Preparation of the Heme Protein P-450 from the Adrenal Cortex and Some of Its Properties

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SUMMARY

Details of a method for the preparation and purification of the heme protein P-450 from bovine adrenal glands are described. This method consists of the isolation of mitochondria, their ultrasonic disruption, followed by lyophilization, and extractions with acetone and 1-butanol in several steps, which remove 60 to 70% of the neutral and phospholipids. The heme protein P-450 is isolated by a fractional extraction with solutions of the neutral detergent Triton X-101, followed by ammonium sulfate precipitation and dialysis. The resulting optically clear preparation can be stored in the frozen state for several months without deterioration.

After reconstitution with the specific FAD-containing reductase and the specific iron sulfur protein, both isolated and partially purified from the adrenal cortex, this P-450 preparation is highly active as steroid 11β-hydroxylase (EC 1.14.1.6) with TPNH as electron donor (pH optimum 6.3 to 6.5, turnover number 5 to 10 min⁻¹ at 25°C). The heme protein is also active in steroid 11β-hydroxylation with artificial electron donors in the presence of the specific iron sulfur protein. In addition, the reconstituted enzyme system catalyzes the side chain cleavage of cholesterol.

The optical absorption spectra of the preparation exhibit Soret maxima at 416, 419, and 448 nm for the Fe³⁺, the Fe²⁺ form, and the CO-compound, respectively. The EPR spectrum shows the characteristic features of the low spin (S = 1/2) form of P-450 (Fe²⁺), with principal components 1.91, 2.24, and 2.42 of the g-tensor.

P-450 accounts for 98% or more, and a P-420 type material for the remaining part of the measured protoheme IX content; these are the only heme proteins detectable. Specific concentration of P-450 in the preparation lies between 1.5 and 2.0 nmoles per mg of protein; the preparation contains also neutral and phospholipids (of the order of 10 to 15%), but negligible amounts of cholesterol (<0.1%).

This enzymatically active material is suitable for optical and other spectroscopic studies as well as detailed investigations of the physical characteristics and the chemical nature of the heme protein P-450. A brief comparison is made with the properties of other particulate or solubilized P-450 preparations, obtained by several investigators from liver microsomes and other mammalian sources, and with the soluble P-450 from Pseudomonas putida.

The steroid 11β-hydroxylase system (EC 1.14.1.6) of bovine adrenocortical mitochondria has been resolved (1-3) into (a) a particulate fraction, designated as P-3, which contains the heme protein P-450; (b) a specific flavin adenine dinucleotide-containing protein, Fp; and (c) an iron sulfur protein, ISP. The flavoprotein (mol wt approximately 60,000) (4) is also referred to as TPNH-iron protein-reductase. The ISP (mol wt approximately 14,200) (5) is also referred to in the literature as non-heme iron protein (6) and as adrenodoxin (7, 8). Recombination of these three components in appropriate proportions restores steroid 11β-hydroxylase activity (9, 10).

The P-3 fraction consists presumably of fragments of mitochondrial membranes to which the heme protein P-450 is firmly attached. It is far superior to microsomes and microsomal preparations (11, 12) in many aspects, especially for spectro-photometric studies of P-450. It contains lesser amounts of other contaminating heme proteins, most importantly of cytochrome b₅. Yet this preparation is not sufficiently pure to allow determination of the optical absorption characteristics and detailed physical and chemical measurements on P-450. Such studies are essential, however, for an understanding of the mechanisms of oxygen activation and substrate hydroxylation catalyzed by this heme protein in steroids and other substrates of mixed function oxidase systems.

In the course of our investigations on the reconstituted steroid

1 The abbreviations used are: Fp, FAD-containing TPNH-iron sulfur protein reductase from adrenal cortex; ISP, the specific iron sulfur protein, isolated from adrenal cortex; DOC, deoxycorticosterone.

