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Purification and characterization of a previously unreported form of cytochrome *P*-448 from the liver of 3-methylcholanthrene-pretreated rats

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At least four hepatic isoenzymes of cytochrome *P*-450 were purified and characterized from rats treated with 3-methylcholanthrene. A monoclonal antibody developed against one of the forms (designated cytochrome *P*-450 MC-B) and polyclonal antibodies against others were used to demonstrate that form MC-B is immunologically distinct from other methylcholanthrene-inducible forms. Limited *N*-terminal amino acid sequencing showed that cytochrome *P*-450 MC-B has a primary structure that differs from the *N*-terminal sequences of other established rat isoenzymes. Cytochrome *P*-450 MC-B has a minimum M_r of 53000, a CO-reduced spectral maximum at 448 nm, a Soret maximum of 417 nm in the absolute oxidized spectrum and a pattern of substrate preferences that differs from those of the other methylcholanthrene-induced forms. The other forms (MC-A, MC-C and MC-D) share characteristics with isoenzymes previously reported by other investigators.

INTRODUCTION

Cytochromes *P*-450 function as the terminal oxidases in the hepatic microsomal mixed-function oxidation system. This system metabolizes a wide variety of endogenous and exogenous compounds, including drugs, steroids and carcinogens (Conney, 1967). The cytochrome *P*-450 hepatic system consists of a family of inducible isoenzymes that possess different spectral and immunological characteristics, substrate preferences and primary protein structures (Guengerich *et al.*, 1982*a*; Ryan *et al.*, 1982). In the present study we have investigated the multiplicity of isoenzymes of cytochrome *P*-450 that are inducible in a rat liver microsomal fraction (referred to below simply as microsomes) after pretreatment of the animals with the polycyclic aryl hydrocarbon 3-methylcholanthrene (3-MC). Three of the isoenzymes that were isolated and characterized appear to be forms previously described by others. A fourth inducible form is shown to have unique biophysical and biochemical properties. This form does not appear to have been previously described by other investigators.

MATERIALS AND METHODS

Chemicals

Chemicals, unless otherwise stated, were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and were reagent grade or better. Tissue-culture media was purchased from Kansas City Biologicals (Lenexa, KS, U.S.A.). Foetal-calf serum was obtained from Sterile Systems (Logan, UT, U.S.A.). Hydroxyapatite was purchased from Bio-Rad Laboratories (Richmond, VA, U.S.A.). DE-52 DEAE-cellulose, DE-53 DEAE-cellulose and CM-52 CM-cellulose were purchased from Whatman (Clifton, NJ, U.S.A.).

Animals

Male Sprague–Dawley rats weighing 175–225 g were obtained from King Animal Laboratories (Oregon, WI, U.S.A.). Rats were housed in wire cages and given Formulab 5008 rat chow (Purina, St. Louis, MO, U.S.A.) and water *ad libitum*. Polyclonal antibodies were raised in adult male New Zealand rabbits. Female Balb/c mice were used for the production of monoclonal antibodies and were purchased from Jackson Laboratories (Bar Harbor, ME, U.S.A.).

Chemical pretreatment of rats was carried out with 3-MC (Aldrich Chemical Co., Milwaukee, WI, U.S.A., and Sigma Chemical Co.) administered intraperitoneally in corn oil, 40 mg/kg body wt. per 24 h for 4 days. Rats were kept without food for 18–24 h after 4 days of treatment and killed by decapitation the following day. Livers were perfused *in situ* with ice-cold phosphate-buffered saline (0.15 M-NaCl/10 mM-potassium phosphate buffer, pH 7.4) and microsomes were isolated by differential centrifugation (Guengerich, 1977). Microsomes were stored at −70 °C until used.

Purification of cytochrome *P*-450 isoenzymes

A scheme of the protocol used in purifying cytochrome *P*-450 isoenzymes from liver microsomes of 3-MC-pretreated rats is shown in Scheme 1. Solubilization and fractionation of microsomal protein and sample concentration and dialysis follow the procedures described previously (Seidel *et al.*, 1984). All purification buffers contained 20% (w/v) glycerol. The initial column used was a 2.5 cm × 60 cm anion-exchange column containing DE-52 DEAE-cellulose resin in the upper half of the column and DE-53 DEAE-cellulose, a higher-capacity anion-exchange resin, in the lower half. Elution of protein from the column with a 1000 ml linear 0–85 mM-KCl

Abbreviations used: MC or 3-MC, 3-methylcholanthrene; cytochrome *P*-450 PB, a major phenobarbital-inducible form of rat liver cytochrome *P*-450 previously purified in our laboratory (Seidel *et al.*, 1984).

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gradient produced two peaks, which absorbed at 417 nm. The earlier-eluted peak contained the cytochrome *P*-450 designated as form MC-D. The later peak contained a mixture of two cytochromes *P*-450, forms MC-B and MC-A (separable on the second DE-53 DEAE-cellulose chromatographic step). Raising the concentration of KCl to 125 mM in the eluting buffer resulted in the elution from the DE-52/DE-53 DEAE-cellulose column of a final peak absorbing at 417 nm, which contained the cytochrome *P*-450 fraction designated as MC-C.

Further purification was carried out on successive DE-53 DEAE-cellulose columns (2.5 cm × 25 cm) and hydroxyapatite columns (2.5 cm × 15 cm), with 1000 ml linear KCl and potassium phosphate gradients respectively (indicated in Scheme 1). Fractions from the hydroxyapatite columns were applied to CM-52 CM-cellulose columns (1.6 cm × 10 cm). Columns were washed with approx. 100 ml of 5 mM-potassium phosphate buffer, pH 7.4, to remove excess non-ionic detergent, and then eluted with 200 mM-potassium phosphate buffer, pH 7.4. Eluted preparations were dialysed overnight against 2 litres of 50 mM-potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol and 0.1 mM-EDTA, concentrated and stored at −70 °C.

Purification of NADPH–cytochrome *P*-450 reductase

The reductase enzyme was purified from rat liver microsomes by using the affinity-chromatography method of Yasukochi & Masters (1976). 2′,5′-Bisphosphoadenosine–Sepharose 4B used in the affinity column purification of the reductase was purchased from Pharmacia (Piscataway, NJ, U.S.A.). The purified reductase preparation was homogeneous when analysed by SDS/polyacrylamide-gel electrophoresis (results not shown).

