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 extraction 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 N-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 (Fe⁴⁺), 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 protoporphyrin IX content; these are the only heme proteins detectable. Specific concentration of P-450 in the preparation lies between 1.5 and 2.0 nmol 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 sometimes 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 spectrophotometric 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 was lost during sonication.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>P-450</th>
<th>P-420</th>
<th>Protein</th>
<th>Ratio of P-450: (P-450 + P-420)</th>
<th>Ratio of P-450: P-450 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondria</td>
<td>μM</td>
<td>%</td>
<td>μM</td>
<td>mg/ml</td>
<td>%</td>
</tr>
<tr>
<td>P-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>S-1</td>
<td>0.39</td>
<td>10.2</td>
<td>0.08</td>
<td>1.32</td>
<td>33.8</td>
</tr>
<tr>
<td>P-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>S-2</td>
<td>0.01</td>
<td>0.3</td>
<td>0.10</td>
<td>0.73</td>
<td>18.6</td>
</tr>
<tr>
<td>P-3</td>
<td>0.080</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 M KCl and subsequent centrifugation. The P-3 fraction was originally employed in the reconstitution of the steroid 11β-hydroxylase (9, 10).

11β-hydroxylase system we found (10) that another particulate fraction obtained from adrenocortical mitochondria, termed P-1,2 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 lip-hydroxylase system. The P-1 preparation to be assayed was added (final concentration of 50 μg/mg protein) to a 100-μl reaction mixture containing 100 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, 5 mM MgCl₂, 0.1 mg/ml albumin from calf serum, 1 mM glucose 6-phosphate (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. Deoxy corticosterone (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 glass-distilled water was used throughout.

1 The P-1 particle preparation is the high speed centrifugation sediment of the mitochondrial sonicate. (For details see Table 1.)

2 The term P-420 does not describe one chemically defined compound but comprises several different heme proteins which form, after reduction, CO-compounds and the hydroxylation reaction started by the addition of the TPNH-generating system at zero time. The

### 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, \( \Delta e_{450} = 9.1 \times 10^4 \text{m}^{-1} \text{cm}^{-1} (450 - 490 \text{ nm}) \), determined originally by Omura and Sato (13) in a microsomal preparation, was used for quantitation.

A rough estimate of the P-420 content1 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, \( \varepsilon_{450} = 2.13 \times 10^5 \text{m}^{-1} \text{cm}^{-1} \) at 420 nm. A value of \( \varepsilon_{450} = -4.1 \times 10^4 \text{m}^{-1} \text{cm}^{-1} \) was used to correct for the (negative) contribution from P-450 in the difference spectra at this wavelength.

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-420 absorption region in the difference spectra. They may 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 acetylation 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 \( \Delta e_{450} = 3.24 \times 10^4 \text{m}^{-1} \text{cm}^{-1} \) at 557 nm (13).

For the flavoprotein (Fp), \( \varepsilon_{450} = 1.13 \times 10^4 \text{m}^{-1} \text{cm}^{-1} \) at 450 nm (20) was used. Quantitation of ISP was based on a molar absorptivity \( \varepsilon_{450} = 1.0 \times 10^4 \text{m}^{-1} \text{cm}^{-1} \) at 415 nm (5, 7). In previous reports from our laboratory (3, 9) a value \( \varepsilon_{450} = 5.6 \times 10^3 (\text{g atom of iron})^{-1} \text{cm}^{-1} \) at the same wavelength was employed, corresponding to the analytical iron content of 2 μg 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): DOC (0.24) as substrate for 11β-hydroxylation, NaCl (58), KCl (44), MgCl₂ (1.3), TPN⁺ (1.0), glycylglycine 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 appropriate ratio of Fp to ISP to P-450 = 1:20:1, unless otherwise stated; this was found to be optimal for 11β hydroxylation (10).
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 (nmoles of product formed) per (mg of protein × min) or as (nmoles of product formed) per (nmoles 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 mitochondria 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 recocling. 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°C. 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° in a vacuum desiccator for at least 2 months with very little loss of P-450 content and enzyme activity.