2 Fp and ISP, both extensively purified, constitute (together with TPNH as the physiological donor) the reducing system for the heme protein P-450 in the adrenocortical steroid 11β-hydroxylase. For the additional specific role of ISP in the hydroxylation reaction see References 32 and 33.
Fractionation of adrenocortical mitochondria

Note that in this preparation about 24% of the protein, 43% of P-450, and a substantial amount of P-420 material were lost during sonication.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>P-450</th>
<th>P-420</th>
<th>Protein</th>
<th>Ratio of P-420: (P-450 + P-420)</th>
<th>Ratio of P-450: P-420 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P-1</td>
<td>3.83</td>
<td>100.0</td>
<td>1.15</td>
<td>3.0</td>
<td>100.0</td>
</tr>
<tr>
<td>S-1</td>
<td>1.80</td>
<td>47.0</td>
<td>0.12</td>
<td>1.65</td>
<td>42.3</td>
</tr>
<tr>
<td>P-2</td>
<td>0.39</td>
<td>10.2</td>
<td>0.08</td>
<td>1.32</td>
<td>33.8</td>
</tr>
<tr>
<td>S-2</td>
<td>0.24</td>
<td>6.2</td>
<td>0.10</td>
<td>0.31</td>
<td>8.0</td>
</tr>
<tr>
<td>P-3</td>
<td>0.01</td>
<td>0.3</td>
<td>0.10</td>
<td>0.73</td>
<td>18.6</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>2.3</td>
<td>0.048</td>
<td>0.084</td>
<td>2.1</td>
</tr>
</tbody>
</table>

* The letters P and S designate the precipitate and the supernatant, respectively, of the centrifugation steps described in Reference 10. The first centrifugation (105,000 × g, 30 min) results in the P-1 preparation, which is used here as starting material for the preparation of the heme protein P-450. P-2 and S-2 are obtained from the supernatant; S-1 is obtained by centrifugation (105,000 × g, 180 min), and the P-3 fraction is obtained from P-2 by sonication in phosphate buffer containing 0.15 mM KCl and subsequent centrifugation. The P-3 fraction was originally employed in the reconstitution of the steroid 11β-hydroxylase (9, 10).

11β-hydroxylase system was found (10) that another particulate fraction obtained from adrenocortical mitochondria, termed P-1', is a better and, in fact, the most suitable source for further purification of P-450 on a preparative scale (cf. Table I). The presence of P-450 in this P-1 preparation has been known (13).

The P-1 preparation contains large amounts of the wanted heme protein and can be stored for extended periods of time without significant loss of either P-450 content or the 11β-hydroxylase activity. This latter fact allows accumulation of sufficient material for an economical and efficient purification of P-450. A somewhat similar preparation has recently been better and, in fact, the most suitable source for further purification of P-450 on a preparative scale (cf. Table I). The presence of P-450 in this P-1 preparation has been known (13).

In this paper we describe details of our preparation procedure for enzymatically active heme protein P-450 from the adrenal cortex and, in addition, include specific information on some of its chemical and physical properties.

**EXPERIMENTAL PROCEDURES**

**Materials**—Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and diithiothreitol (Cleland's reagent) were purchased from Calbiochem, Los Angeles, California, crystalline bovine albumin from Nutritional Biochemicals Corp., Cleveland, Ohio, and the non-ionic surfactant Triton N-101 (registered trademark, nonylphenyl-ether of polyethylene glycol) from Rohm and Haas, Philadelphia, Pa. Deoxycofactorse (DOC, cortexone), TPN+, TPNH, glucose 6-phosphate, and DEAE-cellulose (medium mesh, 0.9 meq per g) were obtained from Sigma, St. Louis, Mo., and corticosterone from the Upjohn Company, Kalamazoo, Mich. All other chemicals were of analytical grade; double distilled water was used throughout.

**Assay Methods**—The concentration of P-450 was determined on the basis of difference spectroscopy of its CO-compound, P-450-CO; the (reduced + CO) minus (reduced) difference spectra were measured in the crude starting material with a Yang Chance type scanning spectrophotometer (16), in all other preparations with a Cary model 14 spectrophotometer, using an optical path length of 1.0 cm. A differential molar absorptivity, Δε420 = 9.1 × 10^4 M^-1 cm^-1 (450 - 490 nm), determined originally by Omura and Sato (13) in a micromolar preparation, was used for quantitation.