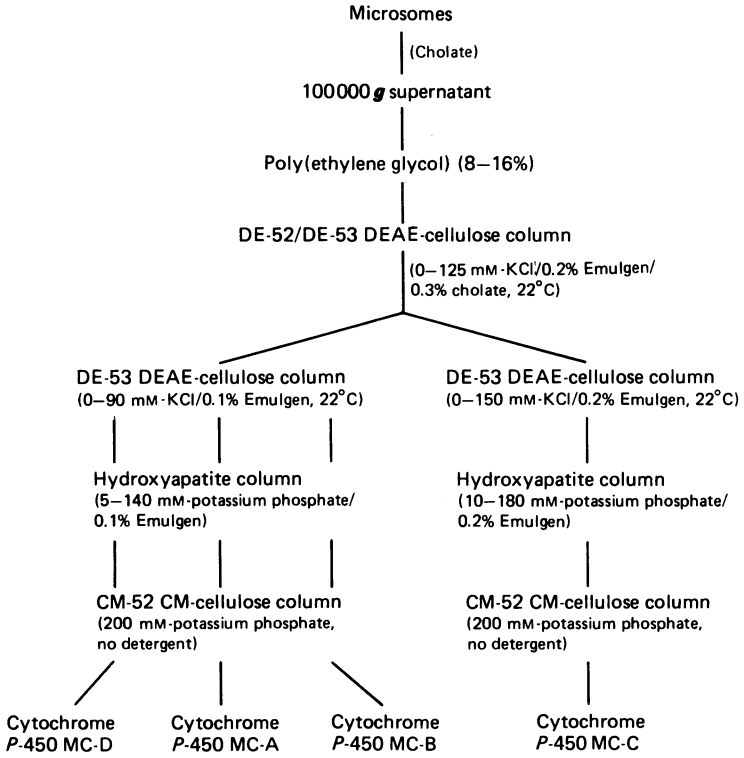
Assay methods

Protein was measured by the method of Lowry *et al.* (1951), with crystalline bovine plasma albumin (Bio-Rad Laboratories) as a standard. Total cytochrome *P*-450 holoenzyme was measured spectrally by using the CO-reduced difference spectral method of Omura & Sato (1964) and an absorption coefficient of 91 $mM^{-1} \cdot cm^{-1}$ for the spectral perturbation in the region 450–490 nm. $Na_2S_2O_4$ was used as the chemical reductant. All spectral determinations were carried out with an Aminco DW-2 spectrophotometer in the split-beam mode.

SDS/polyacrylamide-gel electrophoresis was performed with the discontinuous gel system of Laemmli (1970). Minimum M_r values were estimated by comparison with standard proteins and their published M_r values: bovine liver catalase (M_r 58000) (Calbiochem–Boehringer Corp., La Jolla, CA, U.S.A.); bovine liver L-glutamate dehydrogenase (M_r 53000) (Calbiochem–Boehringer Corp.); ovalbumin (M_r 45000) (Sigma Chemical Co.); horse liver alcohol dehydrogenase (M_r 41000) (Boehringer Mannheim, Indianapolis, IN, U.S.A.).

Enzymic characterization of cytochrome *P*-450 isoenzymes

The catalytic activities of purified cytochrome *P*-450 preparations were determined in a reconstituted system containing 0.1–0.3 nmol of purified cytochrome *P*-450,



Scheme 1. Purification protocol for cytochrome *P*-450 isoenzymes from liver microsomes of 3-MC-pretreated rats

Unless otherwise indicated, all chromatographic procedures were carried out at 0–4 °C. All chromatographic buffers contained 20% (v/v) glycerol and 0.1 mM-EDTA in addition to the components indicated.

600 units of purified NADPH–cytochrome *P*-450 reductase, 30 μM-dilauroyl phosphatidylcholine, 5 mM-glucose 6-phosphate, 1 unit of glucose-6-phosphate dehydrogenase and 0.5 mM-NADPH in 50 mM-Tris/HCl buffer, pH 7.4, containing 3 mM-$MgCl_2$. Substrate concentrations were 1 mM unless otherwise stated. *N*-Demethylation reactions were measured colorimetrically by the formation of formaldehyde. Formaldehyde was determined by the method of Cochin & Axelrod (1959). The dealkylation of 7-ethoxycoumarin to form 7-hydroxycoumarin was measured fluorimetrically by the method of Greenlee & Poland (1978). Aniline hydroxylation forming *p*-aminophenol was determined by the method of Imai *et al.* (1966). Hydroxylation of benzo[*a*]pyrene was determined fluorimetrically by the method of Nebert & Gelboin (1968), with 3-hydroxybenzo[*a*]pyrene (kindly supplied by the National Cancer Institute, Bethesda, MD, U.S.A.) as a standard. Production of *p*-nitrophenol from *p*-nitroanisole was measured as described by Netter & Seidel (1964). Hydroxylated products of testosterone were measured radiochemically by using the t.l.c. method of Waxman *et al.* (1983). 6β-, 7α- and 16α-Hydroxylated testosterone standards were obtained as a gift from Professor D. N. Kirk of the Steroid Reference Collection (London, U.K.). [4-^{14}C]Testosterone (52 mCi/mmol) was purchased from New England Nuclear (Boston, MA, U.S.A.). Benzo[*a*]pyrene, *p*-aminophenol, 7-ethoxycoumarin, 7-hydroxycoumarin and *p*-nitroanisole were purchased from Aldrich Chemical Co.

Amino acid sequencing

N-Terminal amino acid analysis was performed on a Beckman 890C sequencer equipped with a cold trap. A 10 nmol portion of purified sample was retained in a spinning-cup apparatus with 6 mg of precycled Polybrene. The sample was cycled once without phenyl isothiocyanate, and phenyl isothiocyanate was subsequently added in 0.33 M-Quadrol coupling buffer. Conversion into the phenylthiohydantoin derivatives of the cleaved amino acids was carried out with the use of acetyl chloride in methanol (Tarr, 1975). Amino acid derivatives were identified by h.p.l.c. in an Ultrasphere (Altex) C-18 column and a Beckman chromatograph. Standard amino acid phenylthiohydantoin derivatives were obtained from Pierce Chemical Co. (Rockford, IL, U.S.A.).

