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# Purification of cytochromes P-450 derived from liver microsomes of untreated and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-treated marmoset monkeys

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ABSTRACT

The purification of multiple forms of cytochrome P-450 (P450) from 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-treated marmosets using fast protein liquid chromatography (FPLC) is described. The main aim was to achieve a better separation of certain closely related P450 sub-forms from each other than that previously obtained using conventional chromatography. An 8-aminooctyl-Sepharose fraction of cholate-solubilized microsomes was obtained first and, after fast desalting on Sephadex G-25, loaded on to a preparative Mono Q column. Five of the six gradient peaks contained P450 and were each rechromatographed on an analytical Mono Q column. The pass-through peak was fractionated further using a Mono S column. Other HPLC-quality anion- and cation-exchange gels were compared. For removal of excess of non-ionic detergent, five types of hydroxyapatite gels were compared. Seven purified forms of P450 and cytochrome $b_5$ and P420 were isolated and characterized according to PHAST sodium dodecylsulphate–polyacrylamide gel electrophoretic apparent molecular masses, catalytic, spectral and magnetic properties and also TCDD-binding capacity (molar ratio of [$^{14}$C]TCDD to P450). There are at least two sub-forms which appear to be TCDD inducible, one showing a substantial ethoxyresorufin-O-deethylase activity and the other having a high TCDD-binding molar ratio. Two other forms appear to be constitutive, as deduced from comparisons with forms purified from untreated animals.

## INTRODUCTION

The toxicity of polychlorinated dibenzo-*p*-dioxins (PCDDs, "dioxins") has recently been the subject of much investigation [1]. Of all known PCDDs, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to possess the greatest toxic potency, revealing itself in a great diversity of clinical–pathological alterations and over a very wide dosage range [2]. Moreover, toxicity manifestation and dose dependence appear to exhibit some species specificity. TCDD has been found to induce in the rat and other laboratory animals a limited number of cytochrome P-450 forms (P450) equatable to those inducible by the well known class of agents typified by β-naphthoflavone (BNF) or 3-methylcholanthrene (3MC) [3].

The aim of this work was to isolate purified forms of P450 from the marmoset, a non-human primate. Previous initial work in this laboratory involving purification of P450 forms from TCDD-treated marmosets according to established conventional chromatographic procedures for TCDD- and BNF-treated rats [3,4] left open the question as to whether or not with marmosets an adequate separation of certain P450 forms from each other had indeed been achieved. At the same time, the amount of marmoset material available for this purpose was a limiting factor.

In an attempt to provide a better separation and a higher yield of these enzymes, a purification scheme was investigated using fast protein liquid chromatography (FPLC).

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## EXPERIMENTAL

### *Chromatographic gels*

All high-resolution (HR) empty glass columns and, unless indicated otherwise, all prepacked columns were purchased from Pharmacia (Uppsala, Sweden).

### *Animals and animal treatment*

Untreated (UT) female marmosets (*Callithrix jacchus*) and TCDD-induced marmosets of both sexes were used. The animals were 9–10 months old, representing the age of a young adult. A single injection of 300–1000 ng of [$^{14}$C]TCDD per kilogram body mass was given subcutaneously 4 days prior to killing. Each liver weighed between 5 and 10 g.

### *Sample preparation*

All steps were done at 0–4°C. Liver microsomes were prepared and solubilized in sodium cholate as described [5]. 8-Aminooctyl-Sepharose was synthesized following the procedure of Guengerich and Martin [6] and chromatography was carried out as reported previously [7], except that here (after washing with 0.4% cholate) a single elution was effected using Emulgen 911 at a concentration of 0.5% (w/v). The elution pattern was detected by following the absorbance at 405 nm. The collective pool of monooxygenases was concentrated by Amicon PM 30 ultrafiltration and stored at −80°C.

### *Anion-exchange FPLC*

The temperature for this and all further chromatographic steps was 20–25°C, but fractions were collected in a rack filled with ice. Individual portions of the 8-aminooctyl-Sepharose fraction derived from TCDD-induced microsomes (TCDD–P450 8-aminooctyl-Sepharose pool) were desalted on an HR 10/10 column (10 cm × 10 mm I.D. and 10-40-μm particle size) of Sephadex G-25 Superfine or on disposable NAP-25 columns using (as equilibration and elution buffer) Mono Q buffer A, which consisted of 10 m*M* Tris–HCl (pH 7.7), 0.1 m*M* EDTA, 0.5% (w/v) Emulgen 911 and 20% (v/v) glycerol. This sample was loaded on to a Mono Q HR 10/10 column (10-μm particle size) equilibrated with buffer A and, after washing with *ca.* 30 ml, a 115-ml linear gradient of 0–250 m*M* NaCl was applied using 1 *M* NaCl in buffer A (Mono Q buffer B). For this gel, HR 10/10 column size (8 ml) and elution salt anion, this gradient size corresponds to the concentration change of 17.5 m*M*/ml recommended by the manufacturer for HR 5/5 column size (5 cm × 5 mm I.D. = 1.0 ml) [8]. The flow-rate was 0.5–1.0 ml/min in most instances. The pass-through fractions were collected, combined, concentrated and stored as mentioned above until further use.

In place of the Mono Q 10/10 column, a HiLoad 16/10 Q Sepharose HP column (10 cm × 16 mm I.D. and 24–44-μm particle size) was also used. In this instance, selection of the gradient size and flow-rates was based on the scale factors resulting from column volumes and cross-sectional areas, respectively. Peaks eluting from either column during application of the gradient were rechromatographed on a Mono Q HR 5/5 column.

An 8-aminooctyl-Sepharose pool of untreated (UT) P450 was also fractionated on a Mono Q 10/10 column. The HiLoad 16/10 Q Sepharose HP column and rechromatography on Mono Q HR 5/5 were not incorporated here, however.

For the sake of comparison, in a separate experiment, portions of the same 8-aminooctyl-Sepharose pool of UT-P450 were chromatographed on HR 5/5 columns packed in this laboratory [7] with Mono Q, with TSKgel DEAE 5PW (TosoHaas, Stuttgart, Germany) and with LiChrospher 1000 DEAE (Merck, Darmstadt, Germany).

