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Bovine Adrenocortical Microsomal Hemeproteins P-450$_{17\alpha}$ and P-450$_{C\text{-}21}$

ISOLATION, PARTIAL CHARACTERIZATION, AND COMPARISON TO P-450$_{SCC}$*

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A scheme is presented for the simultaneous isolation of P-450$_{17\alpha}$ and P-450$_{C\text{-}21}$ from bovine adrenocortical microsomes and for their purification to specific contents of 19-20 nmol of P-450/mg of protein. Although cholate solubilized only 75% of the total amount of P-450 and 36% of the NADPH-cytochrome *c* (P-450) reductase present in bovine adrenocortical microsomes as compared to Triton N-101, it produced a less complex mixture of microsomal proteins from which most of the P-450 could be removed by a 0-25% cut with polyethylene glycol. The P-450 fraction was subsequently resolved into individual P-450s by column chromatography on ω-aminooctyl-Sepharose; these were then purified to homogeneity by a series of final chromatography steps including DEAE-, CM-, and ω-aminooctyl-Sepharose.

Each of the purified P-450s traveled as a single sharp band in sodium dodecyl sulfate-polyacrylamide gel electrophoresis with apparent M_r = 50,000 and 52,000 for P-450$_{17\alpha}$ and P-450$_{C\text{-}21}$, respectively. The purified preparations were virtually free of P-420 and showed mixed spin absorption spectra. The visible absorption maxima, together with their extinction coefficients, are reported for the ferric and ferrous forms as well as for the substrate and CO complexes. In reconstitution experiments with rabbit liver microsomal NADPH-cytochrome *c* (P-450) reductase and phospholipids, P-450$_{C\text{-}21}$ catalyzed hydroxylation of 17α-hydroxyprogesterone in position 21, forming 45.2 nmol of product/mg of P-450$_{C\text{-}21}$/min. Partially purified P-450$_{17\alpha}$ hydroxylated progesterone to the extent of 5.04 nmol of product/mg of P-450$_{17\alpha}$/h, but the highly purified hemeprotein no longer mediated 17α-hydroxylation. When incubated with adrenodoxin and adrenodoxin reductase instead, it showed activity for the side chain cleavage of cholesterol (~65% of that found with P-450$_{SCC}$). Despite this similarity in enzymatic activities, P-450$_{17\alpha}$ and P-450$_{SCC}$ can be readily and completely separated from each other by chromatography on adrenodoxin-Sepharose. Furthermore, P-450$_{17\alpha}$ was recovered in variable yields, up to 16%, but always greatly exceeding the yield of P-450$_{C\text{-}21}$ (3%). Since the preparation of adrenal microsomes contained only 3% mitochondria the presence of P-450$_{17\alpha}$ in microsomes cannot be accounted for by this contamination. Binding difference spectra with 3-methoxybenzidine, a strong inhibitor of P-450$_{SCC}$, and with 7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalen-1(2H)-one, specific for inhibition of P-450$_{17\alpha}$, show drastic differences in the spectral transitions they elicit in these two hemeproteins. As a further significant distinction P-450$_{17\alpha}$ shows a rapid and fully reversible thermotropic spin state transition, from high to low spin with increasing temperature from 7 to 15 °C, which is not shared by P-450$_{SCC}$. The amino acid compositions of both adrenocortical microsomal P-450s are reported, and that of P-450$_{17\alpha}$ is compared to the known composition of P-450$_{SCC}$. By this criterion again, P-450$_{17\alpha}$ and P-450$_{SCC}$ appear to be closely related, but not identical.

The adrenal cortex is an appealing source material for the isolation, from the same mammalian tissue, of highly specific P-450 hemeproteins, all active in metabolic pathways leading from cholesterol to steroid hormones (1, 2). Although the concentration of these hemeproteins is significantly below that found for P-450s induced in liver microsomes (3), it is sufficient to provide access to four major P-450s. P-450$_{SCC}$[1] and P-450$_{11\beta}$ are located in the inner mitochondrial membrane and have received considerable attention in recent years. Consequently, they are now readily accessible in highly purified form by a number of related procedures (4-10). Much less effort was directed at the isolation and characterization of the monooxygenase systems of bovine adrenocortical microsomes although they also catalyze important biotransformations of the steroid nucleus (11). This paper is addressed primarily to the study of the P-450 hemeproteins of the steroid 17α- and C-21-hydroxylases which represent the two most prominent monooxygenases of bovine adrenocortical microsomes (>75%), and to the comparison of P-450$_{17\alpha}$ to P-450$_{SCC}$.

Hydroxylation of progesterone in position 17α precedes the hydroxylation on carbon 21 in the biosynthesis of cortisol which is the major glucocorticoid in the adrenal glands of man and beef, but not of the rat. To our knowledge, the P-450 nature of the adrenal 17α-hydroxylase, although widely accepted, had never been unequivocally established. Failure to find significant 17α-hydroxylase activity in adrenal microsomes has hampered the study of this enzyme and prompted

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[1] The abbreviations used are: P-450$_{SCC}$, P-450$_{11\beta}$, P-450$_{17\alpha}$, and P-450$_{C\text{-}21}$, cytochromes P-450$_{17\alpha}$, etc., from the respective bovine adrenocortical hydroxylase; PEG, polyethylene glycol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BrCN, cyanogen bromide; EDTA, ethylenediamintetraacetic acid; MOB, 3-methoxybenzidine; SU-10603, 7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalen-1(2H)-one; HPLC, high pressure liquid chromatography.

some investigators to postulate that it is labile or impaired after disintegration of the cellular structure (12, 13), or even that it is a soluble, cytoplasmic enzyme (14). The present study describes the isolation from adrenal microsomes of a P-450 hemeprotein in homogenous form which can catalyze 17α-hydroxylation, although quite inefficiently, while also exhibiting considerable cholesterol desmolase activity. It is our aim to demonstrate that this P-450 hemeprotein is present in significant quantities in bovine adrenocortical microsomes where it accounts for all of the 17α-hydroxylase activity, and that it is distinct from, but closely related to, the mitochondrial $P\text{-}450_{SCC}$.

The 21-hydroxylation of 17α-hydroxyprogesterone has received early recognition because its deficiency leads to congenital adrenal hyperplasia, manifested by virilization in the female and precocious puberty in the male. While its P-450 nature was conclusively demonstrated in 1965 (15), systematic efforts to purify $P\text{-}450_{C\text{-}21}$ have been reported only recently (16–18). Kominami *et al.* (16, 17) have described a workable purification procedure while our investigation was already in progress. We have found this procedure to be readily reproducible, and to yield pure, active enzyme. Hiwatashi and Ichikawa (18) have reported the reconstitution of the steroid 21-hydroxylase from highly purified $P\text{-}450_{C\text{-}21}$ and NADPH-cytochrome P-450 reductase (19). But their preparation of $P\text{-}450_{C\text{-}21}$ appears to differ from that of Kominami *et al.* (16, 17) in its chromatographic characteristics and in other properties, while the $P\text{-}450_{C\text{-}21}$ preparation reported in this communication resembles the hemeprotein described by Kominami *et al.* very closely. Yet we observed a larger molecular weight for $P\text{-}450_{C\text{-}21}$ than was reported by previous investigators.

We describe here an alternate isolation procedure which affords simultaneously and in excellent purity two P-450 hemeproteins, $P\text{-}450_{17\alpha}$ and $P\text{-}450_{C\text{-}21}$. This approach has given us the opportunity to compare $P\text{-}450_{17\alpha}$ and $P\text{-}450_{C\text{-}21}$ and to note that this pair in many respects resembles the corresponding mitochondrial hemeproteins, $P\text{-}450_{SCC}$ and $P\text{-}450_{11\beta}$.

EXPERIMENTAL PROCEDURES[2]

Materials - Triton N-101, Triton X-100, Tween 20, Tween 80, sodium cholate, sodium deoxycholate, SDS, PEG-6000, NADH, NADPH, acrylamide and bisacrylamide, progesterone, 17α-hydroxyprogesterone, Agarose, Type II, and cytochrome c, Type III were all purchased in highest purity from Sigma Chemical Co., St. Louis, MO. Emulgen 913 was a product of Kayo Atlas, Tokyo, Brij-35 was obtained from Pierce Chemical Co., Rockford, IL, and 1-O-n-Octyl-β-D-glucopyranoside was from Calbiochem-Behring Corp., La Jolla, CA. 3-Methoxy benzidine was purchased from Pfaltz and Bauer, Stamford, CT, and 7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalene-1(2H)-one was a product of Ciba-Geigy. Resins, such as Sepharose 4B, DEAE- and CM-Sepharose CL 4B, and hydroxylapatite-silica were bought from Sigma. Radioisotopes, including [1,2-^{3}H],-cholesterol, [1,2-^{3}H]-17α-hydroxyprogesterone, and [4-^{14}C]-progesterone, were obtained from New England Nuclear Corp., Boston, MA. All other chemicals were of reagent grade.