A rough estimate of the P-420 content was made on the basis of the absorption data for a specific P-420 preparation obtained by Omura and Sato (17) with an assumed molar absorptivity of P-420-CO, ε420 = 213 × 10^3 M^-1 cm^-1 at 420 nm. A value of ε420 = -4.1 × 10^3 M^-1 cm^-1 was used to correct for the (negative) contribution from P-450 in the difference spectra at this wave length.

This method represents at best a crude estimate, especially in the earlier stages of purification, since several different heme proteins contribute their absorption to the so-called P-450 absorption region in the difference spectra. They not only differ in the spectral characteristics of their corresponding CO-compounds but also in their physical and chemical properties (18, 19); no attempt was made in this study to separate them analytically. The protoheme IX content of the various preparations was determined spectrophotometrically after acidification and extraction into an organic phase or, more frequently, via the formation of the pyridine hemochrome; quantitation in the latter case was based on the differential molar absorptivity Δε555 = 3.24 × 10^4 M^-1 cm^-1 (555 - 575 nm) of the (Fe^3+-pyridine hemochrome) minus (Fe^2+-pyridine hemochrome) difference spectrum (13).

For the flavoprotein (Fp), ε450 = 1.13 × 10^4 M^-1 cm^-1 at 450 nm (20) was used. Quantitation of ISP was based on a molar absorptivity ε450 = 1.0 × 10^3 M^-1 cm^-1 at 415 nm (5, 7). In previous reports from our laboratory (3, 9) a value ε450 = 5.6 × 10^3 (g atom of iron)^-1 cm^-1 at the same wave length was employed, corresponding to the analytical iron content of 2 g atoms of iron per mole of protein.

Protein was determined by a biuret reaction (21) with bovine serum albumin as standard of reference.

**Steroid 11β-Hydroxylase Activity**—The activity of P-450 preparations in steroid hydroxylation was measured in the reconstituted 11β-hydroxylase system as reported previously (9). In a volume of 2.5 to 3.0 ml, the reaction mixture contained (final concentrations in mM): DOG (0.21) as substrate for 11β-hydroxylation, NaCl (58), KCl (44), MgCl2 (1.3), TPN+ (1.0), glycyglycine buffer (11.5, pH 7.4) and, in addition, bovine serum albumin (7.7 mg per ml), and glucose 6-phosphate dehydrogenase (0.2 i.u. per ml). Fp and ISP isolated from adrenal glands and purified as previously reported (2, 22) were added in an approximate ratio of Fp to ISP to P-450 = 1:20-1, unless otherwise stated; this was found to be optimal for 11β hydroxylation (10).

The P-450 preparation to be assayed was added (final concentration ~1 μM) and the hydroxylation reaction started by the addition of the TPNH-generating system at zero time. The

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1 The P-420 does not describe one chemically defined compound but comprises several different heme proteins which form, after reduction, CO-compounds absorbing maximally near 420 nm in the Soret region. While reproducible for a given type of material, the composition may differ widely from one to the other preparation; any comparison which is based on the content of a P-420 type material can therefore only be a rough estimate.
reaction mixture (final pH 7.2, unless otherwise stated) was incubated at 25° for 10 to 15 min with air as the gas phase. The reaction was terminated by adding 20 ml of ice-cold dichloromethane to the reaction mixture.

The rate of formation of corticosterone was determined fluorometrically by means of slight modification (23) of the method of Mattingly (24). Hydroxylase activity of the P-450 preparation is expressed in terms of (nmol of product formed) per (mg of protein × min) or as (nmol of product formed) per (nmol of P-450 × min), the turnover number.

Preparative Procedures

The freeze-dried P-1 preparation from isolated adrenocortical mitochondria is the starting material for the preparation of P-450. Details of the preparation method are described in Steps I to IV. Step V outlines a method for removal of contaminating P-420-type material. Step VI describes procedures developed to obtain additional P-450 of varied qualities.

All operations are carried out at 4°. The amounts of extractants used are expressed as volume per weight of material. All solutions, except those of Step I, contain dithiothreitol (1 to 10 mM) as protective agent for the preservation of both P-450 and the 11β-hydroxylase activity.