Polyclonal antibodies

Antiserum against purified cytochrome *P*-450 MC-D was raised in New Zealand rabbits. Rabbits were injected subcutaneously with 0.5 mg of purified antigen in Freund's complete adjuvant (Difco Laboratories, Detroit, MI, U.S.A.). Then 4 weeks later the rabbits were boosted with 0.5 mg of antigen injected subcutaneously, and serum was collected 5–7 days later. The immunoglobulin fraction was isolated by $(NH_4)_2SO_4$ fractionation, and the IgG fraction was subsequently obtained by column chromatography on Whatman DE-52 DEAE-cellulose (Sober *et al.*, 1956).

Immuno-blotting

Specificity of antibody preparations for the homologous antigen was determined by using the immuno-blotting method of Towbin *et al.* (1979). Purified cytochrome *P*-450 MC isoenzymes and/or microsomal protein were electrophoresed on SDS/polyacrylamide slab gels and then electro-transferred, with a Hoeffer Transphor electrophoresis unit (Hoeffer, San Francisco, CA, U.S.A.), to nitrocellulose paper (Millipore, Bedford, MA, U.S.A.). The electro-transfer buffer contained 25 mM-Tris, 192 mM-glycine, 20% (v/v) methanol and 0.1% SDS. After electro-transfer, non-specific protein-binding sites on the papers were blocked with 10% (v/v) foetal calf serum and 1% (w/v) bovine serum albumin in phosphate-buffered saline overnight at 0–4 °C. The blocked papers were then incubated in succession with the primary rabbit anti-(rat cytochrome *P*-450) IgG and peroxidase-conjugated goat anti-(rabbit IgG) $F(ab')_2$ fragment (Cappel, Cochranville, PA, U.S.A.) as described previously (Seidel *et al.*, 1984). Immuno-blots were developed by using 4-chloro-1-naphthol as the peroxidase substrate (Hawkes *et al.*, 1982), and the reaction was stopped by displacement with distilled water.

Antigen detection was also carried out by a direct dot binding assay. In this case 0.1–0.2 μg of purified cytochrome *P*-450 was applied directly to the nitrocellulose paper. After the antigen had been allowed to dry, blocking, antibody incubation and detection with 4-chloro-1-naphthol were carried out as described for the electro-transfer procedure.

Immunoadsorption of antibodies

Antibodies present in the rabbit anti-(rat cytochrome *P*-450 MC-D) preparation which recognized antigenic determinants shared by cytochromes *P*-450 MC-D and MC-C were removed by adsorption on a linked MC-C–Sepharose column. This column was prepared from purified cytochrome *P*-450 MC-C and CNBr-activated Sepharose 4B (Pharmacia) according to manufacturer's directions. A 10 mg portion of purified cytochrome *P*-450 MC-C was bound to 1.3 g (4.5 ml gel volume) of CNBr-activated Sepharose and packed in a 10 ml chromatographic column (0.8 mm diam.). The column was washed with the following buffers: (1) 0.1 M-sodium acetate buffer, pH 4.0, containing 1 M-NaCl; (2) 0.1 M-sodium borate buffer, pH 8.3, containing 1 M-NaCl; (3) 10 mM-potassium phosphate buffer, pH 7.4, containing 0.15 M-NaCl (phosphate-buffered saline). Buffers 1 and 2 were washed alternately through the column four times, with 3 column volumes per wash, followed by equilibration of the column with buffer 3. A 15–20 mg portion of IgG was passaged twice over the column, and the eluate peak at 280 nm was subsequently collected. Antibody adsorbed on the column was desorbed by washing the column with 0.2 M-glycine/HCl buffer, pH 2.2, and the column was re-equilibrated with another low-pH–high-pH buffer cycle.

Monoclonal antibody production

Purified cytochrome *P*-450 MC-B (50 μg mixed with Freund's complete adjuvant) was injected subcutaneously into female Balb/c mice. Animals were boosted 2 weeks later with 25 μg of cytochrome *P*-450 MC-B without adjuvant, and again 7 days later. Detection of a positive immune response in the mouse serum utilized a direct dot binding assay in which purified cytochrome *P*-450 MC-B (0.1–0.2 μg) was spotted on nitrocellulose papers, and the assay was performed as described above. Peroxidase-conjugated goat anti-(mouse IgG) $F(ab')_2$ fragment (Cappel) was used as the second antibody. (This assay was also used in the screening of micro-well supernatants

from fused spleen-cell–myeloma cultures for the presence of antibody directed against cytochrome *P*-450 MC-B.) Mice exhibiting high serum antibody titres against cytochrome *P*-450 MC-B in the dot binding assay were used for spleen-cell–myeloma fusions on the third day after the final boost.

The myeloma cell line used in the fusion procedures was the Balb/c-derived P3-X63-Ag/8 line (gift from Dr. G. Dutton, University of Iowa). Fusion procedures were based on the methods described by Scearce & Eisenbarth (1983) and Gard *et al.* (1983). Poly(ethylene glycol) 4000 (Merck, Darmstadt, Germany) was used in the fusions. Here 2×10^8 spleen cells were combined with 2×10^7 myeloma cells for the fusion procedures, and post-fusion cells were subsequently seeded into 96-well flat-bottomed micro-well plates (Corning, Chicago, IL, U.S.A.) along with 2×10^7 normal feeder spleen cells. Individual wells were screened for anti-(cytochrome *P*-450 MC-B) antibody production by direct dot binding assay. Selected colonies were passaged three or more times *in vitro* before being cloned. Soft-agar cloning was carried out to isolate discrete antibody-producing colonies. Anti-(cytochrome *P*-450 MC-B)-antibody-producing cell lines were subsequently replicated in T-75 flasks (Corning), and supernatant containing antibody was collected and stored in small portions at −70 °C. Thawing and refreezing of the supernatant was avoided.

Cloned cell lines were screened to determine immunoglobulin subtype by using a modified dot binding assay. [Immunoglobulin subtyping was performed to confirm homogeneity of the anti-(cytochrome *P*-450 MC-B) antibody.] Purified cytochrome *P*-450 MC-B was spotted on nitrocellulose paper, and the paper was dried and blocked as before. Media supernatants from cloned cell cultures were incubated with the papers for 3 h at room temperature, followed by a wash with phosphate-buffered saline. Rabbit IgG antibodies specific for mouse immunoglobulin subtypes IgA, IgM, IgG, IgG_{2ab}, IgG_{2b} and IgG_3 (Nordic Immunological Laboratories, El Toro, CA, U.S.A.) were incubated with the papers for 2 h at room temperature followed by a wash with phosphate-buffered saline. Peroxidase-conjugated goat anti-(rabbit IgG) $F(ab')_2$ fragment (Cappel) was then incubated with the papers for 2 h at room temperature, and the immune reaction was subsequently detected with 4-chloro-1-naphthol as described above.