Spectral Assays - Optical spectra, unless stated otherwise, were recorded with highly purified P-450 hemeprotein preparations in 50 mM phosphate buffer pH 7.4, containing 100 μM EDTA, 100 μM dithiothreitol and 10% glycerol, using a Varian Cary 210 spectrophotometer with temperature control and gel scanning accessory. Difference spectra resulting from the binding of inhibitors to $P\text{-}450_{17\alpha}$ and $P\text{-}450_{SCC}$ were recorded as described previously (20) except that the Cary 210 spectrophotometer was used. A 2.5 μM hemeprotein solution in buffer was titrated with ethanolic solutions of MOB (5 mM) and SU 10603 (3.8 mM). Equal volumes of ethanol were added to the sample and reference cuvette, respectively.

Analytical Procedures - The heme content of P-450 preparations was determined by the pyridine hemochromogen procedure using an extinction coefficient, reduced minus oxidized, of 20.7 mM^{-1} at 557 nm (21). Identical aliquots of the protein solution were used for protein assays by the biuret method (22) which, in turn, was standardized for P-450 hemeproteins against automated amino acid analysis (23). Amino acid contents were determined for each P-450 preparation on a Beckman Model 120C automatic amino acid analyzer, modified for automatic sample application. To eliminate any influence of detergents and heme on the amino acid composition by breakdown during acid hydrolysis, these components were dissociated from the polypeptide by acid-acetone treatment and subsequent dialysis of the apoprotein against 1% formic acid. Hydrolyses were carried out in duplicate with 5.7 N HCl in sealed tubes under vacuum at 110°C, and aliquots were heated for 24, 48, and 72 h. CyS/2 was determined as $CySO_3H$ after performate oxidation (24) and Trp was calculated from analyses after hydrolysis, for 24 h, with 3 N mercaptoethane sulfonic acid (25).

Gel Electrophoresis - THE SDS-PAGE procedure of Laemmli (26) was carried out with cylindrical gels, using 5–10 μg of purified protein, staining with Coomassie Blue, and addition of Bromphenol Blue as marker dye. After about 1 week of destaining with 7% acetic acid, the protein bands were traced with the gel scanning accessory of the Cary 210 spectrophotometer. Electrophoresis of solubilized BAC microsomes and of chromatographic fractions during the purification procecure were performed in a slab gel with >10 μg of protein in the system of Fairbanks et al. (27).

Enzyme Assays - Succinate dehydrogenase was assayed by a modification of the procedure of Hiatt (28). In a final volume of 1.3 ml, the reaction mixture contained 80 mM phosphate pH 7.5, 0.79 mM phenazine methosulfate, 24 mM KCN, 16 mM succinate, 96 μM dichloroindophenol and 460 μg of protein. The reaction was initiated with succinate and monitored at 600 nm. The molar extinction coefficient of 1.1×10^4 mM^{-1} cm^{-1} was used for dichloroindophenol.

Cytochrome c oxidase was assayed according to the method of Wharton and Tzagoloff (29). The reaction mixture contained 10 mM phosphate, pH 7.0, 1 mM potassium ferricyanide, 0.07% cytochrome c and 9.2 μg protein in a total volume of 1.0 ml. The reaction was initiated with protein and the decrease in absorption at 550 nm was monitored. The millimolar extinction coefficient of 21 mM^{-1} cm^{-1} at 550 nm was used for cytochrome c.

Cholesterol desmolase activity was assayed as previously described (10).

Steroid 21-hydroxylase was measured by the conversion of radioactive progesterone to 11-deoxycorticosterone and by the conversion of radioactive 17α-hydroxyprogesterone to 17α-hydroxy-11-deoxycorticosterone. In the reconstituted system, highly purified rabbit liver microsomal NADPH cytochrome P-450 reductase and BAC microsomal cytochrome $P\text{-}450_{C\text{-}21}$ were used. To introduce the substrate, three drops of glycerol were first placed in the bottom of a test tube. Radioactive progesterone or 17α-hydroxyprogesterone were added followed by 600 nmol of non-radioactive substrate in 60 μl of acetone. The solvents were removed under nitrogen and the remaining components of the assay mixture were added. The final assay mixture in 50 mM K-phosphate buffer, pH 7.2, contained 0.79 nmol cytochrome $P\text{-}450_{C\text{-}21}$, 0.23 units NADPH cytochrome P-450 reductase, 12.0 μmol $MgCl_2$, 6.0 units of glucose-6-phosphate dehydrogenase, 30 μmol of non-radioactive substrate and 0.6 μCi (4-^{14}C) progesterone or 2.1 μCi (1,2-^{3}H) 17-hydroxyprogesterone. The concentration of Emulgen 913 was 0.004% and the total volume was 3 ml. The reaction mixture was preincubated at 37°C and was initiated with NADPH.

Following incubation, 1 ml aliquots were withdrawn and vigorously extracted with methylene chloride. The organic phase was then separated and evaporated to dryness. The separation of product from substrate was achieved by TLC or by HPLC. For TLC separation, the sample was dissolved in 50–100 μl methylene chloride, spotted on a Merck 60-F254 silica gel thin layer chromatography plate (5 cm x 20 cm), and developed using benzene:acetone (80:20). Cold carrier steroids were also chromatographed. Following chromatography, steroids were visualized under UV light. The appropriate zones of the chromatogram were scraped off, transferred into a vial and counted in 5 ml of ACS (Aqueous Counting Scintillant, Amersham) solution with a Packard liquid scintillation counter, model 3380. To assay reaction products by HPLC the dried sample was dissolved in 200 μl of HPLC grade methanol. A 50 μl aliquot was then injected into a high pressure liquid chromatograph, model 204, Waters Associates, Milford, MA, equipped with a model 440 absorbance detector. Steroids were separated on a Waters μBondapak C_{18} column (30 cm x 3.9 mm) in an isocratic system using methanol:water (50:50) at a flow rate of 1 ml/min (30).

A similar system was used to assay steroid 21-hydroxylase activity at the level of the microsomes. For this purpose, 2.9 mg of microsomal protein per ml of reaction mixture were used instead of cytochrome P-450 and NADPH cytochrome P-450 reductase. No detergent was present when steroid 21-hydroxylase activity was determined at the level of the microsomes.

Steroid 17α-hydroxylase activity was assayed in a manner similar to that described above for steroid 21-hydroxylase activity. The final assay mixture, in 50 mM K-phosphate buffer, pH 7.2, contained 0.875 nmol of partially purified $P\text{-}450_{17\alpha}$ and 0.06 units rabbit liver microsomal NADPH cytochrome P-450 reductase, 4.0 μmol $MgCl_2$, 2.0 units glucose-6-phosphate dehydrogenase, 20 μmol glucose-6-phosphate, 200 nmol progesterone, 0.2 μCi (4-^{14}C) progesterone and 2.4 μmol NADPH. The Emulgen 913 concentration was 0.01% and the total volume was 2.0 ml. The assay mixture was preincubated at 37°C for one minute and the reaction was initiated by adding NADPH. Reaction products were extracted and identified by HPLC in the manner described for steroid 21-hydroxylase. Because of the low level of activity observed for steroid 17α-hydroxylase activity, incubations were generally of 1 h duration.

Lipid Extraction - The total lipid extract used in reconstitution experiments was prepared by extraction of BAC microsomes (25 mg protein/ml) in buffer with a mixture of chloroform and methanol (2:1). The organic layer was evaporated to dryness under nitrogen and stored at -20°C until use. Immediately before use, 2 ml of buffer, containing 0.3% cholate, were added to the lipid extract, and the suspension was briefly sonicated, yielding a milky white solution.

Preparation of ω-aminooctyl-Sepharose 4B - Following the method of Cuatrecasas (31), to washed, diluted (1:1 with water) Sepharose finely divided BrCN (25 mg/ml Sepharose) was added at once under stirring and the pH was adjusted to 11 ± 0.5 with 8 N NaOH. The temperature was maintained at 20°C by addition of ice chips. After the pH changes subsided (15–20 min), more ice was added to the slurry which was then transferred quickly to a Buchner funnel and washed, under suction, with 1.5 l of ice water. Per 100 ml of washed Sepharose, 200 nmol of 1,8-diaminooctane were added in cold water, previously adjusted to pH 10 with 12 N HCl. The slurry was gently stirred for 16 h at 4°C, washed with 1.5 L of cold water and stored at 4°C.

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Table I, and Scheme I) are presented in miniprint as prepared by the authors. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 82M-569, cite the authors, and include a check or money order for $3.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

Isolation of P-450 Hemeproteins from BAC Microsomes - The scraped cortical tissue of fresh bovine adrenal glands was homogenized in 4x its volume of 250 mM sucrose for 4 min. at 4° C in a Waring blender. The homogenate was centrifuged at 1,000 x g for 10 min. and the pellet was discarded. The supernatant was then centrifuged at 10,000 x g for 30 min. to obtain the mitochondrial pellet. The postmitochondrial supernatant was centrifuged at 20,000 x g for 15 min. and the pellet consisting of mitochondrial debris was discarded. Microsomes were then sedimented either by ultracentrifugation at 105,000 x g for 1 h or by $CaCl_2$ precipitation, in which case the post- 20,000 x g supernatant was made 10 mM in $CaCl_2$, stirred for 15 min., and centrifuged at 20,000 x g (32). Either microsomal pellet was then resuspended in 200 ml of 0.15 KCl and recentrifuged to remove hemoglobin. The washed microsomal pellet, containing 0.46 nmol P-450/mg protein, was resuspended in 100 mM K-phosphate, pH 7.0, containing 20% glycerol to give 25-30 mg protein/ml. By this procedure 200 g of adrenocortical tissue afforded 4.5 g of microsomal protein.