### *Cation-exchange FPLC*

The pass-through fractions from Mono Q 10/10 or HiLoad 16/10 Q Sepharose HP runs which were desalted on Sephadex G-25 or NAP-25 using Mono S buffer A [20 m*M* morpholinoethanesulphonic acid (MES) (pH 6.5), 0.5% Emulgen 911 and 20% glycerol] were loaded individually on to a Mono S HR 5/5 column equilibrated with the same buffer. For this 1-ml column, a gradient volume of 23 ml incorporating 0–500 m*M* NaCl (recommended concentration change of 21.5 m*M*/ml [8]) was effected using 1 *M* NaCl in buffer A (Mono S buffer B).

Mono S chromatography was also carried out using the Mono Q pass-through fraction resulting from an 8-aminooctyl-Sepharose pool of UT-P450. For comparison, portions of the same Mono S load material (UT-P450 Mono Q pass-through fraction) were also chromatographed separately on HR 5/5

columns laboratory-packed with TSKgel SP 5PW and with LiChrospher 1000 $SO_3^-$.

*Hydroxyapatite FPLC*

P450 fractions were freed from excess of Emulgen 911 on an HR 10/2 column (2 cm × 10 mm I.D.) packed as described [7] with Hydroxylapatite HPLC Grade (Calbiochem, La Jolla, CA, USA). The column was equilibrated with 10 m*M* potassium phosphate (pH 7.4), 20% glycerol and 0.05% sodium cholate and eluted with the same buffer containing 350 m*M* phosphate as first described by Funae and Imaoka [9]. The flow-rate used for this column of Calbiochem gel was 1.0 ml/min and the temperature was 20–25°C.

HR 10/2 columns were also packed with the hydroxyapatite gels Bio-Gel HTP gel (Bio-Rad, Munich, Germany), IBF HA-Ultrogel (Serva, Heidelberg, Germany), TSKgel HA-1000 (TosoHaas) and Granulated Hydroxyapatite synthesized by us according to Mazin *et al.* [10], and tested in comparison.

In these instances, 1-ml columns were packed at the maximum flow-rates recommended by the manufacturers. The sample load consisted of an 8-aminooctyl-Sepharose pool of phenobarbital-induced Wistar rat P450 gel-filtered on NAP-25 cartridges using the hydroxyapatite equilibration buffer described above but containing in addition 0.5% Emulgen 911. Chromatographic runs, all at a constant temperature of 25°C, were then carried out at various flow-rates between 0.02 and 2.0 ml/min. Each sample load was 6.4 nmol. Using the flow-rate for each column that gave the maximum recovery of residual detergent-free P450 eluent, the capacity was measured by loading each column with 100 nmol, washing and eluting as usual.

*Assays*

Assays of methoxy- and ethoxy-O-dealkylase activities were carried out as described [5]. The substrate concentrations were 1.0 and 0.5 $\mu M$, respectively. NADPH-P450 reductase was purified from Wistar rat liver microsomes using, in a final step, gel filtration on an HR 10/30 Superose 12 column (30 cm × 10 mm I.D.) [7]. This preparation had a specific activity of 65 $\mu$mol/min · mg of cytochrome *c* (reduced) when measured in 0.3 *M* potassium phosphate buffer at 30°C [5].

Concentrations of [$^{14}$C]TCDD were calculated from liquid scintillation countings. The specific activity of this sample was 4.51 Bq/pmol, corresponding to 4.22 fmol of TCDD per dpm. Sodium dodecyl sulphate–polyacrylamide gel electrophoretic (SDS-PAGE) analyses were carried out using 4–15% and 10–15% gradient gels with silver staining in an automated PHAST system (Pharmacia) as described previously [5]. P450 contents, protein assays and assays concerning spectral and magnetic properties were also performed as described [5,11].

## RESULTS AND DISCUSSION

*Anion exchange using Mono Q*

Emulgen 911 was used as a non-ionic detergent based on previous results from us for this type of column [11] and from other workers for HPLC-quality DEAE columns [9]. Use of sodium cholate either alone or together with Emulgen 911 routinely led to decreased resolution and increased back-pressure [11]. Of a variety of elution salts tested, NaCl was still found to be the most suitable [11]. Room temperature was used for this and all subsequent columns [12]; a decrease in column temperature during runs in fact led to poorer resolution.

When an 8-aminooctyl-Sepharose pool of TCDD-induced marmoset microsomes was chromatographed on a Mono Q 10/10 column, a profile was obtained as shown in Fig. 1. This appearance was highly reproducible and consisted of one pass-through (PT) peak, representing consistently 50–55% of the total amount of P450 applied to the column, and six gradient peaks (A–F). The designation of peaks here follows the order in which they were first isolated and characterized. All of these peaks contained P450 except for that peak eluting after 250 m*M* sodium chloride, where cytochrome $b_5$ was detected (fraction E). Cytochrome P420 was found, if at all, in the pass-through fraction, as also reported by Roos [13] for Mono Q columns. In this work, the amount of P420 did not exceed that originally present in the column load. Total recoveries of P450 from the Mono Q 10/10 column prior to removal of residual detergents were routinely about 70%. We found that the resolution, especially of the smaller peaks, was enhanced when 10 m*M* instead of 20 m*M* Tris buffer was employed.