RESULTS

Purification of cytochrome *P*-450 isoenzymes

Individual cytochrome *P*-450 forms were purified by using a series of ion-exchange chromatographic columns. Scheme 1 indicates the general scheme used in the separation of different forms of cytochrome *P*-450 from liver microsomes of 3-MC-pretreated rats. Solubilization of microsomes with cholate and poly(ethylene glycol) fractionation followed procedures previously described (Seidel *et al.*, 1984). The first column contained DE-53 DEAE-cellulose in the lower half of the column and DE-52 DEAE-cellulose in the upper half. The use of DE-53 DEAE-cellulose, which has a higher binding capacity, in combination with DE-52 DEAE-cellulose was found to improve the recovery of cytochromes *P*-450 in preliminary purification steps. The use of a shallow KCl gradient (0–85 mM-KCl, 1000 ml) aided the resolution of the peak containing cytochrome *P*-450 MC-D from that containing cytochromes *P*-450 MC-A and MC-B. The fraction containing the cytochrome *P*-450 MC-C fraction was eluted in a step gradient from 85 mM- to 125 mM-KCl. The use of a linear 0–125 mM-KCl gradient or a series of shallow steps in KCl concentration did not alter the elution profile of this peak containing cytochrome *P*-450 MC-C, and hence these protocols were discontinued in favour of a more-rapid elution of this peak from the room-temperature column.

In an average purification, the final recoveries of cytochromes *P*-450 were 4.0% (MC-C), 1.6% (MC-D), 0.5% (MC-B) and 0.3% (MC-A) of the starting amount (8000 nmol generally) of total microsomal cytochrome *P*-450. These final molar proportions were not significantly different from the relative proportions of individual isoenzymes obtained in fractions from initial chromatographic separation procedures (results not shown). The greatest percentage of the total recoverable cytochrome *P*-450 was contained in the cytochrome *P*-450 MC-C preparation. Cytochrome *P*-450 MC-D contributed moderately to the total recoverable pool of cytochromes *P*-450, and cytochromes *P*-450 MC-A and MC-B represented minor contributors.

Characterization by SDS/polyacrylamide-gel electrophoresis

The degree of purity of each of the cytochrome *P*-450 MC preparations was investigated by using SDS/polyacrylamide-gel electrophoresis. Cytochromes *P*-450 MC-A, MC-B, MC-C and MC-D were electrophoresed on SDS/8%-polyacrylamide slab gels (Fig. 1). Results of these studies indicated that cytochromes *P*-450 MC-A, MC-B and MC-D were electrophoretically homogeneous. Cytochrome *P*-450 MC-C, however, exhibited two bands in the 50000–56000-M_r region. (We were unsuccessful in obtaining a homogeneous cytochrome *P*-450 MC-C preparation despite the use of several additional ion-exchange chromatographic columns.) Minimal M_r values established for each of these cytochrome *P*-450 preparations were M_r 48000 for form MC-A, M_r 53000 for form MC-B, M_r 54000 for form MC-D and M_r 53500 and 55500 for the two bands of preparation MC-C (Fig. 1). Nomenclature for the cytochrome preparations was established on the basis of their migration towards the anode, with the lower-M_r forms alphabetized first. Each was prefaced with 'MC' to indicate induction with 3-MC.

Spectral characteristics

Table 1 summarizes spectral characteristics for each of these cytochrome *P*-450 MC preparations. Mean specific contents obtained were 15.2 nmol (MC-A), 13.1 nmol (MC-B), 15.1 nmol (MC-D) and 17.6 nmol (MC-C) of cytochrome *P*-450/mg of protein. These data are based on the quantitative determination of holoenzyme in each preparation, by using the CO-reduced difference spectral method of Omura & Sato (1964), and hence do not take into account haemless cytochrome *P*-450, which may be present in the preparations. Spectral Soret maxima were determined for each cytochrome *P*-450 MC under absolute oxidized and reduced conditions and for the CO-reduced difference spectra. Cytochrome *P*-450 MC-D exhibited an absolute oxidized Soret maximum at 392 nm, indicating that this is a high-spin ferric cytochrome. The high-spin character of this isoenzyme

was determined in the presence of 0.2% Emulgen, a non-ionic detergent, and 0.5% sodium cholate, and did not appear to be altered by the presence or the absence of these detergents. A similar high-spin cytochrome *P*-448 has been previously reported in livers of rats pretreated with 3-MC (Wolf & Oesch, 1983), isosafrole (Ryan *et al.*, 1980) or polychlorinated biphenyls (Goldstein *et al.*, 1982).

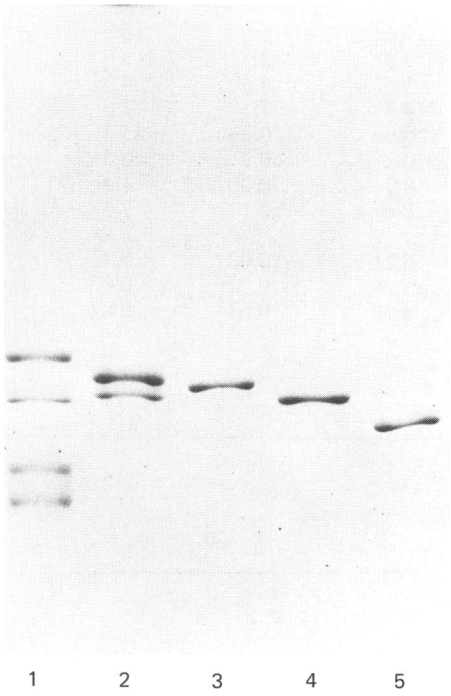


Fig. 1. SDS/polyacrylamide-gel electrophoresis of cytochromes *P*-450 purified from liver microsomes of 3-MC-pretreated rats

Electrophoresis was from top to bottom on an 8% slab gel. Proteins were detected with Coomassie Blue stain. Lane 1, M_r standard proteins: catalase (M_r 58000), L-glutamate dehydrogenase (M_r 53000), ovalbumin (M_r 45000) and alcohol dehydrogenase (M_r 41000). Lane 2, 1.5 μg of cytochrome *P*-450 MC-C. Lane 3, 1 μg of purified cytochrome *P*-450 MC-D. Lane 4, 1 μg of purified cytochrome *P*-450 MC-B. Lane 5, 1 μg of purified cytochrome *P*-450 MC-A.