To this amount of resuspended microsomes (4.5 g protein), 13.5 g of sodium cholate (or other detergent) were added, the solution was made 100 µM in EDTA and 100 µM in dithiothreitol, then stirred at 4°C for 1 h and centrifuged at 105,000 x g for 1 h. The pellet was discarded. To the supernatant was added an equal volume of 50% PEG-6000 in the same buffer, which had been dissolved at 60-70°C and cooled to R.T., to bring the temperature of the resulting mixture to 10°C. This mixture was then stirred for 30 min. at 4°C and centrifuged at 12,000 x g for 60 min. The yellow supernatant was discarded and the pellet was resuspended in 100 ml of the same buffer, containing 3% cholate, and dialyzed overnight against 50 mM phosphate, pH 7.0, containing 20% glycerol, 100 µM EDTA and 100 µM dithiothreitol. After dialysis the undissolved material was removed by centrifugation at 12,000 x g for 15 min. and the supernatant was applied to a column of ω-aminooctyl-Sepharose for chromatographic resolution.

Column chromatography on ω-aminooctyl-Sepharose - About 280-300 ml of crude P-450 solution, recovered from the preceding step and containing 1400-1500 nmol P-450 were dialyzed overnight against 50 mM K-phosphate, pH 6.9, containing 20% glycerol, 100 µM dithiothreitol, 100 µM EDTA and 0.3% cholate, and then applied to a column of ω-aminooctyl-Sepharose equilibrated with this buffer (1.5 cm x 20 cm). The effluent contained hemoglobin, NADPH-cytochrome c (P-450) reductase and a small amount of cytochrome P-450. After washing the column further with ∿100 ml buffer to bring the Soret absorption to 0.01 absorbance, elution was continued with 1 L of buffer containing 0.2% Emulgen 913 to recover $P\text{-}450_{17\alpha}$ within 250 ml. The next 750 ml gradually eluted $P\text{-}450_{C\text{-}21}$. To recover cytochrome b_5 the buffer was further augmented with 1.0% cholate and 0.5 M KCl (about 400 ml). For further purification or storage at -20°C, the various peak fractions were pooled and reduced in volume by ultrafiltration to 10-15 ml.

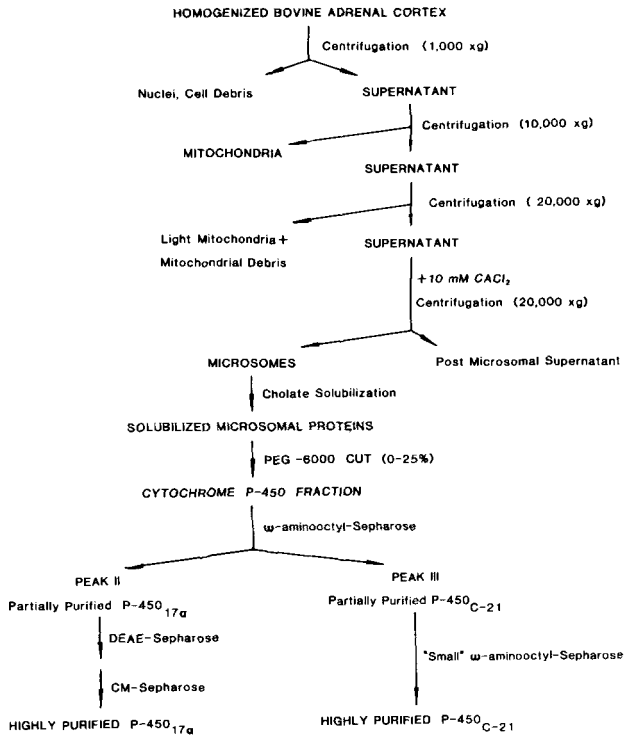
Purification of $P\text{-}450_{17\alpha}$ - After dialysis (2 x) against 1 L of 10 mM K-phosphate, pH 7.6, containing 20% glycerol, 0.2% Emulgen 913, 0.3% cholate, 100 µM EDTA and 100 µM dithiothreitol, the concentrated, partially purified $P\text{-}450_{17\alpha}$ preparation was applied to a column of DEAE-Sepharose (1.5 cm x 29 cm) equilibrated with the same buffer and gradually eluted with the same buffer. The peak fractions were pooled and concentrated by ultrafiltration (Amicon PM-30 filter) to 5 ml. This material was either stored at -20°C or dialyzed against 1 L of K-phosphate buffer, 10 mM, pH 6.9, containing 20% glycerol, 0.2% Emulgen 913, 100 µM EDTA and 100 µM dithiothreitol, and applied to a column of CM-Sepharose (1.5 cm x 6.0 cm) equilibrated with the same buffer. After application the column was first washed with 15 ml of equilibration buffer which did not move the P-450 band and then with 80 ml of the same buffer but containing 0.3% cholate. The P-450 was finally eluted in a highly concentrated band (3-4 ml) by raising the concentration of this buffer to 100 mM. During the last two chromatographic steps this protein was gradually converted to the low-spin form. This highly purified preparation exhibited a specific content of 20 nmol P-450/mg protein and was used for amino acid analysis, SDS-PAGE, and other analytical tests.

Purification of $P\text{-}450_{C\text{-}21}$ - The concentrated fractions containing P-450 were dialyzed against 1 L of 50 mM K-phosphate, pH 7.0, containing 20% glycerol, 100 µM EDTA, 100 µM dithiothreitol and 0.3% cholate and then applied to a smaller column of ω-aminooctyl-Sepharose (1.5 cm x 10 cm) equilibrated with the same buffer. This column was eluted with a linear gradient, 0 - 0.2% Emulgen 913 in the equilibration buffer, 200 ml, followed by 400 ml of this buffer containing 0.2% Emulgen 913. Fractions containing P-450 were pooled and concentrated by ultrafiltration. $P\text{-}450_{C\text{-}21}$ so purified showed a specific content of 19 nmol P-450/mg protein and could be stored at -20°C. Again, this protein was at this point predominantly in the low-spin form. This preparation was used for amino acid analysis, SDS-PAGE, and other analytical tests.

Affinity Chromatography on Adrenodoxin-Sepharose - Adrenodoxin-Sepharose 4B was prepared as described previously (10) using adrenodoxin with $A_{280}/A_{414} = 1.2$. A column of Adrenodoxin-Sepharose (0.9 x 12 cm) was equilibrated with 20 mM K-phosphate, pH 7.3, 100 µM EDTA, 100 µM dithiothreitol, and 10% glycerol. $P\text{-}450_{SCC}$ of $A_{280}/A_{393} = 1.6$ and $P\text{-}450_{17\alpha}$, eluted from the first ω-aminooctyl-Sepharose column, were mixed and applied in a volume not exceeding 2 ml. The column was washed with 50 ml of the same buffer. $P\text{-}450_{17\alpha}$ was then eluted sharply with 70 ml of 100 mM K-phosphate, pH 7.3, 100 µM EDTA, 100 µM dithiothreitol, and 10% glycerol containing 100 mM KCl. $P\text{-}450_{SCC}$ was finally stripped off with 130 ml of the 100 mM K-phosphate buffer containing 500 mM KCl.

Detergent Removal - Detergents were removed from P-450 by column chromatography on CM-Sepharose or hydroxylapatite-silica gel (Sigma). Aliquots of P-450 were dialyzed against 10 mM K-phosphate buffer, pH 7.0, containing 20% glycerol, 100 µM EDTA and 100 µM DTT. Following dialysis, P-450 was applied to a small CM-Sepharose (0.9 cm x 10.0 cm) or hydroxylapatite-silica gel (0.9 cm x 10.0 cm) column. The column was then washed with the same buffer (75 - 100 ml) to remove Emulgen 913. Either column could be eluted sharply with 300 mM K-phosphate, pH 7.2, containing 20% glycerol, 100 µM EDTA and 100 µM DTT. For the reconstitution assays 0.004% Emulgen 913 was included in this elution buffer. Because P-450 is prone to convert to inactive P-420 forms in the absence of Emulgen 913, the detergent free preparation should be used immediately.

An overview of the purification procedures of the bovine adrenocortical microsomal P-450 hemeproteins is given by the flow sheet in SCHEME I. The essential steps in the first part of this scheme are careful preparation of microsomes by differential centrifugation, including an additional spin of the postmitochondrial supernatant at 20,000 x g for 15 min. to remove residual mitochondria and mitochondrial debris. The purified microsomes were then collected either by ultracentrifugation or by low speed centrifugation after precipitation with $CaCl_2$ (32) and tested for mitochondrial contamination by comparing several characteristic enzymatic activities. As summarized in Table I, the first pellet



SCHEME I. Flow Sheet Showing The Sequence of Steps Used in the Purification of the BAC Microsomal Hemeproteins, $P\text{-}450_{17\alpha}$ and $P\text{-}450_{C\text{-}21}$.

obtained at 20,000 x g from the post-mitochondrial supernatant, prior to ultracentrifugation or $CaCl_2$ treatment, was found to contain mitochondrial material (16-30%) but no contamination was detected in the purified microsomal fraction. Succinate dehydrogenase, cytochrome c oxidase, and cholesterol desmolase, which are considered to be markers for mitochon-

TABLE I

SELECTED ENZYME ACTIVITIES

Subcellular Fraction	Succinate Dehydrogenase	Cytochrome C Oxidase	Cholesterol Desmolase	Steroid 17α-Hydroxylase	Steroid C-21-Hydroxylase
	nmoles / min / mg protein				
Mitochondria	38.8	1.554	7.6	0.0	0.0
20,000 x g Pellet	15.5 (40%)	517 (38%)	N.D.	N.D.	N.D.
Microsomes	0.0	47 (3%)	0.22 (3%)	0.25[1]	2.60[2]
Microsomes + Inhibitor of 17α-Hydroxylation	0.0	45 (3%)	0.20 (3%)	0.0	2.58[2]
Microsomes + AdX + AdX Reductase	N.D.	N.D.	4.8 (63%)	N.D.	N.D.