Adrenocortical Mitochondria and Freeze-dried P-1 Preparation—One hundred grams of adrenal cortex tissue, a quantity obtained from about 20 steers, yields a mitochondrial preparation containing between 1.0 and 1.5 g of protein. Details of this procedure have been given elsewhere (25). A suspension of fresh adrenocortical mitochondria in distilled water, 60 to 80 mg of protein per ml, is sonicated (ultrasonic disintegrator, Measuring and Scientific Equipment, Inc., Westlake, Ohio; 20 kHz, 60 watts sonic output) for a total of 15 min in an ice-cooled vessel, with frequent interruption and recooling. Subsequently, the sonicate is centrifuged at 105,000 × g for 30 min (Spinco model L, rotor No. 40). The sediment, the so-called P-1 fraction (10), is stored directly in the centrifuge tubes at −20°. It serves as the starting material for purification of P-450 from the adrenal cortex.

Sediments accumulated from four to five separate batch preparations, corresponding to 400 to 500 g of adrenocortical tissue, are thawed, combined, and homogenized in 40 to 50 ml of ice-cold glass-distilled water in 5-ml portions; then the suspensions are lyophilized. The freeze-dried powder can be stored at 4° and Scientific Equipment, Inc., Westlake, Ohio; 20 kHz, 60 watts sonic output) for a total of 15 min in an ice-cooled vessel, with frequent interruption and recooling. Subsequently, the sonicate is centrifuged at 105,000 × g for 30 min (Spinco model L, rotor No. 40). The sediment, the so-called P-1 fraction (10), is stored directly in the centrifuge tubes at −20°. It serves as the starting material for purification of P-450 from the adrenal cortex.

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Step I: Defatting—The freeze-dried P-1 preparation is washed with acetone (20 ml per g of freeze-dried material) to remove traces of moisture which might have been readorsorbed during handling. The acetone-washed material is extracted once with 1-butanol in the ratio of 20 ml per g and then twice again with 1-butanol in the ratio of 10 ml per g. This is followed by three consecutive extractions with acetone (10 ml per g). Each time the sediment is recovered by centrifugation in the cold, and the supernatants are discarded. The extracted material is spread directly in the centrifuge tubes at −20°. It serves as the starting material for purification of P-450 from the adrenal cortex.

Step II: Extraction with 0.02% Triton N-101—The defatted material from Step I is suspended in a solution containing 0.02% (w/v) Triton N-101 and dithiothreitol (10 mM) in phosphate buffer (50 mM, pH 7.0). The ratio of eluant volume to amount of defatted material is 20 ml per g. The resulting suspension is centrifuged at 105,000 × g for 30 min. The supernatant contains some P-450, trace amounts of hemoglobin (if any), small amounts of ISP, and rather large quantities of P-420, as well as various non-CO-combining heme proteins (0.02% Triton extract No. 1).

The residue is re-extracted with the same solution in the ratio of 10 ml per g of material. After high speed centrifugation, this second supernatant contains some P-450 and additional small quantities of P-420-type compounds of an undetermined chemical nature (0.02% Triton extract No. 2). Details of the preparation method are described in Steps I to IV. Step V outlines a method for removal of contaminating P-420-type material. Step VI describes procedures developed to obtain additional P-450 of varied qualities.

Step III: Extraction of P-450—The sediment of Step II is suspended in phosphate buffer (50 mM, pH 7.0) containing 0.2% (w/v) Triton N-101 and dithiothreitol (10 mM); the ratio of eluant volume to amount of defatted material is 10 ml per g. The resulting suspension is centrifuged at 105,000 × g for 30 min. The amber-colored supernatant (0.270 Triton extract No. 2) contains some P-450, trace amounts of hemoglobin (if any), small amounts of ISP, and rather large quantities of P-420, as well as various non-CO-combining heme proteins (0.02% Triton extract No. 1). After high speed centrifugation, this second supernatant contains some P-450 and additional small quantities of P-420-type compounds of an undetermined chemical nature (0.02% Triton extract No. 2).