Cytochromes *P*-450 MC-A, MC-B and MC-C were predominantly low-spin in character, possessing absolute oxidized Soret maxima between 416 and 417 nm. Analysis of their CO-reduced difference spectra shows that three of these cytochrome *P*-450 MC forms belong to the 'cytochrome *P*-448' class of isoenzymes, these being the MC-B (448 nm), MC-C (446 nm) and MC-D (447 nm) forms. The MC-A form has a CO-reduced difference spectral maximum at 452 nm. A form of cytochrome *P*-450 possessing a CO-reduced difference spectral maximum at 452 nm, which is modestly inducible in rat liver by 3-MC pretreatment, has been described by others (Guengerich *et al.*, 1982*a*; Ryan *et al.*, 1982). However, the presence of more than two forms of rat hepatic cytochrome *P*-450 with CO-reduced difference spectral maxima occurring at 448 nm or less does not appear to have been previously reported in similarly induced rat liver microsomes.

Substrate activities

Substrate preferences of purified cytochromes *P*-450 are often used to detect similarities shared with previously reported forms. This method has shown, for example, preferences of a major form of 3-MC-inducible cytochrome *P*-448 for hydroxylation of testosterone at the 6β-position and for metabolism of benzo[*a*]pyrene and 7-ethoxycoumarin (Lu & West, 1980; Ryan *et al.*, 1982).

Catalytic activities of each of the cytochrome *P*-450 MC isoenzymes were determined in a reconstituted system containing saturating amounts of NADPH, dilauroyl phosphatidylcholine and purified NADPH–cytochrome *P*-450 reductase. The results of these studies are shown in Table 2. The cytochrome *P*-450 MC-C preparation exhibited the highest substrate turnover for 7-ethoxycoumarin, *p*-nitroanisole and benzo[*a*]pyrene, and was active in the hydroxylation of testosterone at the 6β-position. Cytochrome *P*-450 MC-A was most active in catalysing the hydroxylation of testosterone at the 7α-position. In contrast, neither of the cytochrome *P*-448 isoenzymes MC-D or the newly identified MC-B exhibited a strong preferential activity towards any of the substrates examined.

Table 1. Spectral characteristics of cytochrome *P*-450 MC isoenzymes

For the determination of absolute spectra of oxidized and reduced cytochromes *P*-450 the reference cuvette contained sample buffer, i.e. 100 mM-potassium phosphate buffer, pH 7.4, containing 20% (v/v) glycerol, 0.1 mM-EDTA, 0.2% Emulgen 911 and 0.5% sodium cholate. Reductions were done chemically with $Na_2S_2O_4$. Specific contents were determined by CO-reduced difference spectral quantification of holoenzyme by using an absorption coefficient of 91 $mM^{-1} \cdot cm^{-1}$. Values represent means for three or four separate purifications, with the exception of MC-A, which was purified only once.

Cytochrome *P*-450	Mean specific content (nmol/mg of protein)	Soret wavelength maxima (nm)		
		Oxidized	Reduced	CO-reduced difference
MC-A	15.2	416	410	452
MC-B	13.1	417	410	448
MC-C	17.6	416	409	446
MC-D	15.1	392	408	447

Table 2. Catalytic activities of cytochrome *P*-450 MC isoenzymes determined in a reconstituted system

For experimental details see the Materials and methods section.

Substrate	Reaction	Cytochrome *P*-450...	Product (nmol/min per nmol of cytochrome *P*-450) MC-A	MC-B	MC-C	MC-D
Benzphetamine	*N*-Demethylation		19.0	8.5	15.8	15.7
NN-Dimethylnitrosamine*	*N*-Demethylation		3.9	3.5	3.6	5.0
Ethylmorphine	*N*-Demethylation		5.5	4.3	4.3	4.5
Aminopyrine	*N*-Demethylation		14.7	7.3	12.0	9.9
7-Ethoxycoumarin	*O*-Dealkylation		0.9	0.2	65.3	1.1
Aniline	*p*-Hydroxylation		0.2	0.2	0.5	0.5
Benzo[*a*]pyrene†	3- and 9-Hydroxylation		0.2	0.2	1.4	0.2
p-Nitroanisole	*O*-Demethylation		<0.1	<0.1	7.0	<0.1
Testosterone‡						
6β-Hydroxylation			0.21	0.02	0.42	0.03
7α-Hydroxylation			15.76	0.22	0.07	0.10
16α-Hydroxylation			0.10	0.02	0.02	0.10

* 100 mM-Dimethylnitrosamine.
† 80 μM-Benzo[*a*]pyrene.
‡ 0.12 mM-Testosterone + 10 nmol (0.5 μCi) of [^{14}C]testosterone.

Rat cytochrome *P*-450	*N*-Terminal sequence 1 10 20
a (452 nm)	M L D T G L L L V V I L A S L S V M L L
b (450 nm)	M E P S I L L L L A L L V G F L L L L V
c (447 nm)	P S V Y G F P A F T S A T E L L L A V
e (450 nm)	M E P S I L L L L A L L V G F L L L L V
f (447.5 nm)	M D L V T F L V L T L S S L I L L S L W
g (447.5 nm)	M D P V V V L L L S L F F L L
h (451 nm)	M D P V L V L V L T L S S L L L L S L W
i (449 nm)	M D P F V V L V L S L S F L L L L Y ? W
PB-1 (450 nm)	M D L V M L L V L T L T ? L I L L (S) I W
RLM 3 (449 nm)	M D P V V V L L L M L F F L L L L
RLM 5 (451 nm)	M D P V L V L V L T L L L L L
d (447 nm)	A F S Q Y I S L A P E L L L A T A I F
MC-D (447 nm)	A F S G Y I P L A P E L L L A
MC-B (448 nm)	M D L L P A L

Fig. 2. *N*-Terminal sequence data for cytochromes *P*-450 MC-B and MC-D, and comparison with published sequences of rat liver cytochrome *P*-450 isoenzymes

The *N*-terminal sequences obtained for cytochromes *P*-450 MC-D and MC-B (see the text) are shown at the bottom of the Figure. Parenthetical data indicate the CO-reduced difference spectral maximum reported for each purified cytochrome *P*-450 isoenzyme.