[1] 17α-Hydroxylation of Progesterone

[2] 21-Hydroxylation of 17α-Hydroxyprogesterone

dria, show levels at or below 3% and the microsomal activities of 17α- and C-21 hydroxylation of steroids are present as expected. While C-21 hydroxylase activity was measured with progesterone as substrate, forming 1 nmol product/mg protein/min, and with 17α-hydroxyprogesterone, giving 2.6 nmol product/mg protein/min, 17α-hydroxylation was rather low (0.25 nmol product/mg protein/min) and could be completely inhibited by addition of SU-10603, a specific inhibitor for $P\text{-}450_{17\alpha}$[4]. Most importantly, while the assay for cholesterol desmolase[3] activity (10) using [1,2-^{3}H]-cholesterol yielded equally low values for the final microsomal preparation, after addition of the typical mitochondrial proteins, adrenodoxin and adrenodoxin reductase, much higher levels of desmolase activity were found.

The microsomal preparation was then solubilized with sodium cholate. The selection of this detergent was based on the extent of solubilization of P-450 hemeproteins (and of the NADPH-cytochrome c (P-450) reductase) with ten different detergents. Triton N-101 and Emulgen 913 were the most efficient of these detergents for the solubilization of P-450 (100%) and the reductase (∿70%) but cholate produced a less complex mixture of proteins while still solubilizing the major portion of P-450 (∿75%) which could be readily fractionated with PEG-6000 to yield 69-70% of a crude but active P-450 preparation (0-25% cut). Fractionation with ammonium sulfate, on the other hand, caused low yields and formation of the inactive P-420 form.

RESULTS AND DISCUSSION

Purification of P-450 Hemeproteins—The crude P-450 preparation contained 69% of the CO complex-forming material present in the microsomes as well as other proteins of interest to this investigation, such as the NADPH-cytochrome *c* (P-450) reductase and cytochrome b_5 and its reductase. These proteins were subsequently resolved into four distinct peaks (I–IV) by chromatography on ω-aminooctyl-Sepharose (1.5 × 20 cm), using a stepwise elution of the components by addition of detergents and salt to the equilibration buffer (Fig. 1) as described under "Experimental Procedures." The elution program was designed to take advantage of the unusual properties of this support material which combines general hydrophobic interaction, some affinity interactions, and anion exchange characteristics. The first fraction (peak I, 0–300 ml) contained residual hemoglobin and NADPH-cytochrome *c* (P-450) reductase which were not retained at all. Barely resolved from this fraction followed within the next 60 ml, a small amount of P-450, as yet unidentified, which accounts for 10% or less of the total P-450 (peak IA). Continued washing with 30–40 ml brought the Soret absorption to well below 0.01 *A*. Upon addition of 0.2% Emulgen 913 to the buffer, P-$450_{17\alpha}$ started to elute sharply (peak II, 200 ml) followed and partially overlapped by NADH-cytochrome b_5 reductase (peak IIA, 100 ml). After these components P-$450_{C\text{-}21}$ eluted very slowly and in rather dilute form (750 ml) but as a well resolved peak (peak III). Addition of salt was able to sharpen the P-$450_{C\text{-}21}$ peak to enhance the yield of this hemeprotein considerably but at the expense of purity due primarily to contamination by cytochrome b_5. Finally, cytochrome b_5 can be eluted as a sharp peak (IV) by addition of 1.0% cholate and 0.5 M KCl to the preceding buffer as shown in Fig. 1. However, better yields are obtained by gradient elution chromatography, from 0.3% cholate, 0 mM KCl to 1.0% cholate and 0.5 M KCl. This column chromatography step offers a broad spectrum of partially purified hemeproteins and reductases all of which are sufficiently resolved for further, individual purification.

The pooled, dialyzed, and concentrated fractions constituting peaks II (P-$450_{17\alpha}$) and III (P-$450_{C\text{-}21}$) were then applied to separate columns and purified to homogeneity. P-$450_{17\alpha}$, which is relatively hydrophilic yielded to ion exchange chromatography on DEAE-Sepharose followed by CM-Sepharose (Fig. 2), while P-$450_{C\text{-}21}$ was most efficiently purified by repeated chromatography on ω-aminooctyl-Sepharose using very small resin columns and concentrated P-450 solutions to minimize losses. The resulting purified preparations were essentially free of P-420 and had specific P-450 contents of 20 and 19 nmol of P-450/mg of protein, respectively. By this procedure P-$450_{17\alpha}$ and P-$450_{C\text{-}21}$ are afforded in yields, up to 16 and 3%, respectively, from cholate-solubilized microsomes, based on the assumption that these two hemeproteins account for the entire CO-binding capability of the microsomes. But since additional P-450 hemeproteins have been noted, especially in peak IA of the ω-aminooctyl-Sepharose column, these values could be corrected upward by 10%.

Our highly purified P-$450_{17\alpha}$ and P-$450_{C\text{-}21}$ preparations showed single sharp bands in SDS-PAGE (Fig. 3). No trace contaminants were detected. In comparison to the mobilities of marker proteins separated under identical conditions, P-$450_{17\alpha}$ traveled with an apparent M_r = 50,000 while P-$450_{C\text{-}21}$ appeared to be slightly larger, close to 52,000. It is important to note that under these conditions P-450_{SCC} shows a mobility comparable to that of P-$450_{17\alpha}$ although its polypeptide weight appears to be significantly smaller (10).

Spectral Properties of Purified P-450 Hemeproteins—The spectral characteristics of the purified P-450 hemeproteins are illustrated in Fig. 4 (*A* and *B*). As shown in Fig. 4*A*, ferric P-$450_{17\alpha}$ has its Soret peak at 417 nm and a shoulder at 644 nm indicative of residual high spin; the α and β peaks are at 568 and 534 nm, respectively. Upon reduction the Soret peak shifts to 414 nm and a peak at 546 nm appears replacing the

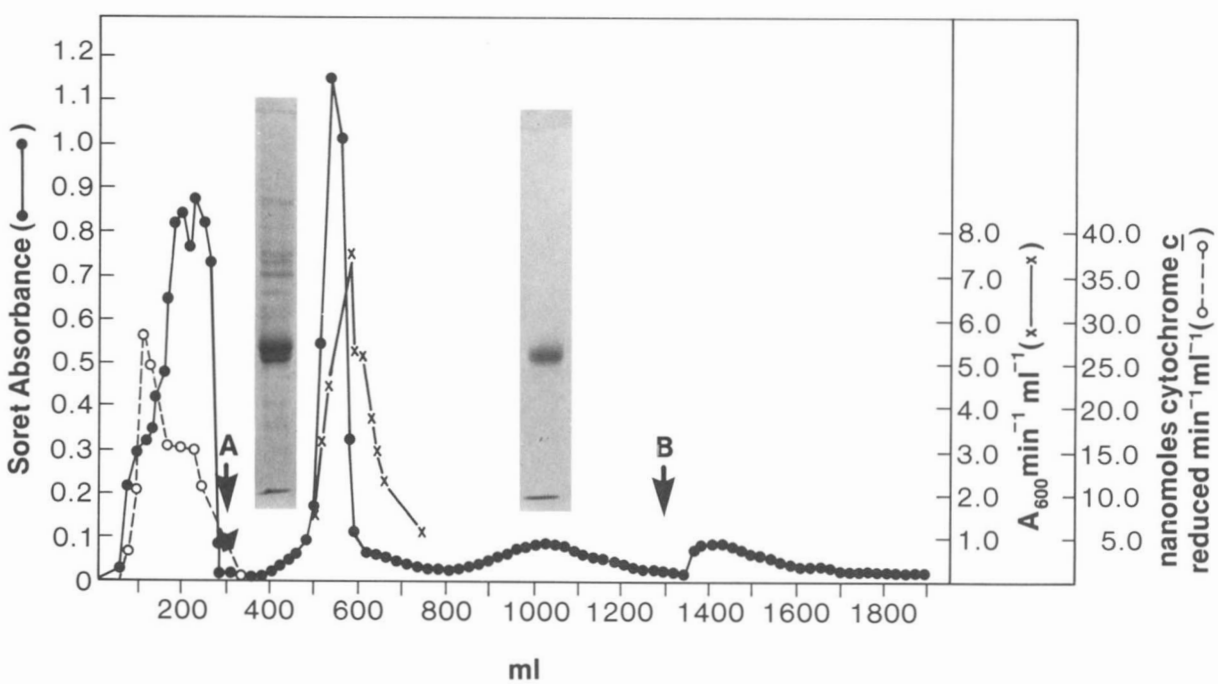


FIG. 1. **First ω-aminooctyl-Sepharose chromatography—resolution of crude bovine adrenocortical microsomal P-450 hemeprotein mixture.** Following cholate solubilization and PEG-6000 fractionation, ~1400 nmol of P-450 in 50 mM K-phosphate buffer, pH 6.9, containing 20% glycerol, 100 μM EDTA, 100 μM dithiothreitol, and 0.3% cholate were applied to an ω-aminooctyl-Sepharose column (1.5 × 20.0 cm) equilibrated with the same buffer. The column was eluted with 1 liter of buffer containing 0.2% Emulgen 913 (*arrow A*). The buffer was then further augmented with 1.0% cholate and 0.5 M KCl (*arrow B*). The *insets* are SDS-PAGE tests of partially purified cytochrome P-$450_{17\alpha}$ (*left*) and P-$450_{C\text{-}21}$ (*right*).