An increase in resolution was also found when

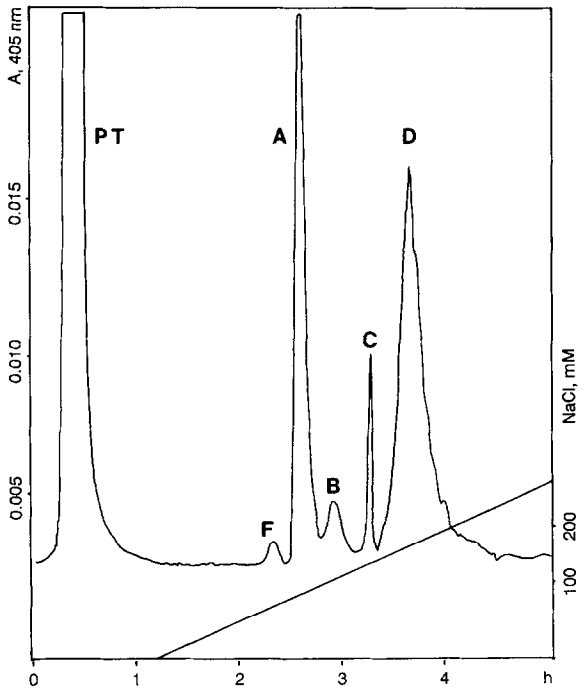


Fig. 1. Anion-exchange chromatography on Mono Q. Column: HR 10/10. Sample load: 17.6 nmol of TCDD-treated marmoset P450, prefractionated on 8-aminooctyl-Sepharose and desalted on Sephadex G-25. Buffer A: 10 m*M* Tris–HCl (pH 7.7), 20% glycerol, 0.1 m*M* EDTA, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–250 m*M* NaCl. Flow-rate: 0.5 ml/min. Detection: 405 nm with 0.02 a.u.f.s. The fractions isolated are designated PT (pass-through), A, B, C, D and F. Yield in all fractions: 68%.

lower flow-rates but not when flatter gradients were used. Thus, flow-rates down to 1.0 or 0.5 ml/min were beneficial, but concentration changes of less than 2.2 m*M*/ml were not.

Finally, the best separations of peaks C (from D) and F (from A) were reproducibly obtained on continuous gradients from 0 to 200 or 250 m*M* chloride. Step runs of 0–40–250 m*M* as used previously [7] were disadvantageous for this purpose. Detailed, segmented gradients as exploited by Roos [13], however, may improve the separation of individual peaks.

*Mono Q 10/10 versus HiLoad 16/10 Q Sepharose HP*

Because in comparison with Mono Q 10/10, HiLoad 16/10 Q Sepharose HP is 2.5 times larger and at the same time significantly less expensive, this column was also considered. The highest flow-rate used for Mono Q 10/10 was 2.5 ml/min, resulting in a back-pressure of 3.5–4.0 MPa. HiLoad 16/10 Q Sepharose HP, in contrast (with its 2.5-fold cross-sectional area), could not be operated at a flow-rate higher than 2.5 ml/min. The back-pressure in this instance was 0.3 MPa.

For fast separations of P450 forms eluting in the pass-through fraction, both columns gave similar results, as judged by SDS-PAGE. However, for the isolation of minor forms, notably those in peaks C and F, or for the isolation of C-free peak D, only Mono Q 10/10 was of value. This type of finding was also reported by Beissmann and Reisener [14], who compared Mono Q and Q Sepharose Fast Flow during the purification of a glycoprotein elicitor. It should be noted that the Fast Flow gel has a particle size range of 45–165 μm and that this gel and the Q Sepharose HP gel are both more hydrophilic than Mono Q.

We observed that, in comparison with Mono Q 10/10 runs, Q Sepharose HP separations were also not as reproducible, especially with regard to the retention and elution of fractions B, C and F.

*Comparison of various HPLC-quality anion exchangers*

HR 5/5 columns of laboratory-packed Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE gel were compared for their ability to separate forms of UT-P450. Despite the fact that these gels are all derived from different manufacturers, a first inspection of possible differences due to gel type (strong basic or weak basic anion exchanger) and/or particle size (10 or 5 μm) seemed justified (Table I). One of the aims of this work was to increase the resolution and yield.

Fig. 2 shows the chromatographic profiles of such runs with corresponding SDS-PAGE lanes of individual fractions. The relative sizes of the pass-through fractions in the chromatograms expressed as area under the curve (AUC) for total protein detection (for Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE of 23, 37 and 39%, respectively) indicate that Mono Q retained the most protein applied. In comparison with both DEAE columns, Mono Q showed in the electrophoretograms of the pass-through fractions much less protein, but with a noticeable restriction more to the

TABLE I

CHARACTERISTICS OF THREE HPLC-QUALITY ANION-EXCHANGE GELS

| Gel designation | Gel source | Anion exchanger type | Functional group | Particle size (μm) | Pressure[a] (MPa) |
|---|---|---|---|---|---|
| Mono Q | Pharmacia | Strong basic | $-CH_2N^+(CH_3)_3$ | 10 | 2.6–3.4 |
| TSKgel DEAE 5PW | TosoHaas | Weak basic | $-(CH_2)_2NH^+(CH_2CH_3)_2$ | 10 | 1.2–1.4 |
| LiChrospher 1000 DEAE | Merck | Weak basic | $-(CH_2)_2NH^+(CH_2CH_3)_2$ | 5 | 2.6–3.6 |

[a] Back-pressure at 0.5 ml/min found for a 5 mm I.D. column. For sample load and buffer, see Experimental.