Step IV: Ammonium Sulfate Precipitation—P-450 is precipitated from the supernatant of Step III by the addition of solid ammonium sulfate to a final half-saturation. The precipitated material is collected by centrifugation at 15,000 × g for 10 min. The supernatant is discarded. The sediment is dissolved in a minimal volume of phosphate buffer (50 mM, pH 7.0) and the resulting P-450 solution is dialyzed against this buffer solution for 12 hours to remove ammonium sulfate. (Similar procedures are applied to other detergent extracts, as for example those of Step VI.) Oxygen is excluded at this final stage of preparation by bubbling a steady stream of nitrogen through the dialysis setup. After dialysis the clear solution contains between 15 and 35 nmol of P-450 per ml, depending on the volume of buffer solution used to suspend the protein precipitate. A slight opal-
ence appears sometimes at the highest P-450 concentrations (>30 mM), but it can be removed by centrifugation. The P-450 solution is routinely stored at -20°C with but little loss of hydroxylase activity and only an insignificant conversion of P-450 into P-420 even after several months. The preparation can also withstand storage at 2-4°C for several days. Repeated freezing and thawing, however, impairs drastically enzymatic activity, optical qualities (i.e., the material becomes turbid), and the P-450 content.

Step V: Removal of P-420-type Material—Some residual P-420 can be removed by an additional purification step consisting of rapid successive passage of the diazylated P-450 solution from Step IV through one or two short DEAE-cellulose columns (e.g., 1 × 3 cm) which are equilibrated with phosphate buffer (50 mM, pH 7.0). The eluate can be concentrated as needed by ultrafiltration.

Step VI: Recovery of Additional P-450—Additional amounts of P-450 can be recovered from the residue of Step III by extraction with a solution of 10% (w/v) Triton N-101 in phosphate buffer (10% Triton extract). In general, the procedures described in Steps III and IV are followed. On centrifugation at 110,000 × g for 90 min a dark-colored heavy sediment, a reddish middle layer, and a clear yellow-brown supernatant are obtained. Half of the extracted P-450 is found in the yellow-brown supernatant fraction; additional quantities can be obtained from the middle layer. The spectroscopic qualities and the steroid hydroxylase activity of the material obtained from the supernatant of the 10% Triton Extract, after ammonium sulfate precipitation and dialysis, are only slightly inferior to those of the P-450 preparation obtained in the regular Steps III to V. Additional extractions of the residue with detergent solution seem feasible, but the quality of the extracted material, in terms of its heme content, as well as the ratio of P-450 to protein, decreases rapidly with further extractions.

Some P-450 of lower quality can also be obtained from the 0.02% Triton N-101 extracts of Step II after ammonium sulfate precipitation (cf. Table II for quantitative details). While less suitable for chemical studies on P-450, this material is still highly active in the reconstituted steroid 11β-hydroxylase and in cholesterol-related hydroxylation reactions.

RESULTS

The method described here was specifically developed to isolate enzymatically active P-450 from the adrenal cortex in a form which is suitable for detailed investigations of its chemical nature. The obtained soluble preparation possesses excellent qualities for spectrophotometry in the ultraviolet, visible, and near infrared regions of the spectrum (20). It is free of the other components of the steroid hydroxylase multienzyme system (18, 27), Ep and ISP, and is nearly free of contaminating heme proteins and additional chromophoric material which would interfere with the measurements. For quantitative data on the enzyme activity throughout the preparation procedure see Table III.

In the development of the various steps, we have considered the preservation of a functional P-450 to be of greater importance than enzyme purification measured in more conventional units as a specific P-450 concentration (nmoles of P-450 per mg of protein), or a specific enzyme activity (rate of product formation per mg of protein). Consequently, no special attempts were made to remove inert protein from the preparation, a combination of all of these measured quantities, however, was used as a guideline throughout, as shown in Tables II and III and in the text.

<table>
<thead>
<tr>
<th>Material</th>
<th>P-450</th>
<th>11β-Hydroxylase activity (corticosterone formed)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmoles/mg protein</td>
<td>nmoles/mg protein X min</td>
</tr>
<tr>
<td>Freeze-dried P-1</td>
<td>0.73</td>
<td>6.65</td>
</tr>
<tr>
<td>Defatted P-1 (Step 1)</td>
<td>0.65</td>
<td>5.25</td>
</tr>
<tr>
<td>P-450 (Steps III + IV)</td>
<td>1.09</td>
<td>6.35</td>
</tr>
<tr>
<td>DEAE-cellulose (Step V)</td>
<td>1.09</td>
<td>9.18</td>
</tr>
<tr>
<td>After ultrafiltration</td>
<td>1.65</td>
<td>9.25</td>
</tr>
</tbody>
</table>

* After chromatography on a single DEAE-cellulose column.