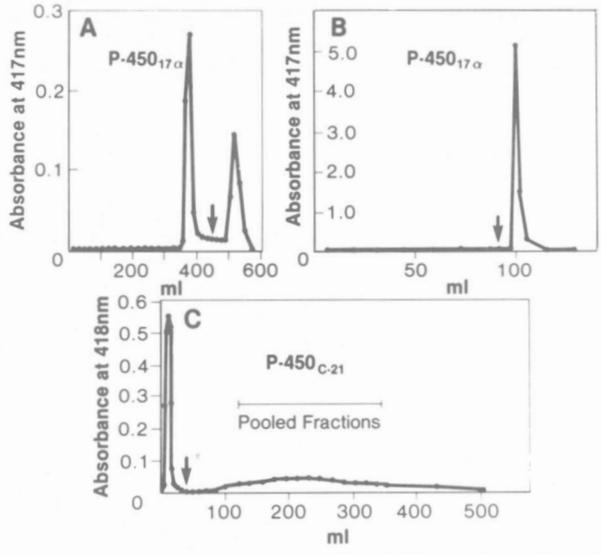


FIG. 2. **Final purification steps for P-450$_{17\alpha}$ (*A* + *B*) and P-450$_{C\text{-}21}$ (*C*).** *A*, DEAE-Sepharose chromatography of P-450$_{17\alpha}$. The column (1.5 × 29 cm) was equilibrated with 10 mM K-phosphate, pH 7.6, 20% glycerol, 0.2% Emulgen 913, 0.3% cholate, 100 μM EDTA, and 100 μM dithiothreitol. The fractions corresponding to the P-450$_{17\alpha}$ peak in Fig. 1 were dialyzed against this buffer, concentrated to 5 ml, applied to the column, and eluted. These steps were carried out with the same buffer and led to the recovery of P-450$_{17\alpha}$. The second peak emerged only after application of buffer containing 500 mM KCl and contained only small amounts of P-450. *B*, CM-Sepharose chromatography of P-450$_{17\alpha}$. The peak fractions of the DEAE-Sepharose chromatography were again concentrated by ultrafiltration to ~5 ml and applied to the column (1.5 × 6 cm) which had been equilibrated with 10 mM K-phosphate, pH 6.9, 20% glycerol, 0.2% Emulgen 913, 100 μM EDTA, and 100 μM dithiothreitol. After sample application the column was washed with 15 ml of this buffer, and then with 80 ml of buffer to which 0.3% cholate were added. Sharp elution of highly purified P-450$_{17\alpha}$ in 3–4 ml was then accomplished by raising the concentration of the last buffer to 100 mM. *C*, second ω-aminooctyl-Sepharose chromatography of P-450$_{C\text{-}21}$. After dialysis against 50 mM K-phosphate, pH 7.0, 20% glycerol, 100 μM EDTA, 100 μM dithiothreitol, and 0.3% cholate the P-450$_{C\text{-}21}$-containing fractions of the first ω-aminooctyl-Sepharose column were concentrated to about 5 ml and applied to a second column of this resin (1.5 × 10 cm), equilibrated with this buffer. The column was then eluted with a 200-ml linear gradient, from 0–0.2% Emulgen 913 in the same buffer, followed by 400 ml of the 0.2% Emulgen 913-containing buffer. The first peak contains a mixture of contaminants together with a small amount of P-450 while the second peak contains homogeneous P-450$_{C\text{-}21}$.

α and β bands. The reduced CO complex exhibits a Soret maximum at 449 nm and a maximum at 548 nm. It appears that P-450$_{17\alpha}$ may be present in the membrane in the high spin state but that it loses its high spin character progressively during the isolation and purification process although a trace of high spin persists. It remains unresolved whether the enzyme, once converted to the low spin form, resists the reintroduction of the substrate, even by the mixed micelle procedure used for P-450$_{SCC}$ (10), or whether the enzyme accepts the substrate without change in spin state. This effect could be due to the very high affinity of Emulgen 913 for P-450$_{17\alpha}$, evidenced by its pronounced influence on the position of the Soret maximum which is shifted from 393 to 396 nm, and on the apparent spin state. There is some evidence that a low spin complex is formed first under the influence of Emulgen 913 which, in turn, facilitates the release of substrate at a later point. Ferric P-450$_{C\text{-}21}$ (Fig. 4*B*), in the low spin state, has a Soret maximum at 418 nm, but despite repeated efforts we were unable to completely remove a shoulder at 645 nm suggesting a trace of residual high spin. The absorption maxima of the α and β bands of this ferric hemeprotein are at 570 and 537 nm, respectively. Addition of the substrate, 17α-hydroxyprogesterone, shifts the Soret band to 396 nm. No α and β bands are noticed but the band with maximum at 645 nm is more prominent. Upon reduction with sodium dithionate the Soret maximum shifts to 414 nm and a single broad band with maximum at 549 nm replaces the peaks in the α-β region.

Since P-450$_{17\alpha}$ exhibits a unique temperature-dependent high to low spin transition when the temperature is raised from 7 to 15 °C, the spectral characteristics of this hemeprotein were also studied in this temperature range. As illustrated in Fig. 5, the mixed spin state at this temperature reveals the presence of two Soret peaks, at 396 and 417 nm, respectively. A temperature increase to 15 °C converts the protein to the low spin form, showing an isosbestic point at 406 nm as evidence that only one P-450 hemeprotein is involved. The conversion to the low spin state is rapid, and almost complete within 15 min (*inset A*) while reversion occurs only slowly and takes about 4 h to reach the mixed spin state observed at 7 °C. Since repeated spectral conversions to the high and low spin forms can be carried out with the same aliquot of enzyme solution, producing neither qualitative nor quantitative changes in the behavior of the enzyme, we conclude that the substrate must still be present even in the apparently low spin

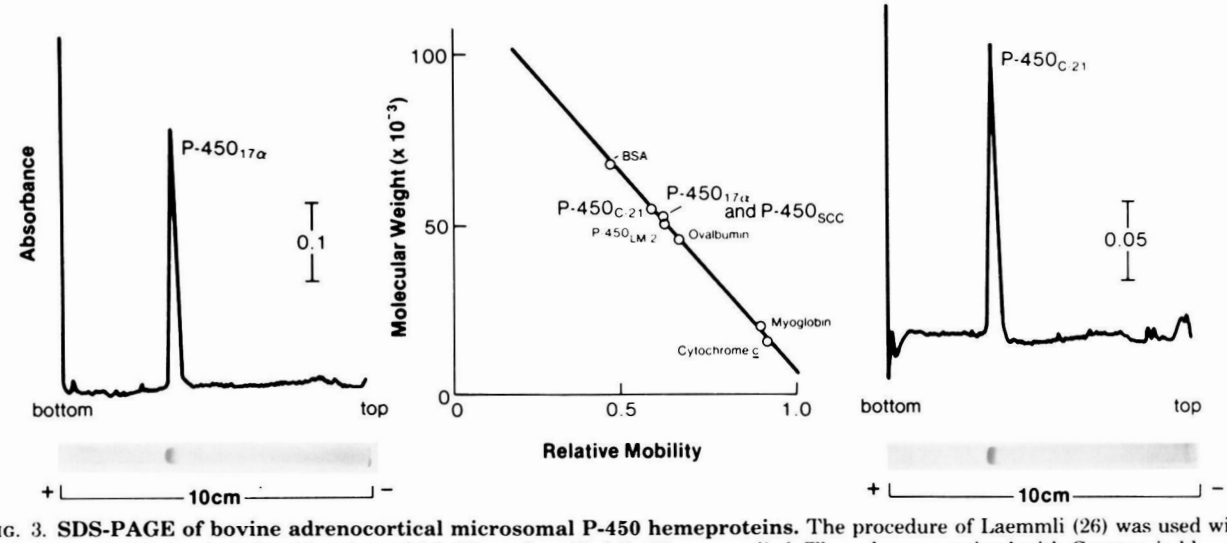


FIG. 3. **SDS-PAGE of bovine adrenocortical microsomal P-450 hemeproteins.** The procedure of Laemmli (26) was used with 7.5% cross-linked cylindrical gels (0.5 × 10 cm) to which 10 μg of purified P-450 was applied. The gels were stained with Coomassie blue and the destained gels were scanned at 600 nm. *BSA*, bovine serum albumin.

form with Soret maximum at 417 nm. However, this complete reversibility applies only within the time and temperature range stated. Temperature increases to above 15 °C for several hours lead to irreversible changes and progressive loss of substrate. This extensive thermotropic spin state transition is, to our knowledge, unique for P-450$_{17\alpha}$ and is not shared by P-450$_{SCC}$ (data not shown). It also goes into the opposite direction to the spin state transitions observed with P-450$_{CAM}$, P-450$_{LM-2}$, or P-450$_{C-21}$.