N-Terminal amino acid sequencing

Limited *N*-terminal sequencing was carried out on the two homogeneous forms of cytochrome *P*-448, cytochromes *P*-450 MC-B and MC-D. These were compared with sequences reported for other rat liver isoenzymes to determine what relationship, if any, was present (Fig. 2). With reference to Fig. 2, the sequences for cytochromes *P*-450a, *P*-450c and *P*-450f–*P*-450i were reported by Haniu *et al.* (1984), with cytochromes *P*-450f–*P*-450i

representing isoenzymes purified from untreated rat liver microsomes (Ryan *et al.*, 1984). Cytochrome *P*-450a is inducible to a minor extent by both phenobarbital and 3-MC, and cytochrome *P*-450c is the major isoenzyme reportedly inducible by 3-MC (Botelho *et al.*, 1979; Ryan *et al.*, 1982). Cytochrome *P*-450 PB-1 is a phenobarbital-inducible isoenzyme described by Waxman & Walsh (1983). Cytochromes *P*-450b and *P*-450e were sequenced by Yuan *et al.* (1983) and are phenobarbital-inducible forms. Forms similar to cytochromes *P*-450b and *P*-450e have also been partially sequenced by Waxman & Walsh (1982). The constitutive isoenzymes, cytochromes *P*-450 RLM 3 and RLM 5, were reported by Cheng & Schenkman (1983). The sequence for cytochrome *P*-450d was reported by Botelho *et al.* (1982).

As shown, the sequence for cytochrome *P*-450 MC-D is homologous at 13 out of 15 residues with the sequence reported for cytochrome *P*-450d. Both of these isoenzymes are high-spin forms of cytochrome *P*-448. The minor points of departure for cytochrome *P*-450 MC-D from the referenced sequence for cytochrome *P*-450d are at positions 4 and 7, these being glycine and proline respectively for cytochrome *P*-450 MC-D. The observed minor differences in *N*-terminal sequences for these two purified isoenzymes may be a reflection of a degree of sequence microheterogeneity appearing between the different strains of rats used in the studies [Long Evans immature males (Botelho *et al.*, 1982) and Sprague–Dawley mature males (present work)].

Comparison of the limited sequence obtained for purified cytochrome *P*-450 MC-B with sequences reported for other rat liver microsomal isoenzymes indicates that this isoenzyme has not been previously described. Comparison of the sequence for cytochrome *P*-450 MC-B with sequences obtained for other cytochromes *P*-448 from rat hepatic microsomes (Fig. 2) shows little homology among the cytochrome-*P*-448-type isoenzymes, again suggesting that they originate in different structural genes.

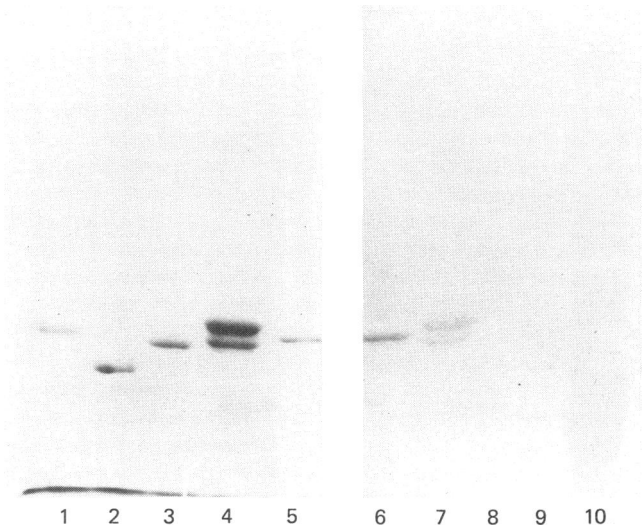


Fig. 3. Immuno-blot recognition of purified cytochrome *P*-450 isoenzymes by rabbit anti-(rat cytochrome *P*-450 MC-D) antibody

Purified isoenzymes were electrophoresed on SDS/polyacrylamide gels and subsequently electro-transferred to nitrocellulose paper. Incubation of antibodies was performed as described in the Materials and methods section. Antibody binding was detected by using 4-chloro-1-naphthol as the substrate for the peroxidase-conjugated second antibody. Lanes 1–5, SDS/polyacrylamide-gel electrophoretograms stained for protein with Coomassie Blue: lane 1, cytochrome *P*-450 PB (1 μg); lane 2, cytochrome *P*-450 MC-A (1 μg); lane 3, cytochrome *P*-450 MC-B (1 μg); lane 4, cytochrome *P*-450 MC-C (2 μg); lane 5, cytochrome *P*-450 MC-D (1 μg). Lanes 6–10, immuno-blot of isoenzymes electro-transferred to a similar gel: lane 6, cytochrome *P*-450 MC-D; lane 7, cytochrome *P*-450 MC-C; lane 8, cytochrome *P*-450 MC-B; lane 9, cytochrome *P*-450 MC-A; lane 10, cytochrome *P*-450 PB.

Immunological characterization

Immunological characterization of the purified cytochromes *P*-448 MC-B and MC-D was pursued to characterize these isoenzymes further. A polyclonal antibody against purified cytochrome *P*-450 MC-D was raised in rabbits. The IgG fraction from this antiserum was tested for cross-reaction with all of the forms of cytochrome *P*-450 purified from liver microsomes of 3-MC-pretreated rats and with a phenobarbital-inducible form (cytochrome *P*-450 PB) which was previously purified and characterized in our laboratory (Seidel *et al.*, 1984). Fig. 3 indicates that this polyclonal anti-(cytochrome *P*-450 MC-D) antibody recognized the MC-D isoenzyme and both bands present in the cytochrome *P*-450 MC-C preparation. None of the remaining cytochrome *P*-450 isoenzymes was recognized by this antibody. These results indicate that the cytochrome *P*-450 MC-D isoenzyme shares one or more antigenic determinants with both of the polypeptides present in the cytochrome *P*-450 MC-C preparation. (Judged from these results and the high specific content of cytochrome *P*-450 per mg of protein in the MC-C preparation, it therefore seems probable that the cytochrome *P*-450 MC-C preparation contains two isoenzymes of rat liver microsomal cytochrome *P*-450 that bear similar antigenic determinants.)