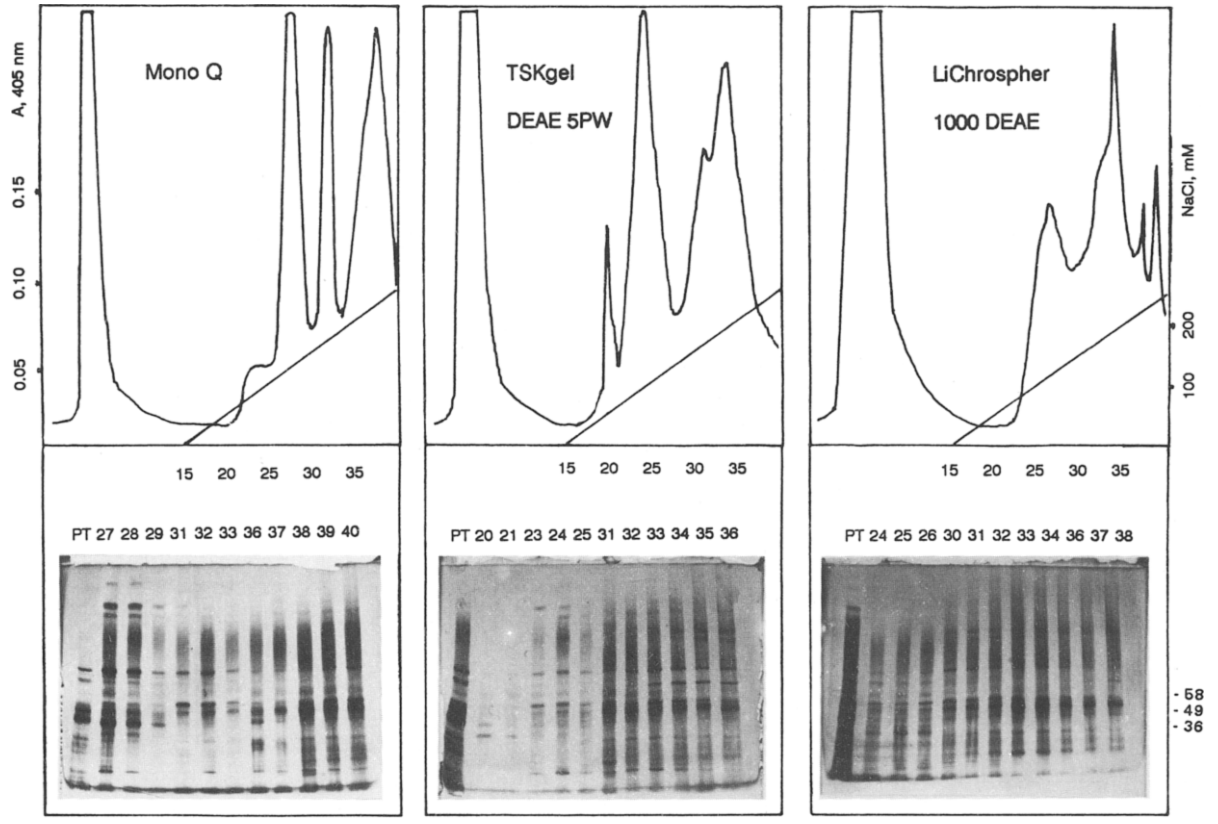


Fig. 2. Comparison of chromatographic profiles and SDS-PAGE patterns of three different HPLC-quality anion exchangers. HR 5/5 columns were laboratory packed with Mono Q, TSKgel DEAE 5PW and LiChrospher 1000 DEAE gels. Sample load: 2.86 nmol of untreated female marmoset P450 prefractionated on 8-aminooctyl-Sepharose and desalted on Sephadex G-25. Buffer A: 10 m*M* Tris (pH 7.7), 20% glycerol, 0.1 m*M* EDTA, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–250 m*M* NaCl. Flow-rate: 0.5 ml/min. Yield in all fractions: 65–75%. Detection: 405 nm with 0.25 a.u.f.s. During the chromatographic runs, 0.5-ml fractions were collected (upper row of numbers beneath chromatographic profiles) and the pass-through (PT) fraction and gradient peak fractions were then chosen for electrophoresis (lower row of numbers). Samples of 0.3 μl of those fractions were electrophoresed using 4–15% gradient gels and silver staining in the automated PHAST system [5]. On the right of these are indicated the positions of marker proteins (with $M_r \times 10^{-3}$) catalase (58), fumarase (49) and lactate dehydrogenase (36).

P450 region between relative molecular mass ($M_r$) *ca.* 50 000 and 52 000 (Fig. 2).

Each gradient region consists basically of three main peaks. Regarding the first main peak here in each run, Mono Q apparently contains the highest concentration of forms in the 49–50-kilodalton range (Fig. 2). The second peaks are all basically similar, whereas among the third peaks Mono Q includes a fraction showing somewhat higher homogeneity (at $M_r \approx 52\,000$).

As might have been expected, the differences between the two DEAE gels themselves appeared to be less striking; the 5-$\mu$m LiChrospher 1000 DEAE material possibly yields slightly more homogeneous fractions, especially towards higher salt concentrations. Total recoveries of P450 for all three columns were consistently 65–75%. Judging from all of the above findings, Mono Q was adopted for routine purifications of these enzymes.

*Cation exchange using Mono S*

Portions of the Mono Q 10/10 pass-through fraction derived from TCDD material were loaded on to a Mono S HR 5/5 column and eluted with a continuous gradient. A typical profile is shown in Fig. 3. The high absorbance of the Mono S pass-through fraction reflects the high concentration of Emulgen 911 present in the Amicon PM 30 retentate of the column load; no P450 was ever detected here. This fraction contained, however, large concentrations of TCDD (57 ± 3% of applied TCDD load, expressed as the average of three separate experiments).

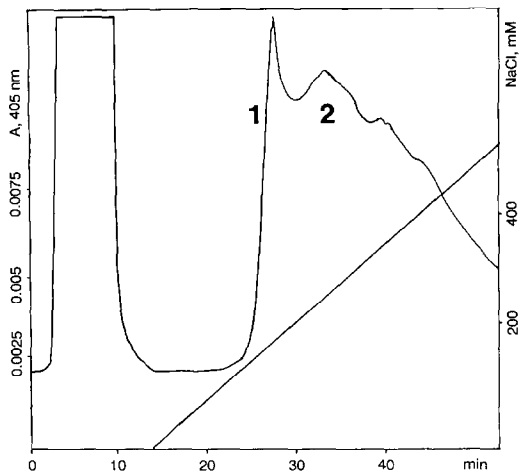


Fig. 3. Cation-exchange chromatography on Mono S. Column: HR 5/5. Sample load: 2.4 nmol of TCDD-treated marmoset P450 derived from the Mono Q pass-through fraction as shown in Fig. 1. Buffer A: 20 m*M* MES (pH 6.5), 20% glycerol, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–500 m*M* NaCl. Flow-rate: 0.7 ml/min. Detection: 405 nm with 0.02 a.u.f.s. The fractions isolated are designated 1 and 2. Yield in all fractions: 87%.