Because pure P-450$_{17\alpha}$ has never been obtained in a predominantly high spin form, the trace for the substrate complex of this hemeprotein was omitted from Fig. 4*A*, although it has the same Soret maximum and roughly the same relationship to the Soret band of the ferric low spin form as that shown for P-450$_{C-21}$ (Fig. 4*B*).

Table II lists some of the extinction coefficients for highly purified P-450$_{17\alpha}$ and P-450$_{C-21}$. Except for gradual conversion to low spin upon storage, both pure P-450$_{17\alpha}$ and pure P-450$_{C-21}$ preparations appear to be spectrally stable at −20 °C for several months and show no significant P-420 formation in the presence of small amounts of Emulgen 913. Complete removal of Emulgen 913 by chromatography on hydroxylapatite causes progressive formation of P-420. Thus, a proper correlation between Soret and protein absorptions as a measure of purity cannot be given and a ratio of A_{280}/A_{396} is not applicable to the bovine adrenocortical microsomal proteins during purification, although their molar extinction values in the UV region (278–280 nm) are not much different from those of P-450$_{SCC}$ and P-450$_{11\beta}$.

Enzymatic Properties of Purified P-450 Hemeproteins—In reconstitution experiments with highly purified P-450$_{C-21}$ rabbit liver microsomal NADPH-cytochrome *c* (P-450) reductase was quite effective. When supplemented with total phospholipid extract, [1,2-^{3}H]17α-hydroxyprogesterone and NADPH, a specific activity of 45.2 nmol of 17α-hydroxy-11-deoxycorticosterone/mg of P-450$_{C-21}$/min was measured, and in the case of [4-^{14}C]progesterone, a value of 14.4 nmol of 11-deoxycorticosterone/mg of P-450$_{C-21}$/min was obtained as described under "Experimental Procedures." Fig. 6 demonstrates that in the presence of the rabbit liver microsomal reductase, the P-450$_{C-21}$-CO complex was better than 95% reduced by NADPH within 8 min, as confirmed by addition of a few grains of dithionate to the reaction mixture after an apparent plateau had been reached in the Soret absorption at 450 nm. On the other hand, reconstitution experiments with P-450$_{17\alpha}$, as shown in Fig. 7, had to be carried out with partially purified hemeprotein because of our inability to reform the substrate complex from highly purified P-450$_{17\alpha}$ which had lost almost all of its substrate. Thus, it is not surprising that small amounts of P-450$_{C-21}$ were still present in this preparation as evidenced by formation of 1.44 nmol of radiolabeled 11-deoxycorticosterone/mg of protein/h and 0.5 nmol of radiolabeled 11-deoxy-17α-hydroxycorticosterone/mg of protein/h in addition to the formation of 5.04 nmol of radiolabeled 17α-hydroxyprogesterone/mg of protein/h. Although this activity is unexpectedly low, it should be stressed that no measurable 17α-hydroxylase activity was detected when P-450$_{SCC}$ was substituted for P-450$_{17\alpha}$ in the assay. On the other hand, reconstitution of P-450$_{17\alpha}$ with bovine adrenocortical mitochondrial adrenodoxin and adrenodoxin reductase produced cholesterol desmolase activity of 11.68 nmol of product/mg of protein/min which is about two-thirds the activity reported for P-450$_{SCC}$ (33).

Amino Acid Compositions of P-450 Hemeproteins—The amino acid compositions of the two major bovine adrenocortical microsomal P-450 hemeproteins are reported in Table III. The compositions were calculated on the basis of 9 and 10 Met residues for P-450$_{C-21}$ and P-450$_{17\alpha}$, respectively, as compared to 11 residues of Met in both P-450$_{SCC}$ and P-450$_{11\beta}$. The choice of these dividing factors is further supported by the fact that they cause a coincidence of 13 residues of His and 51 residues of Glx in both microsomal proteins and the occurrence of integral values for Cys/2 and Trp in both. The resulting formula weights for the respective polypeptide

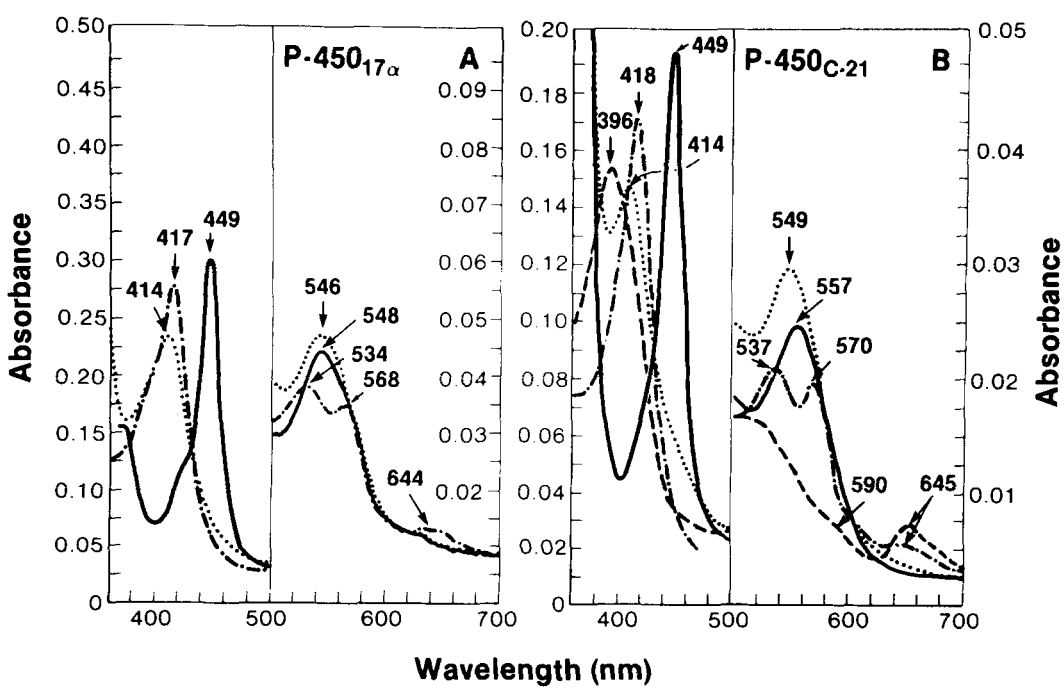


FIG. 4. **Absorption spectra of bovine adrenocortical microsomal P-450 hemeproteins.** *A*, P-450$_{17\alpha}$. The spectra were recorded in 50 mM K-phosphate, pH 7.0, 20% glycerol, 100 μM EDTA, 100 μM dithiothreitol, and 0.3% cholate at 20 °C. *B*, P-450$_{C-21}$. Buffer and temperature were the same as for *A* except that the buffer also contained 0.04% Emulgen 913. The substrate complex was formed by addition of 10 μl of 20 mM 17α-hydroxyprogesterone in acetone to the ferric P-450.