Immunoabsorption of polyclonal antisera against a solid-phase-linked heterologous antigen for removal of cross-reacting antibody has been utilized successfully for distinguishing various forms of cytochrome *P*-450 (Reik *et al.*, 1982). We chose this method to determine whether the polyclonal rabbit anti-(cytochrome *P*-450 MC-D) antibody could be made 'monospecific' for the MC-D form alone. For this study we linked the heterologous antigens of cytochrome *P*-450 MC-C to CNBr-activated Sepharose (Pharmacia) and passaged the rabbit anti-(rat cytochrome *P*-450 MC-D) IgG over a column of the immobilized cytochrome *P*-450 MC-C antigen. After this, the immunoadsorbed anti-(cytochrome *P*-450 MC-D) antibody was tested for recognition of cytochromes *P*-450 MC-C and MC-D, and for polypeptide recognition in immuno-blots of liver microsomes of 3-MC-pretreated rats. The results shown in Fig. 4 indicate that the immunoadsorbed polyclonal anti-(cytochrome *P*-450 MC-D) antibody was selective only for cytochrome *P*-450 MC-D, did not recognize either polypeptide in cytochrome *P*-450 MC-C, and reacted with a single polypeptide in the microsomes of 3-MC-pretreated rats. These findings indicated that cytochrome *P*-448 MC-D contains antigenic determinants that are unique to this isoenzyme.

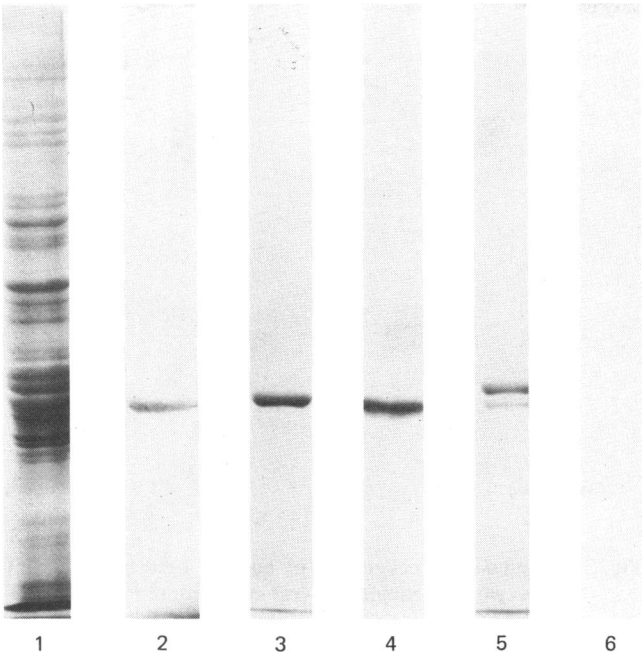


Fig. 4. Immuno-blot of 3-MC-induced microsomes, purified cytochrome *P*-450 MC-D and cytochrome *P*-450 MC-C with rabbit anti-(rat cytochrome *P*-450 MC-D) antibody pre-adsorbed against cytochrome *P*-450 MC-C

Lanes 1, 3 and 5, SDS/polyacrylamide-gel electrophoretogram lanes stained for protein with Coomassie Blue: lane 1, liver microsomal protein from 3-MC-pretreated rats (15 μg); lane 3, purified cytochrome *P*-450 MC-D (1.5 μg); lane 5, cytochrome *P*-450 MC-C (1.5 μg). Lanes 2, 4 and 6, electrophoretic transfer of protein from gels, followed by immunological staining with rabbit anti-(rat cytochrome *P*-450 MC-D) specific antibody and 4-chloro-1-naphthol as the peroxidase substrate for the peroxidase-conjugated second antibody: lane 2, immuno-blot of microsomal protein (15 μg); lane 4, immuno-blot of cytochrome *P*-450 MC-D (1.5 μg); lane 6, immuno-blot of cytochrome *P*-450 MC-C (1.5 μg).

Immunological characterization of purified cytochrome *P*-450 MC-B was carried out with a monoclonal antibody (designated MAb MC-B/17-5) developed from Balb/c mouse spleen cells and the P3-X63-Ag/8 mouse myeloma cell line. MAb MC-B/17-5 antibody was characterized as belonging exclusively to the IgG_1 immunoglobulin subclass as described in the Materials and methods section. This monoclonal antibody was screened for recognition of a panel of purified cytochrome *P*-450 isoenzymes by a direct dot binding assay, with 0.2 μg each of the purified cytochrome *P*-450 isoenzymes (Fig. 5*a*). The monoclonal anti-(cytochrome *P*-450 MC-B) antibody recognized only its homologous antigen, showing no cross-reactivity with cytochromes *P*-450 MC-A, MC-C and MC-D, or the previously purified (Seidel *et al.*, 1984) phenobarbital-inducible form, cytochrome *P*-450 PB. Fig. 5(*b*) shows the results of an immuno-blot of liver microsomes of MC-induced rats with the anti-(cytochrome *P*-450 MC-B) MAb MC-B/17-5 as the primary antibody. Only one polypeptide band was identified by the monoclonal anti-(cytochrome *P*-450 MC-B) antibody, also indicating that it recognizes an antigenic determinant unique to a single protein in these microsomes.