Success of this preparation method depends to a large extent on a carefully controlled breakage of the isolated mitochondria which, after centrifugation, yields the P-1 preparation (10); the latter is then freeze-dried. (A quantitative evaluation of these and related steps is made in Table I.) The superiority of the P-1 preparation over the previously studied P-3 fraction (9) in terms of preparative yields of P-450 and also in terms of its purity is evident. However, the P-1 preparation could only be utilized after the earlier difficulties had been overcome to release and extract an enzymatically active P-450 rather than P-420-type material. The ratio of P-450: (P-450 + P-420) (cf. Table I) is used empirically to present the P-450 content of the various preparations in relation to the content of other CO-combining heme proteins. In calculating this expression a somewhat arbitrary molar absorptivity ε420 = 2.13 × 10^4 cm^-1 at 420 nm was assumed for the undoubtedly inhomogeneous population of P-420-CO.

Freeze-drying and subsequent "defatting," that is the treatment of the water-free P-1 preparation with acetone and 1-butanol, are essential steps of the method; they help to prepare the material for the fractional extractions with detergent solution. Large amounts of neutral and phospholipids are removed, resulting in a 35 to 40% loss of dry weight. However, this "defatting" process is not complete; the P-450 preparation obtained in Steps I to V still contains lipids and phospholipids (of the order of 10 to 15% by weight, varying seasonally with the composition of the adrenal glands) but negligible amounts (<0.1%) of cholesterol. As yet, the residual lipid material could not be removed without loss of the enzyme activity; detailed investigations of the "defatting" process are in progress.

The neutral detergent Triton N-101 as extracting medium was chosen after an extended study of the effect of a large number of both neutral and other detergents on adrenal microsomes and related materials and measurements of the associated steroid 21-hydroxylase activity (28). The detailed extraction conditions were determined empirically by systematic variation until a relatively pure (in terms of heme content and ratio of P-450 to protein) and enzymatically active preparation (turnover number with respect to P-450 concentration and specific activity with respect to protein) was obtained.

In the two consecutive extractions with 0.02% Triton N-101...
typical for material carried through Steps I to V, a P-450 content is present in the better preparations. In an example oxide.
frequently omitted.
stability of the resulting preparation. Therefore, this step is often outweighed by substantial losses and a generally lower higher purity of the heme protein achieved in this step is, however, separation through the chromatography described in Step V. The P-450 purity or steroid hydroxylase activity.
mentions outlined in Step VI, with comparatively little loss of either considerable increase in the yield of P-450 can be achieved by the treat-
This fact makes further extraction advisable; a considerable increase in the yield of P-450 can be achieved by the treat-
Removal of P-420 material is possible by carrying the prepar-
Removal of P-420 material is possible by carrying the prepar-
Some Spectroscopic Properties—Detailed spectroscopic data of this P-450 preparation, together with a number of physical and chemical properties, have been reported earlier (10, 19, 20, 31). However, a brief spectroscopic characterization of the preparation should be of interest here.
Fig. 1 shows the difference spectrum of the CO-compound, P-450-CO, formed with excess carbon monoxide after chemical reduction; this spectrum is characterized by a strong positive “peak” at 449 nm and a complex negative “trough” at about 405 nm. A small amount of P-420-CO (cf. legend to Fig. 1 and Footnote 4) is seen as a shoulder at 423 nm. Rather nondistinctive changes in the optical absorption occur in the region of the conventional α- and β-bands (see Reference 32 for details).
The optical absorption spectrum of the isolated preparation, P-450 (Fe⁺⁺), exhibits absorption bands at 280, 356, and 416 nm, rather shallow β- and α-bands around 541 and 570 nm, respectively, and very weak absorption around 625 to 630 nm (Fig. 2, solid line). The position of the Soret maximum at 416 nm and, in particular, the absence of any detectable shoulder at shorter wavelengths (290 to 410 nm) characterize this P-450 preparation as a low spin (S = ½) ferric heme protein at room temperature. No significant change of the absorption spectrum is observed on cooling, except for the expected general sharpening. This observation and the failure to detect significant amounts of high spin heme proteins by EPR spectroscopy at temperatures as low as 4.2° K (for details see References 19, 31, and 32) clearly establish the nature of this preparation as a low spin (S = ½) system also at cryogenic temperatures. The results show no evidence for the occurrence of a "thermal spin state equilibrium"; for a discussion of this concept and related properties see Reference 33.