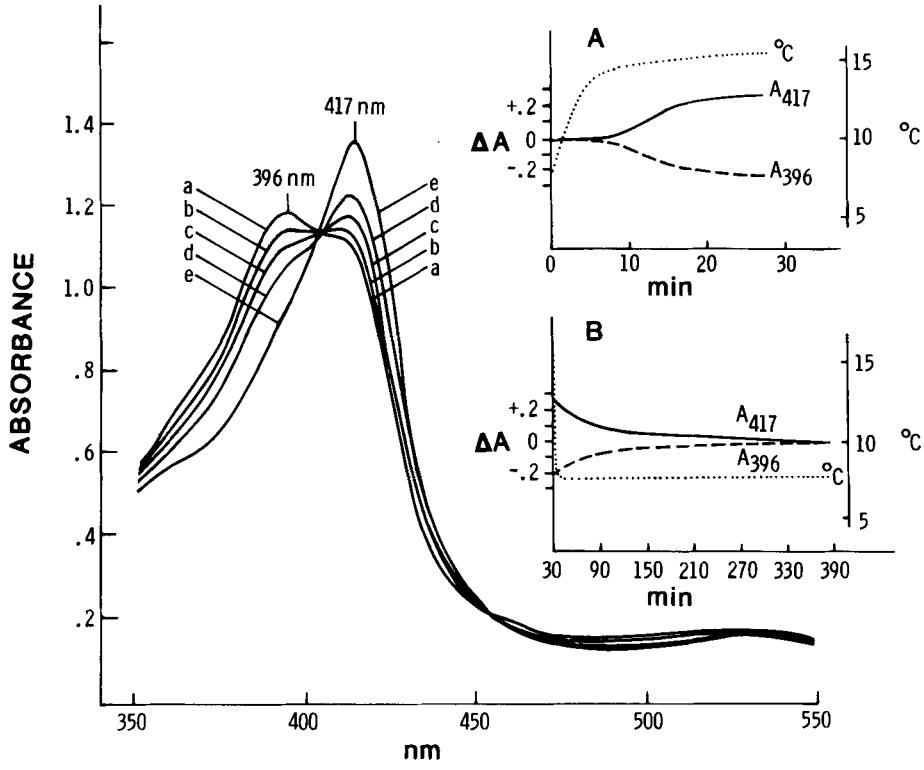


FIG. 5. **Temperature-induced spin state transition of P-450$_{17\alpha}$.** The sample cuvette contained purified P-450$_{17\alpha}$, 100 mM K-phosphate, pH 7.0, 20% glycerol, 100 μM EDTA, 100 μM dithiothreitol, 0.3% cholate, and 0.2% Emulgen 913 while the same buffer was in the reference cuvette, both equilibrated at 7.4 °C by circulating water. *Curves a–e* refer to increased temperatures after the water bath was turned off, as follows: *a*, 7.4 °C; *b*, 14.3 °C; *c*, 14.5 °C; *d*, 14.7 °C; *e*, 15.4 °C. *Inset A* represents the absorbance changes at 417 and 396 nm as a function of time as the temperature is allowed to rise from 7.4 to 15.4 °C. When the water bath was turned on, the spectra slowly reverted back to the initial form, as shown in *inset B*.

TABLE II

Optical properties of adrenocortical microsomal hemeproteins (25 °C)

	P-450$_{17\alpha}$		P-450$_{C\text{-}21}$	
	γ	ε	γ	ε
	nm	*mM*	*nm*	mM
Oxidized form	417	98	418	118
	534	12.3	537	13.1
	568	11.2	570	13.8
Oxidized substrate complex	396	N.D.	396	97.5
	645	N.D.	644	4.7
Reduced form	414	82.5	414	84.4
	546	14.0	549	16.4
Reduced CO complex	449	94.7	449	113.8
	548	15.8	557	15.6
Reduced CO difference	449	91	449	91

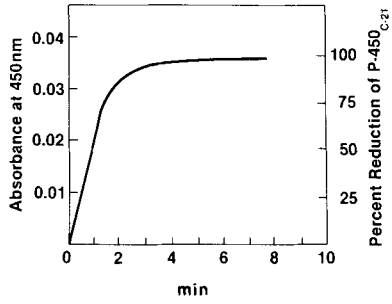


FIG. 6. **Reduction of bovine adrenocortical microsomal P-450$_{C\text{-}21}$-CO complex.** After a base-line of equal light absorbance was recorded with 1-ml aliquots containing 0.385 nmol of P-450$_{C\text{-}21}$ and 0.45 unit of rabbit liver microsomal reductase in 50 mM K-phosphate, pH 7.2, 20% glycerol, 100 μM EDTA, 100 μM dithiothreitol, 0.3% cholate, and 0.3% Emulgen 913, and an O_2-scavenging system, consisting of 20 mg of glucose, 70 units of glucose oxidase, and 1400 units of catalase, the stoppered sample cuvette was equilibrated with CO by bubbling a gentle stream of CO through the solution for 5 min at room temperature. NADPH (10 μl of a solution containing 1 mg in 250 μl of buffer) was used to initiate the reaction and the change in absorbance at 450 nm was monitored for 8–10 min.

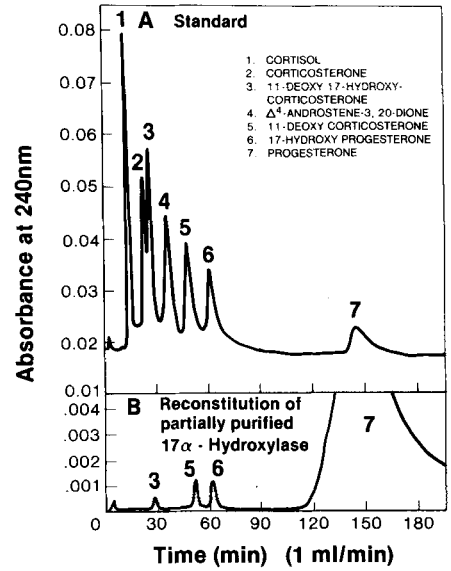


FIG. 7. **Resolution of steroids by HPLC.** *A*, standard of seven steroids. A methanolic solution containing 5 nmol of each of seven common steroids was applied to a Waters μBondapak C_{18} column equilibrated with methanol/water, 50:50 (30). The chromatography was performed isocratically at room termperature with a Waters HPLC system, using 2500 p.s.i. to give a flow rate of 1 ml/min, and a chart speed of 5 min/inch. B, resolution of reaction products from reconstitution of 17α-hydroxylase with partially purified P-450$_{17\alpha}$. A 50-μl aliquot of a solution of steroids in methylene chloride, resulting from extraction of the reconstitution mixture, were applied and chromatographed under the conditions given for *A*.

chains of 46,720 for P-450$_{17\alpha}$ and 48,870 for P-450$_{C\text{-}21}$ are in reasonable agreement with the mobilities of these hemeproteins in SDS-PAGE which correspond to apparent M_r = 50,000 and 52,000, respectively. The difference between the polypeptide weight and the SDS-PAGE values is readily accounted for by the weight for the single heme prosthetic group and a carbohydrate moiety of 6–8 saccharide units

TABLE III

Amino acid composition of the major P-450 hemeproteins of bovine adrenocortical microsomes

After careful removal of heme and detergents, the apoproteins were hydrolyzed with 5.7 N HCl in vacuum-sealed tubes at 110 °C for 24, 48, and 72 h. The values listed represent the average of duplicate analyses, corrected according to the projection of the time course of hydrolysis, and calculated for integral values of 9 and 10 residues of Met in P-450$_{C-21}$ and P-450$_{17\alpha}$, respectively, and of 13 residues of His in both proteins.

	P-450$_{17\alpha}$	P-450$_{C-21}$
Cyso$_3$H	5	7
Asx	36	28
Thr	18	19
Ser	20	24
Glx	51	51
Pro	26	30
Gly	25	33
Ala	26	30
Val	27	37
Met	10	9
Ile	25	16
Leu	51	63
Tyr	17	9
Phe	26	17
His	13	13
Lys	29	19
Arg	25	29
Trp	3	5
Total	433	439
Protein weight	46,720	48,870
SDS-PAGE	50,000	52,000

which is as yet undetermined. The compositions of both proteins show a preponderance of hydrophobic and dicarboxylic amino acid residues. P-450$_{C-21}$ seems to be the most hydrophobic P-450 hemeprotein so far analyzed. The amino acid composition of P-450$_{17\alpha}$ is very similar to that of P-450$_{SCC}$, especially with regard to the contents of Asx, Thr, Ser, Glx, Pro, His, and Lys (10), with the other residue numbers being noticeably larger presumably due to a longer polypeptide chain of P-450$_{17\alpha}$. Except for a few comparable values our amino acid composition disagrees with the composition listed by Hiwatashi and Ichikawa (18) for their P-450$_{C-21}$ preparation which rather resembles our P-450$_{17\alpha}$. In fact, this resemblance also applies to the elution characteristics, in that their P-450$_{C-21}$ elutes earlier and sharper from the ω-aminooctyl-Sepharose column than that reported by Kominani *et al.* (16). This behavior is again rather comparable to that of our P-450$_{17\alpha}$ preparation.

Our amino acid composition of P-450$_{C-21}$ is somewhat reminiscent of that of P-450$_{11\beta}$,[3] both in its pronounced hydrophobic character and in the content of certain amino acid residues despite the relatively large differences in size (45,000 *versus* 52,000).

Comparative Binding Studies with P-450$_{17\alpha}$ and P-450$_{SCC}$—Since P-450$_{17\alpha}$ can also strongly interact with adrenodoxin and adrenodoxin reductase, we investigated its affinity of binding for adrenodoxin by chromatography on adrenodoxin-Sepharose (Fig. 8). In this experiment it is clearly demonstrated that P-450$_{17\alpha}$ binds to adrenodoxin-Sepharose tightly at low ionic strength. But by gradually increasing the ionic strength of the elution buffer, P-450$_{17\alpha}$ can be eluted sharply at an intermediate ionic strength so that it emerges from the column completely resolved from P-450$_{SCC}$ which requires a much higher ionic strength for elution from this column. We conclude that these two hemeproteins are readily distinguishable because P-450$_{SCC}$ has a significantly higher affinity for adrenodoxin, at least at moderate to high ionic strengths.

[3] K. M. Dus, unpublished results.

