Similar findings were reported by Guengerich *et al.*[19] for rat cytochrome P-450, where it was clearly shown that the concentration levels of different UT cytochromes are affected to different extents upon induction, and that the effects are also different for PB and BNF inductions.

Mono S and combined elution

When Mono S was used in place of Mono Q, recoveries were routinely found to be about 97%, *i.e.* 10% higher than on Mono Q. On Mono Q roughly 80% of the total yield could be found in the fractions (Figs. 2, 5 and 7), whereas with PB-induced

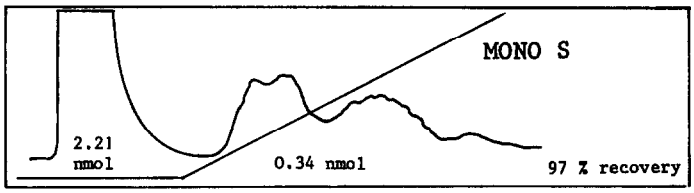

Fig. 8. Cation-exchange chromatogram on Mono S. Sample, cholate-solubilized phenobarbital-induced marmoset liver microsomes, filtered through Sephadex G-75. Sample size, 2.6 nmol P-450 = 4.0 mg protein; column size, 50 × 5 mm; flow-rate, 0.5 ml/min. Buffer A, 10 m*M* potassium phosphate (pH 6.5)–20% glycerin–0.1 m*M* EDTA–0.5% Emulgen 911; gradient, 0–250 m*M* in 25 ml; detection, 405 nm at 0.01 a.u.f.s.

microsomes on Mono S, less P-450 was found to bind to the column (Fig. 8). This is in contrast to observations on rat P-450, chromatographed on an SP column[2]. However, this may reflect the differences between the two animal species rather than between the two column types, since the latter differ only in the spacer group, which is methylene for Mono S and propyl for SP. No essential differences between Mono S and SP with regards to selectivity, efficiency or capacity have been reported[21].

None of the three Mono S fractions was found to be as electrophoretically homogeneous as the unretained fraction of Mono Q[10]. However, apparent electrophoretic homogeneity was obtained[10] when the Mono Q pass-through fraction was eluted with a stepwise gradient of phosphate (0–120 m*M*) from Mono S (Fig. 8).

CONCLUSIONS

Various chromatographic parameters were tested for their effect on resolution and recovery of P-450 obtained from marmoset monkeys. Since no data on the chromatography of this material had previously been reported, this study may form the basis for further work, in which fractions are tested by electrophoreses and specific contents are determined. Optimal results were obtained with Tris buffer (pH 7.7, 18°C) for FPLC on Mono Q, with potassium phosphate buffer (pH 6.5) for FPLC on Mono S, and with 0.5% Emulgen 911 and sodium chloride gradients at a flow-rate of 0.5 ml/min for both columns.

From the present data, it is evident that changes in chromatographic procedures can dramatically affect the elution profiles and the number of peaks eluting from the column. An increase in the number of peaks may be due to the resolution of a larger number of isozymes[20], but it may also be caused by the separation of a single isozyme which exists in various degrees of homogeneity[18]. Therefore, it must be concluded that claims of heterogeneity of cytochromes P-450 based on the observation of multiple peaks, as well as claims of homogeneity in the case of a single peak, must be considered carefully.

Comparisons of elution profiles require a high degree of reproducibility. The columns investigated here were found to fulfill this requirement, even after 1000 h of use. It was considered a distinct disadvantage that elution buffers containing cholate cannot be used on Mono Q. On the other hand, Mono Q and Mono S are extremely inert column materials, which allow washing with many acids, bases, and organic solvents.

Columns of 100 × 10 mm were used to allow an increase in the sample loads of up to a factor 10. The 50 × 5 mm columns were found to permit injection of up to 30 mg of protein, and thus can be used for semi-preparative work.

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