The gradient portion revealed an ill-defined pattern consisting of two or more individual, broadly overlapping peaks all containing P450. P420 corresponding to that originally present in the load material eluted at salt concentrations above 300 m*M*. Variations of equilibration buffers and elution salts had only a slight effect on the apparent resolution over the whole gradient area as judged by the absorbance profile. MES as equilibration buffer gave slightly sharper peaks than did sodium or potassium phosphate, and sodium or potassium chloride as elution salts gave sharper peaks than did sodium acetate. The latter salt has been used elsewhere for cation exchange involving sulphopropyl columns [9]. SDS-PAGE in our hands revealed, however, that differences in separation of a lower molecular mass P450 fraction at the gradient start from a high-molecular-mass fraction in the middle using different buffer–elution salt combinations were considerable. MES–NaCl was therefore chosen as the most suitable pair (results not shown).

As we had no knowledge of any possible effects of MES itself on P450, the following checks were made. P450 was not seen spectrophotometrically to be converted into P420 or into any other degradation products, as has indeed been shown to occur elsewhere with other less widely used buffers or elution agents [15]. Substrate-binding spectra likewise were not observed. Finally, no loss of methoxy- or ethoxy-O-resorufin dealkylase activity was seen when TCDD-induced marmoset microsomes were incubated in the presence or absence of MES.

In Fig. 3, the two P450 fractions have been designed 1 and 2. Electrophoreses of such separations showed that within the chromatographic profile, homogeneous fractions in region 2 (as judged by SDS-PAGE) could be obtained. These results are not shown here but are comparable to those in Fig. 4, which depicts a fractionation of UT-marmoset P450. Region 1, however, consisting of a lower-molecular mass fraction, was always contaminated with fraction 2. Use of detailed step gradients here,

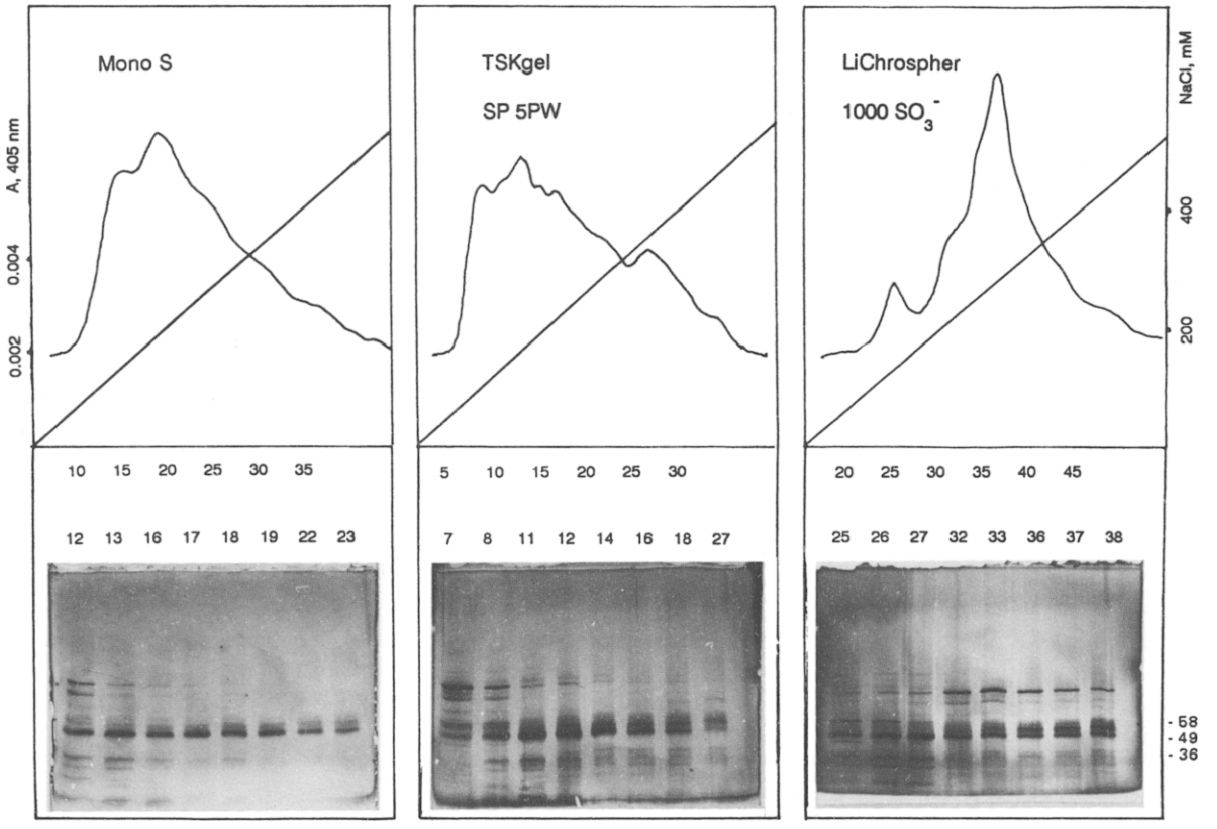


Fig. 4. Comparison of chromatographic profiles and SDS-PAGE patterns of three different HPLC-quality cation exchangers. HR 5/5 columns were laboratory packed with Mono S, TSKgel SP 5PW and LiChrospher 1000 $SO_3^-$ gels. Sample load: 1.32 nmol of untreated female marmoset P450 prefractionated as the pass-through fraction of Mono Q and desalted on Sephadex G-25. Buffer A: 20 m*M* MES (pH 6.5), 20% glycerol, 0.5% Emulgen 911. Buffer B: 1 *M* NaCl in buffer A. Gradient: 0–500 m*M* NaCl. Flow-rate: 0.5 ml/min. Yield in all fractions: 80–90%. Detection: 405 nm with 0.01 a.u.f.s. During the chromatographic runs, 0.5-ml fractions were collected (upper row of numbers beneath chromatographic profiles) and gradient peak fractions were then chosen for electrophoresis (lower row of numbers). Samples of 1.0 $\mu$l of those fractions were electrophoresed using 4–15% gradient gels and silver staining in the automated PHAST system [5]. On the right of these are indicated the positions of marker proteins (with $M_r \times 10^{-3}$) catalase (58), fumarase (49) and lactate dehydrogenase (36).