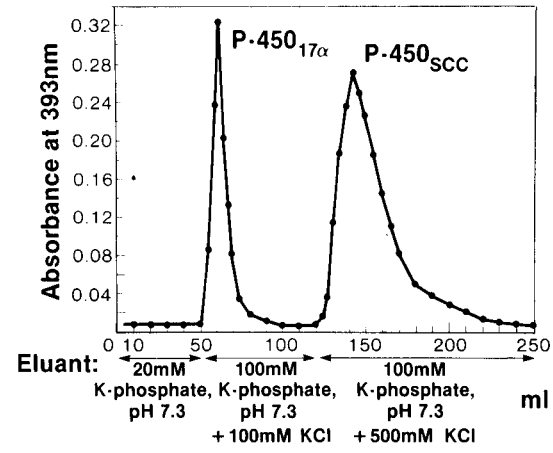


FIG. 8. **Affinity chromatography on adrenodoxin-Sepharose.** Partially purified P-450$_{17\alpha}$ and P-450$_{SCC}$, 50–60 nmol each in a total volume of 2 ml, were applied to a column of adrenodoxin-Sepharose (0.9 × 12 cm) equilibrated with 20 mM K-phosphate, pH 7.3, 100 μM EDTA, 100 μM dithiothreitol, and 10% glycerol. The column was developed by stepwise elution as indicated and the effluent was monitored for Soret absorption at 393 nm.

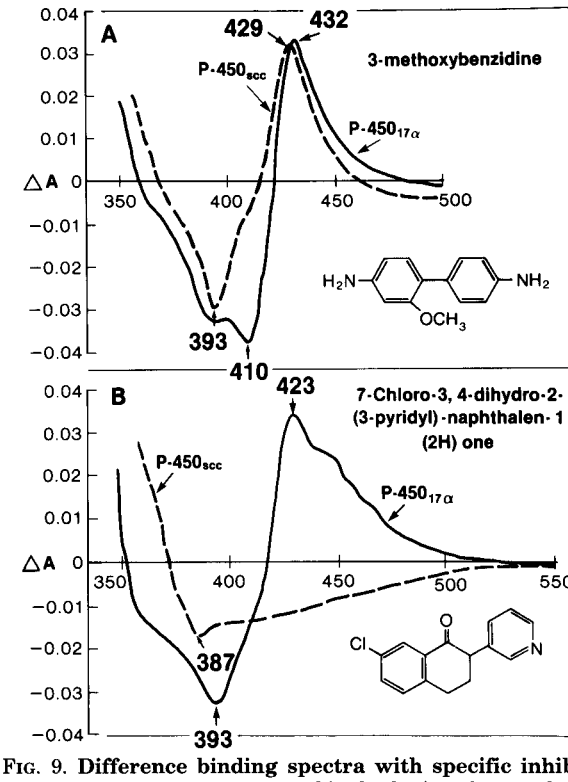


FIG. 9. **Difference binding spectra with specific inhibitors.** P-450$_{17\alpha}$ and P-450$_{SCC}$ were prepared in the ferric, substrate-free form by metabolic conversion of the substrate and subsequent chromatography on adrenodoxin-Sepharose as described previously (10). After a base-line of equal light absorbance was recorded with 1 ml of a 2.5 μM P-450 solution in 50 mM K-phosphate, pH 7.3, 10% glycerol, in each cuvette, aliquots of an ethanolic solution of 3-methoxybenzidine or SU-10603 and an equal volume of ethanol were added to the sample and reference cuvette, respectively.

The close similarity of P-450$_{17\alpha}$ to P-450$_{SCC}$ as well as its own unique character are re-emphasized by the type II difference spectra obtained from binding studies with the specific

inhibitors shown in Fig. 9. 3-Methoxybenzidine was introduced by Duval and Vickery (34) as a highly effective and specific inhibitor for P-450$_{SCC}$. Our spectral titration has indicated apparent K_D values of ~10 and ~19 μM for P-450$_{SCC}$ and P-450$_{17\alpha}$, respectively. As demonstrated in Fig. 9*A*, 3-methoxybenzidine binding to P-450$_{17\alpha}$ causes a minimum at 393 nm which is almost identical to that found with P-450$_{SCC}$, but, in addition, there is a second, deeper minimum at 410 nm. Fig. 9*B*, on the other hand, shows the binding of 7-chloro-3,4-dihydro-2-(3-pyridyl)-naphthalen-1(2H)-one (SU-10603). This reagent, introduced by Neher and Kahnt (35) was shown to be a specific inhibitor of steroid 17α-hydroxylation in human fetal adrenal mitochondria and microsomes.[4] It binds very tightly to P-450$_{17\alpha}$ (apparent K_D ~7 μM) giving rise to a pronounced minimum at 393 nm and a maximum at 423 nm. It also effectively inhibits all 17α-hydroxylase activity in the microsomes (Table I) (36). However, addition of this inhibitor to P-450$_{SCC}$ produces a completely featureless difference spectrum, and no significant inhibition of cholesterol side chain cleavage was observed. From these results we conclude that SU-10603 is indeed a specific inhibitor of bovine adrenocortical microsomal 17α-hydroxylation and that P-450$_{17\alpha}$ which mediates this hydroxylation is a unique microsomal hemeprotein.

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REFERENCES

1. Brooks, R. V. (1979) in *The Adrenal Gland* (James, V. H. T., ed) pp. 67–92, Raven Press, New York
2. Tait, J. F., Tait, S. A. S., and Bell, J. B. G. (1980) in *Eassys in Biochemistry* (Campbell P. N., and Marshal, R. O., eds) Vol. 16, pp. 19–174, Academic Press, New York
3. Haugen, D. A., and Coon, M. J. (1976) *J. Biol. Chem.* **251,** 7929
4. Sato, H., Ashida, N., Suhara, K., Itagaki, E., Takemori, S., and Katagiri, M. (1978) *Arch. Biochem. Biophys.* **190,** 307
5. Suhara, K., Gomi, T., Sato, H., Itagaki, E., Takemori, S., and Katagiri, M. (1978) *Arch. Biochem. Biophys.* **190,** 290
6. Shikita, M., and Hall, P. F. (1973) *J. Biol. Chem.* **248,** 5598–5604
7. Tilley, B. E., Watanuki, M., and Hall, P. F. (1977) *Biochim. Biophys. Acta* **488,** 330
8. Watanuki, M., Tilley, B. E., and Hall, P. F. (1978) *Biochemistry* **17,** 127
9. Seybert, D. W., Lambeth, J. D., and Kamin, H. (1978) *J. Biol. Chem.* **253,** 8355–8358
10. Hanukoglu, I., Spitsberg, V., Bumpus, J. A., Dus, K. M., and Jefcoate, C. R. (1981) *J. Biol. Chem.* **256,** 4321–4328
11. Vinson, G. P., and Whitehouse, B. J. (1970) *Adv. Steroid Biochem. Pharmacol.* **1,** 161–342
12. Rosenthal, O., and Narasimhulu, S. (1969) *Methods Enzymol.* **15,** 636–638
13. Hofmann, F. G. (1969) *Biochim. Biophys. Acta* **37,** 566
14. Young, R. B., Bryson, M. J., and Sweat, M. L. (1965) *Arch. Biochem. Biophys.* **109,** 233
15. Cooper, D. Y., Levin, S. S., Narasimhulu, S., Rosenthal, O., and Estabrook, R. W. 1965) *Science (Wash. D. C.)* **147,** 400
16. Kominami, S., Mori, S., and Takemori, S. (1978) *FEBS Lett.* **89,** 215
17. Kominami, S., Ochi, H., Kobayashi, Y., and Takemori, S. (1980) *J. Biol. Chem.* **255,** 3386–3394
18. Hiwatashi, A., and Ichikawa, Y. (1981) *Biochim. Biophys. Acta* **664,** 33
19. Hiwatashi, A., and Ichikawa, Y. (1979) *Biochim. Biophys. Acta* **580,** 44
20. Swanson, R. A., and Dus, K. M. (1979) *J. Biol. Chem.* **254,** 7238–7246
21. Porra, R. J., and Jones, D. T. G. (1963) *Biochem. J.* **87,** 181
22. Gornall, A. G., Bardawill, C. J., and David M. M. (1949) *J. Biol. Chem.* **177,** 751
23. Dus, K., Katagiri, M., Yu, C.-A., Erbes, D. L., and Gunsalus, I. C. (1970) *Biochem. Biophys. Res. Commun.* **40,** 1423
24. Hirs, C. H. W. (1956) *J. Biol. Chem.* **219,** 611–621
25. Penke, B., Ferenczi, R., and Kovacs, K. (1974) *Anal. Biochem.* **60,** 45
26. Laemmli, U. K. (1970) *Nature (Lond.)* **227,** 680
27. Fairbanks, G., Steck, T. L., and Wallach, D. F. H. (1971) *Biochemistry* **10,** 2606
28. Hiatt, A. J. (1961) *Plant. Physiol.* **36,** 552
29. Wharton, D. C., and Tzagoloff, A. (1967) *Methods Enzymol.* **10,** 245
30. Kramer, R. E., Simpson, E. R., McCarthy, T. L., and Waterman, M. R. (1982) *Endocrinology*, in press
31. Cuatrecasas, P. (1970) *J. Biol. Chem.* **245,** 3059–3065
32. Schenkman, J. B., and Cinti, D. L. (1978) *Methods Enzymol.* **52,** 83
33. Hanukoglu, I., and Jefcoate, C. R. (1980) *J. Chromatogr.* **190,** 256
34. Duval, T. F., and Vickery, K. E. (1980) *Steroids* **36,** 473
35. Neher, R., and Kahnt, F. W. (1965) *Modifiers of Adrenocortical Function*, p. 209, Czechoslovak Medical Press
36. Gower, D. C. (1974) *J. Steriod Biochem.* **5,** 501

[4] J. Ian Mason, private communication.