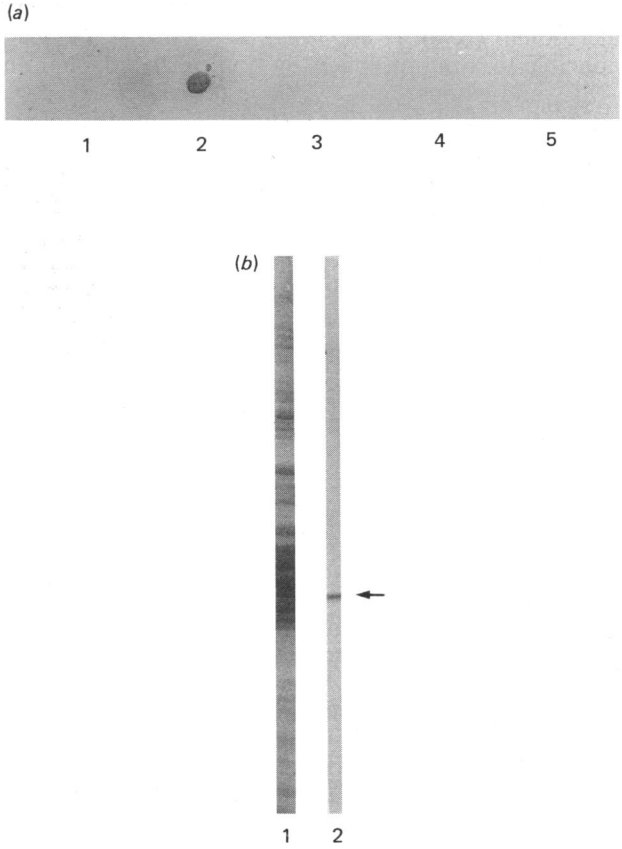


Fig. 5. (*a*) Direct dot binding assay of purified cytochrome *P*-450 isoenzymes with mouse monoclonal MAb MC-B/17-5 anti-(cytochrome *P*-450 MC-B) antibody and (*b*) immuno-blot of liver microsomes of 3-MC-pretreated rats with mouse monoclonal MAb MC-B/17-5 anti-(cytochrome *P*-450 MC-B) antibody

(*a*) 1, Cytochrome *P*-450 MC-A (0.2 μg); 2, cytochrome *P*-450 MC-B (0.2 μg); 3, cytochrome *P*-450 MC-C (0.2 μg); 4, cytochrome *P*-450 MC-D (0.2 μg); 5, cytochrome *P*-450 PB (0.2 μg). 4-Chloro-1-naphthol was the peroxidase substrate. (*b*) Lane 1, 15 μg of microsomal protein electrophoresed on an SDS/8.0%-polyacrylamide gel and stained with Coomassie Blue. Lane 2, microsomal protein electro-transferred to nitrocellulose paper and immunologically stained with MAb MC-B/17-5 antibody to detect cytochrome *P*-450 MC-B. 4-Chloro-1-naphthol was the peroxidase substrate.

DISCUSSION

The present study has characterized at least four distinct isoenzymes of cytochrome *P*-450 present in liver microsomes of 3-MC-pretreated rats. One of these, cytochrome *P*-450 MC-B, is an inducible isoenzyme of the 'cytochrome *P*-448' phenotype that has not been reported previously. It was purified to homogeneity and is immunologically distinct from the other forms of cytochrome *P*-450 described in the present paper. Cytochrome *P*-450 MC-B exhibits a minimum M_r on SDS/polyacrylamide-gel electrophoresis that is distinct from those of the other 3-MC-inducible isoenzymes. It also differs with respect to its substrate activity profile in a reconstituted system. Most importantly, *N*-terminal

sequence data obtained for cytochrome *P*-450 MC-B show that the primary amino acid sequence of this isoenzyme is unique with respect to those reported for other previously described rat liver cytochrome *P*-450 isoenzymes (Fig. 2). Review of the literature does not indicate prior reports of identification of a cytochrome *P*-450 form similar to MC-B in other species, or in non-hepatic tissues of the rat.

In addition to cytochrome *P*-450 MC-B, three other cytochromes *P*-450 were purified or partially purified from liver microsomes of 3-MC-pretreated rats. These have served primarily as a basis for comparison of physicochemical characteristics with cytochrome *P*-450 MC-B. We noted that a number of characteristics possessed by these isoenzymes appear to be similar to those of forms reported in the literature. Cytochrome *P*-450 MC-A is similar, in possessing a CO-reduced difference spectral maximum at 452 nm, a minimum M_r of 48000 on SDS/polyacrylamide-gel electrophoresis and a marked preference for the 7α-hydroxylation of testosterone, to cytochrome *P*-450a described by Ryan *et al.* (1982) and cytochrome *P*-450 UT-F described by Guengerich *et al.* (1982*a*). Cytochrome *P*-450 MC-D, purified to homogeneity as a high-spin cytochrome-*P*-448-type of isoenzyme, closely resembles an isoenzyme or isoenzymes reported by several investigators as being present after treatment of rats with 3-MC (Wolf & Oesch, 1983), polychlorinated biphenyls (Goldstein *et al.*, 1982) or isosafrole (Ryan *et al.*, 1980). Polyclonal antibodies generated against these purified high-spin isoenzymes in the investigating laboratories have typically exhibited immunological recognition of a major form of cytochrome *P*-450 inducible by 3-MC (Reik *et al.*, 1982). We have also noted in the present work that a polyclonal antibody raised in rabbits against cytochrome *P*-450 MC-D recognizes the isoenzyme(s) constituting cytochrome *P*-450 MC-C, which contained most of the cytochrome *P*-450 recoverable from microsomes of 3-MC-pretreated rats. The most convincing evidence for correlation of cytochrome *P*-450 MC-D with previously reported high-spin rat isoenzymes is the extensive homology seen in the *N*-terminal sequence obtained for cytochrome *P*-450 MC-D with that reported by Botelho *et al.* (1982) for their high-spin cytochrome *P*-450d.

Correlation of the cytochrome *P*-450 MC-C preparation with major forms of cytochrome *P*-448 characterized by other investigators has been obscured by the presence of a second polypeptide in the otherwise homogeneous preparation. The two proteins consistently co-purified during all chromatographic separation protocols despite numerous modifications of the procedures. It may be noted that both of these polypeptides are recognized by a polyclonal antibody generated against highly purified cytochrome *P*-450 MC-D. It may be that these two polypeptides represent two distinct isoenzymes of cytochrome *P*-450. Whether both of these belong to the major cytochrome *P*-448 metabolic phenotype remains to be elucidated.

The identification of the previously unreported 3-MC-inducible cytochrome *P*-450 MC-B (and to a certain extent even the co-purification of two immunologically related proteins in cytochrome *P*-450 MC-C) underlines the fact that an increasing number of cytochromes *P*-450 are being purified after chemical pretreatments once thought to induce only a single major isoenzyme of cytochrome *P*-450. This multiplicity has been observed for several inducing agents, including 3-MC (Ryan *et al.*, 1982) and β-naphthoflavone and phenobarbital (Guengerich *et al.*, 1982*b*). The functional significance of such patterns of multiple isoenzyme induction remains, however, to be fully elucidated.

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