The absorption spectrum of P-450 (Fe⁺⁺) (Fig. 2, dashed line) exhibits an unusually broad Soret absorption band at 418.5 to 419 nm; only a single unstructured absorption band remains near 555 nm.

Enzymatic reduction of P-450 (Fe⁺⁺) in the reconstituted steroid 11β-hydroxylase system, with even a large excess of TPNH, is incomplete (10, 18). In accordance with the previously reported oxidation-reduction potential of P-450 from the adrenal cortex (Fe⁺⁺ = -400 to +10 mv) for the Fe⁺⁺/Fe⁺ couple at 25° (18, 27), only a partial decrease in amplitude and an incomplete red-shift of the Soret band are observed with this reductant. In the presence of suitable ligands, on the other hand, such as CO, pyridine-containing bases, or molecular oxygen, the maximal amounts of the liganded forms of P-450 (Fe⁺⁺) are formed, as discussed elsewhere (10, 18, 32).

EPR spectroscopy of this preparation (Steps I to IV or V) shows the characteristic features of the low spin (S = ½) form of P-450 (Fe⁺⁺⁺) with rhombic symmetry, and with principal components of the g-tensor 1.91, 2.24, and 2.42 (19) at 77° K. Unlike the situation in hepatic microsomal preparations, there is no indication of any heterogeneity of P-450 (Fe⁺⁺⁺) in this preparation.

Steroid 11β-hydroxylase activity at successive stages of the purification procedure is shown in Table III. In general, the changes in the specific activity and the specific P-450 concentration during Steps I to V move in the same direction. In the example listed, the over-all increase in the specific P-450 concentration from the freeze-dried P-1 preparation as starting material to the final P-450 preparation (Steps I to V) was 2.26-fold. This value must be compared with a corresponding increase in the specific hydroxylase activity by a factor of 1.39 and an actual decrease by 38% in the turnover number, from 9.0 to 5.6 nmoles of corticosterone per nmole of P-450 × min at 25°.

The reconstituted steroid 11β-hydroxylase system shows a pronounced narrow pH optimum between pH 6.3 and 6.5 (cf. Fig. 3); loss of half of the enzyme activity is found at pH 7.5 and slightly below pH 6.0. (The latter value is somewhat uncertain since the preparation precipitates rapidly at or near pH 6.0.)

**Discussion**

The P-450 preparation from the adrenal cortex has been investigated in our laboratory for several years and a substantial amount of information has already been obtained. Its optical absorption (10, 26, 32) and EPR characteristics (19, 31), its properties as a Fe⁺⁺⁺/Fe⁺⁺ oxidation reduction couple (26, 27) and associated kinetic parameters (18), its interaction (in the Fe⁺⁺⁺ form) with ligands (32), with steroids and other "substrate" molecules (31), and its reactivity (in the Fe⁺⁺⁺ form) towards carbon monoxide, nitrogenous bases, and oxygen (18, 32, 37) have been reported. The preparation is suitable for studies related to the specificity of the hydroxylation reaction (34). Based on the availability of all components of the steroid hydroxylase multienzyme system and the use of artificial electron donors, we have demonstrated a specific requirement for the ISP in the hydroxylation reaction (35).

This fact has subsequently been confirmed by Gunsalus et al., for the hydroxylation of camphor catalyzed by P-450CAM from Pseudomonas putida (36). The experimental separation of the two different one-electron reduction steps required by the stoichiometry of the steroid hydroxylation reaction has recently been achieved with our preparation (32, 37).