TABLE II

CHARACTERISTICS OF THREE HPLC-QUALITY CATION-EXCHANGE GELS

| Gel designation | Gel source | Cation exchanger type | Functional group | Particle size ($\mu$m) | Pressure[a] (MPa) |
|---|---|---|---|---|---|
| Mono S | Pharmacia | Strong acidic | $-CH_2SO_3^-$ | 10 | 1.6–2.1 |
| TSKgel SP 5PW | TosoHaas | Strong acidic | $-(CH_2)_3SO_3^-$ | 10 | 1.0–1.1 |
| LiChrospher 1000 $SO_3^-$ | Merck | Strong acidic | $-(CH_2)_3SO_3^-$ | 5 | 3.2–4.9 |

[a] Back-pressure at 0.5 ml/min found for a 5 mm I.D. column. For sample load and buffer, see Experimental.

as employed previously with untreated marmosets [7], did not improve the separation in the sense of increasing the apparent homogeneity, but did, of course, improve the yield of either fraction.

The appearance of more gradient peaks and SDS-PAGE bands here than those found by Funae and Imaoka [9] for Sprague–Dawley rats may possibly indicate for marmosets the presence of a greater multiplicity of P450 forms.

*Comparison of various HPLC-quality cation exchangers*

As explained above for anion exchangers, three cation exchangers were also examined (Table II and Fig. 4). The profiles consist mainly of two peaks and are all much less well defined than any of those seen for anion exchangers. In spite of such overlapping profiles, SDS-PAGE revealed that the production of some homogeneous P450 fractions was still possible. Here, it is evident that all three columns delivered a P450 fraction with $M_r \approx 50\,000$–$51\,000$.

Surprisingly, Mono S and TSKgel DEAE 5PW are apparently more similar in that a greater degree of P450 homogeneity is seen in the main, middle regions of the chromatograms, whereas for LiChrospher 1000 $SO_3^-$ this holds true for the very first peak. The back-pressures encountered for the 5-$\mu$m gel require either lower flow-rates than those used for the other gels (0.5 ml/min for an HR 5/5 column) or HPLC pumps capable of delivering pressures exceeding 5.0 MPa.

Overall, under these conditions, Mono S appeared to provide the electrophoretically purest P450 fractions and, in addition, the largest number of these per run.

*Detergent removal and comparison of various hydroxyapatite gels*

Prior to analyses of different forms of purified P450, residual amounts of the non-ionic detergent Emulgen 911 were removed on hydroxyapatite. This also effects a lower concentration of TCDD, where decreased stoichiometric ratios of TCDD to P450 were observed (see below). This is also the case with cation-exchange chromatography, where some detergent (but no P450) is eliminated in the pass-through fraction. In the rat, such ratios (for P450 1A2 [16]) were mostly found to approach unity and the binding was shown to be very tight [17,18]. The significance of the ratio and the strength of TCDD binding for these primate enzymes is at present not clear, however.

Examination of four commercially available gels and one that we synthesized according to the literature [10] was undertaken because, as pointed out earlier, we had to rely on good yields. In past experience [5,7,11], we have found that however high the yields of P450 subsequent to ion-exchange and hydrophobic interaction chromatography may be, final removal of excess of non-ionic detergent via hydroxyapatite can result in P450 losses of up to 50%. The present experiment was done to ascertain optimum flow-rates (resulting in the highest recoveries of detergent-free P450) and capacities (derived from optimum flow-rates) for each gel (Table III). Recovery in this sense concerns the concentration of spectroscopically intact enzyme determined from difference spectra.

For this purpose, optimum flow-rates and the absolute values of the recoveries themselves were found to differ enormously (Fig. 5). Lower recoveries at lower flow-rates and prolonged contact with the gel are presumably due to more thermal degradation and/or loss of haeme [19]. All back-pressures for all columns at all flow-rates remained constant with the exception of Bio-Gel HTP gel, which repeatedly resulted in strong gel compression and increased back-pressure ($>5$ MPa), thus allowing only one run per column filling. For these enzymes and at this temperature, Hydroxylapatite HPLC Grade (Calbiochem) gave the highest recoveries of P450 applied. These were apparently but reproducibly found to reach 100%. It may be noted that excellent recoveries were also obtained at flow-rates 5–10 times that recommended by the manufacturer (Fig. 5 and Table III).

Capacities also varied considerably and are given in Table III. The highest value was found for TSKgel HA-100 and represented 1.5–3 times that found for other hydroxyapatite gels.

*Properties of purified P450 forms*

Anion- and cation-exchange chromatography on Mono Q and Mono S columns resulted in the isolation of seven different fractions of P450 from liver microsomes of TCDD-induced marmosets (Table IV). The total recoveries among these forms were found to vary considerably, this probably

TABLE III

CHARACTERISTICS OF VARIOUS TYPES OF HYDROXYAPATITE USED FOR REMOVAL OF RESIDUAL NON-IONIC DETERGENT FROM P450 FRACTIONS

For sample source and detergent, see Experimental.

| Gel designation | Gel source | Particle size (μm) | Recommended linear flow-rate[a] (ml/h · cm²) | Recommended flow-rate[b] (ml/min) | Optimum flow-rate found[c] (ml/min) | Recovery found[d] (%) | Capacity found[e] (nmol/ml) |
|---|---|---|---|---|---|---|---|
| Bio-Gel HTP | Bio-Rad | 10 | 25–100 | 0.3–1.3 | 1.5 | 53 | 18 |
| Hydroxyapatite HPLC Grade | Calbiochem | 20 | 15 | 0.2 | 0.5–1.0 | 100 | 38 |
| HA-Ultrogel | IBF | 60–180 | 5–30 | 0.07–0.4 | 0.1 | 48 | 20 |
| Granulated hydroxyapatite | Mazin *et al.* [10] | 200–250 | 80 | 1.0 | 0.5–1.0 | 73 | 29 |
| TSKgel HA-1000 | TosoHaas | 5 | 60–120 | 0.8–1.6 | 1.5 | 70 | 57 |

[a] By manufacturer for elution purposes.
[b] For a 10 mm I.D. column.
[c] For 6.4 nmol of P450 loaded on to a 1.27 cm × 10 mm I.D. (1-ml) column; flow-rate giving the highest recovery of detergent-free P450 shown in Fig. 5.
[d] Recovery at optimum flow-rate; column and load as above.
[e] Amount of detergent-free P450 in nmoles per ml of gel elutable after 100 nmol were loaded and washed.

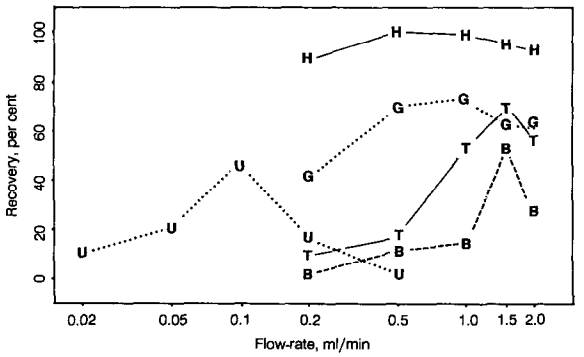


Fig. 5. Removal of excess of non-ionic detergent via hydroxyapatite. Five types of hydroxyapatite gels were laboratory-packed in HR 10/2 columns (1.27 cm × 10 mm I.D. = 1.0 ml), equilibrated, loaded, washed and eluted as described under Experimental. Sample load for each run: 6.4 nmol of an 8-aminooctyl-Sepharose pool of phenobarbital-induced Wistar rat P450 gel filtered on NAP-25 cartridges using the hydroxyapatite equilibration buffer containing in addition 0.5% Emulgen 911. After successive runs, each at flow-rates between 0.02 and 2.0 ml/min, the recovery of detergent-free P450 in percent (based on the load of P450 applied) as a function of flow-rate was determined. Temperature for all runs: 25°C. Symbols: B = Bio-Gel HTP; G = Granulated Hydroxyapatite; H = Hydroxylapatite HPLC Grade; T = TSKgel HA-1000; U = HA-Ultrogel.

reflecting the wide variation in the relative contents of each form originally present. Such diversity has already been found for HPLC-purified P450s isolated from rats treated with similar inducing agents such as 3-methylcholanthrene [9,20].

The degree of purity expressed as specific contents among the P450 forms found in the present work varied between 9.2 and 16.1 nmol per milligram of protein (Table IV). Fig. 6 shows SDS-PAGE results for these forms, where it is evident that at this stage forms A, F and 1 are not homogeneous. As mentioned above, fraction E was not included in this or other assays as it had been identified spectroscopically as cytochrome $b_5$.

$M_r$ values ranged between 49 000 and 56 000 (Table V). Hence there is no striking difference to the $M_r$ ranges found in different laboratories for a number of different rat P450s [4,9,19].

Marmoset P450s having the lowest apparent values for absorption maxima in CO–dithionite difference spectra (447 and 448 nm) may possibly correspond to one or both of those main forms P450 1A1 and 1A2 inducible in the rat which have the

TABLE IV

P450 FORMS PURIFIED FROM TCDD-TREATED AND UNTREATED MARMOSETS

P450 forms A, B, C, D and F were derived from Mono Q gradient fractions. Fraction E was cytochrome $b_5$. Forms 1 and 2 were derived from Mono S chromatography of the Mono Q pass-through (PT) fraction. Residual non-ionic detergent was removed from all fractions via hydroxyapatite chromatography. AO = 8-Aminooctyl-Sepharose.

| Source | Total protein (mg) | Total content (nmol) | Specific content (nmol/mg) | Recovery (%) |
|---|---|---|---|---|
| *TCDD-treated microsomes* | 7110 | 775 | 0.109 | 100 |
| AO pool | 874 | 682 | 0.78 | 88 |
| Mono Q | | | | |
| PT | 70 | 333 | 4.9 | 43 |
| Form A | 2.5 | 23.2 | 9.2 | 3.0 |
| Form B | 0.57 | 8.5 | 14.8 | 1.1 |
| Form C | 0.34 | 4.7 | 13.7 | 0.6 |
| Form D | 2.3 | 31.0 | 13.5 | 4.0 |
| Form F | 1.4 | 17.8 | 12.4 | 2.3 |
| Mono S | | | | |
| Form 1 | 2.6 | 40.6 | 15.8 | 5.2 |
| Form 2 | 4.4 | 71.5 | 16.1 | 9.3 |
| *Untreated microsomes* | 828 | 77.0 | 0.093 | 100 |
| AO pool | 101 | 55.4 | 0.55 | 72 |
| Mono Q | | | | |
| PT | 6.1 | 20.0 | 3.3 | 26 |
| Form A | 0.89 | 7.7 | 8.7 | 10 |
| Form D | 0.46 | 5.1 | 11.1 | 6.6 |
| Form F | 0.64 | 2.8 | 4.4 | 3.7 |
| Mono S | | | | |
| Form 2 | 0.32 | 4.6 | 14.4 | 6.0 |