A brief comparison of this P-450 preparation with other particulate or solubilized P-450 preparations from mammalian sources, as well as some bacterial P-450 preparations seems appropriate. A variety of experimental approaches has been used to obtain P-450, especially from liver microsomes after drug induction for increased yields; this includes proteolysis with trypsin or bacterial proteases (38, 39), solubilization with sodium cholate or deoxycholate (15), and treatments with detergents (40). The supporting media contain in most cases glycerol or other polyols in concentrations of 20% or higher; their presence seems to stabilize the solubilized material at least temporarily. In the case of microsomes, large quantities of cytochrome b₅ have to be removed without affecting P-450. Many of the preparations described so far are left inactive after these treatments.

Coon and Lu (41, 42) have recently succeeded in solubilizing a P-450 preparation from liver microsomes, which is capable of enzyme activity in the ω-hydroxylation of fatty acids, the hydroxylation of hydrocarbons, and the demethylation of drugs (e.g. benzphetamine, turnover number 22 min⁻¹ at 25°), when supplemented with a microsomal reductase fraction and an ad-
ditional heat-stable factor. The latter has been identified as phosphatidylcholine (43).

Mitani and Horie (15) have described a solubilization procedure for P-450 from bovine adrenocortical mitochondria, which employs extraction with sodium cholate in several concentrations, combined with ammonium sulfate fractionation. Jefcoate et al. (44) have recently employed a "defatting" process somewhat similar to the one described here, but with isoctane as solvent. P-450 was extracted with sodium cholate in the presence of glycerol and fractionated with ammonium sulfate, followed by dialysis against distilled water. A partial separation of 11β-hydroxylase activity from the side chain cleavage activity (leading from cholesterol to pregnenolone) was reported.

Bacterial P-450-type heme proteins have been isolated and studied by several groups. The best known example is the so-called P-450$_{CAM}$, the soluble heme protein component of a 5-exomethylene hydroxylase system that can be induced in P. putida by growth on d-camphor (cf. Reference 36). This soluble heme protein has recently been crystallized in the form of its camphor complex in the presence of mercuriosuccinate as protecting agent (45). Soluble bacterial preparations have also been obtained by Appleby (46), from Rhizobium japonicum.

The optical absorption spectrum of the adrenal P-450 preparation described here (Fig. 2) is nearly identical with that of the substrate-free low spin (S = 1/2) form of P-450$_{CAM}$ as reported by Gunsalus et al. (36, 47) and, more recently, by Peterson (48), except for a higher unspecified ultraviolet absorption which corresponds to the lower specific heme concentration. It is also nearly identical with the absorption of low spin forms of Rhizobium P-450 (especially Rhizobium P-450 a; cf. Reference 46).

Neither the P-450 preparation described here nor other P-450 preparations from mammalian tissues described in the literature have so far resulted in a substantial increase of the specific P-450 concentration above the level in the starting material. Final values for our preparation are found in the range of 1.5 to 2.0 nmoles per mg of protein, with no clearcut correlation to the enzymatic activity. By the method described here, a nearly complete separation from contaminating heme proteins, as well as from the other enzyme components of the steroid 11β-hydroxylase system, is achieved. At the same time, the intactness of the heme protein is maintained, and it remains active in the reconstituted hydroxylase systems. As mentioned, no particular attempts have been made in this preparation procedure to remove inert protein in order to increase the specific P-450 concentration.

Higher specific P-450 concentrations, with or without associated enzyme activities, have only been reported for bacterial preparations of P. putida and R. japonicum. Of these, P-450$_{CAM}$ from P. putida is free of lipids, but contains glucosamine (0.6 residue per mole; mol wt approximately 46,000) and an additional carbohydrate component of unknown nature. The specific P-450 concentration of this material can reach values of up to 21.7 nmoles per mg of protein.

Currently, attempts are being made in our laboratory to achieve further improvements in the preparation of P-450 from the adrenal cortex by: (a) a more complete removal of the lipid components and of the remaining traces of cholesterol and other steroids, and by (b) a more extensive removal of inert proteins without impairing the enzyme activities. Our efforts to study the specificity of the steroid hydroxylase system and to achieve further separation of individual enzyme activities (cf. References 30 and 34) are being continued.

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