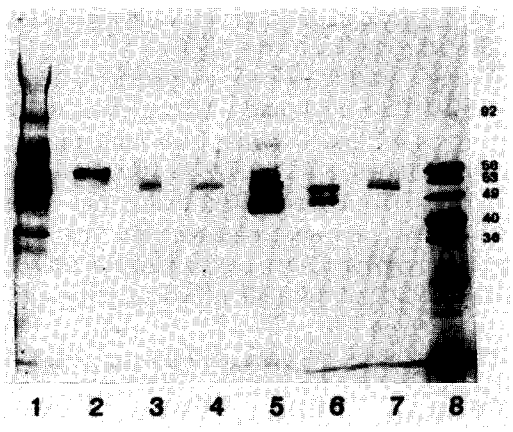


Fig. 6. SDS-PAGE gel of purified fractions of TCDD-induced marmoset P450s. The anode is at the bottom, and the 10–15% gradient gel was stained with silver [5]. Lanes: 1 = form A; 2 = form B; 3 = form C; 4 = form D; 5 = form F; 6 = form 1; 7 = form 2; 8 = marker proteins (with $M_r \times 10^{-3}$) phosphorylase *a* (92), catalase (58), glutamate dehydrogenase (53), fumarase (49), aldolase (40), lactate dehydrogenase (36).

same absorption values. As deduced from the absolute spectra, the contribution to the atypical high-spin state for TCDD marmoset form 2 may possibly relate this form to 1A2 forms found in rats [20] and rabbits [21]. Other forms in the marmoset may, however, correspond more closely to those of other gene families such as P450 3, as has been suggested for a form purified from polychlorinated biphenyl (PCB)-treated crab-eating monkeys [22].

Catalytic activities measured in reconstituted systems containing rat NADPH P450 reductase [5] and the substrates 7-ethoxyresorufin and 7-methoxyresorufin are also shown in Table V. These substrates have been shown to exhibit some specificity towards rat P450 forms 1A1 and 1A2, respectively [23,24]. In this work, it can be seen that forms C and D both have the highest values for these O-dealkylation rates. We therefore gather, for the time being, that one or both of C and D may possibly correspond to 1A1 and/or 1A2.

TCDD binding was monitored by liquid scintillation counting of the fractions, as it is known that a wide variety of inducing agents such as TCDD, pentachlorodibenzofuran (PCDF) and 3MC not only induce P450 but also bind tightly to it [17,18,25]. It was shown there that the highest values obtainable for (detergent-free) rat material were almost unity. Stoichiometric ratios of TCDD to (detergent-free) marmoset P450 are also given in Table V, where it can be seen that the highest value is 0.48 (for form C). Previous work in our laboratory with TCDD-treated rats and marmosets indicated, however, that ratios as high as 6.3:1 could be registered in solutions still containing some excess of non-ionic detergent (results not shown). As yet, we have no explanation for this phenomenon. Over and above the obvious difference in species, it may be conceivable that the dosage of TCDD-like inducing agents utilized plays a role; in this work, for marmosets (and in our previous work on rats), the doses used were 300–1000 ng/kg body mass, whereas for rats treated elsewhere [17,18,20] the doses were 320 μg/kg, 1 mg/kg and 5 mg/kg, respectively. The fact that three different inducing agents are compared (TCDD, pentachlorodibenzofuran and 3-methylcholanthrene) certainly also plays a role. We have found that, in contrast to rats, at doses of 300–1000 ng TCDD per kilogram body mass, an apparent plateau in inductive capacity of EROD activity was reached. Higher doses for marmosets were therefore not used.

Finally, it was considered important to discuss which forms of P450 isolated from TCDD microsomes may not have been TCDD induced but were already present in untreated microsomes. Comparisons of the chromatographic profiles of separations of TCDD-treated and untreated material indicated in a reproducible manner that forms D, F and 2 eluted in the same positions and in basically comparable yields. These three UT forms also had SDS-PAGE molecular masses and difference spectra absorption maxima equivalent to those of TCDD forms. Judging from such biophysical parameters, we conclude that in TCDD-treated marmosets, forms D, F and 2 are probably constitutive. Of all the UT fractions, only UT form D had an appreciable EROD activity (39 pmol of product/min · nmol of P450) which is, however, only one tenth of that seen for the TCDD form D (Table V).

We have recently found that monoclonal antibodies raised against purified rat P4501A2 reacted with both TCDD-D and UT-D forms (results to be published elsewhere). Accordingly, this type of P450 form could be classified as constitutive although inducible. A similar phenomenon has been reported

TABLE V

ELECTROPHORETIC, CATALYTIC, SPECTRAL, MAGNETIC AND TCDD-BINDING PROPERTIES OF P-450 FORMS PURIFIED FROM TCDD-TREATED MARMOSETS

For P450 form designations, see Table IV. Residual non-ionic detergent was removed from all fractions via hydroxyapatite chromatography. Abbreviations and explanations: apparent molecular masses were as judged by SDS-PAGE; MROD, EROD = rate of methoxy- or ethoxyresorufin O-dealkylation in pmol of product/min · nmol of P450 from a reconstituted system containing NADPH–P450 reductase; the concentration of reductase was 10 μmol (cytochrome *c* reduced)/min per nmol P450; absorption maximum (in nm) of iron(II) carbonyl complex; L, H = low or high magnetic spin state as deduced from absolute spectrum of oxydized solution; TCDD binding = molar ratio of TCDD-to-P450.

| P450 form | Molecular mass | MROD | EROD | Absorption maximum | Spin state | TCDD binding |
|---|---|---|---|---|---|---|
| A[a] | 50 000–54 000 | 15.1 | 5.3 | 448–449 | L | 0.03 |
| B | 56 000 | 2.9 | 28.5 | 450 | L | 0.10 |
| C | 53 000 | 101 | 125 | 447 | L | 0.48 |
| D | 52 000 | 282 | 431 | 447 | L | 0.12 |
| F[a] | 48 000 | 2.3 | 27.7 | 452 | L | 0.09 |
| 1[a] | 49 000 + 51 000 | <0.2 | <0.2 | 448–451 | L | 0.003 |
| 2 | 51 500 | 1.1 | 37.0 | 448 | L+H | 0.096 |

[a] Non-homogeneous.

for human liver, where a large amount of the microsomal P450 was shown to comprise P450 1A2 [26]. Altogether, adult [27] and prenatal human livers [28] have been shown to contain a great diversity of P450 forms.

Control over induction in humans is, of course, difficult or impossible in most instances, and to this end we consider the marmoset a potential model for assessing the treatment of primates by xenobiotics.

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