Purification of P-450

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 readsorbed 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 over a large surface area, dried in a vacuum desiccator over silica gel, and stored at 4°. Starting from 100 g of the freeze-dried P-1 preparation, a yield of 60 to 85 g of defatted material is obtained.

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).

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 suspension is centrifuged at 105,000 × g for 30 min. The amber-colored supernatant (0.2% Triton extract) contains relatively pure P-450; it is decanted and treated as described in Step IV. The residue is saved for further extractions (cf. Step VI); additional, although somewhat less pure, P-450 can be obtained in this way (cf. also data in Table II).

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 protein is centrifuged at 105,000 × g for 30 min. The resulting P-450 solution is dialyzed against this buffer solution (50 mM, pH 7.0) to remove ammonium sulfate and any other contaminating material. After dialysis, the clear solution contains about one-third of both P-450 and protein, as determined in an aliquot sample.

Step V: Purification of P-450 from freeze-dried P-1 preparation

Starting material, freeze-dried “defatted” P-1 preparation, contained 5.56 nmoles of P-450, 5.18 nmoles of P-420, and 9.1 g of protein. The ratio of P-450: (P-450 + P-420) was 0.63; P-450 to protein was 0.61 nmoles per mg.

All extracts were subjected to the procedure described in Step IV, i.e. ammonium sulfate precipitation and dialysis of the dissolved precipitate. About one-third of both P-450 and total protein is precipitated under these conditions in the extracts of Step II, essentially all is precipitated in the extract of Step III, and about one-half of both P-450 and protein is precipitated in the 1.0% Triton N-101 extract, Step VI.

<table>
<thead>
<tr>
<th>Extracting solution</th>
<th>Step I</th>
<th>P-450</th>
<th>P-420</th>
<th>(P-450 + P-420)</th>
<th>Protein</th>
<th>Ratio of P-450 to (P-450 + P-420)</th>
<th>Ratio of P-450 to protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.02% Triton (No. 1)</td>
<td>II</td>
<td>108</td>
<td>25</td>
<td>133</td>
<td>734</td>
<td>1.9</td>
<td>0.81</td>
</tr>
<tr>
<td>0.06% Triton (No. 2)</td>
<td>II</td>
<td>34</td>
<td>?</td>
<td>108</td>
<td>0.6</td>
<td>0.31</td>
<td>1.24</td>
</tr>
<tr>
<td>0.2% Triton</td>
<td>IV</td>
<td>329</td>
<td>4.8</td>
<td>334</td>
<td>266</td>
<td>5.9</td>
<td>0.98</td>
</tr>
<tr>
<td>1.0% Triton</td>
<td>VI</td>
<td>536</td>
<td>17</td>
<td>553</td>
<td>585</td>
<td>9.6</td>
<td>0.97</td>
</tr>
</tbody>
</table>

* Yield of P-450 refers to the amount of P-450 present in the starting material, as determined in an aliquot sample.

Purification of P-450 (P-450 + P-420) was 60 to 65 g of defatted material 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 P-450 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° 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° 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 dialyzed P-450 solution from Step IV through one or two short DEAE-cellulose columns (e.g. 1 x 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 1.0% (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 x 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 1.0% 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 (26). It is free of the other components of the steroid hydroxylase multienzyme system (18, 27), Fp 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 III

<table>
<thead>
<tr>
<th>Material</th>
<th>P-450</th>
<th>11β-Hydroxylase activity (cortisosterone 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 I)</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 ε_410 = 2.13 x 10^4 ac cm^-1 at 420 mm 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 concentration is present in the better preparations. In an example, a heterogeneous P-420 material is extracted. Part of the extracted heme protein is not capable of combining with CO, but does have the usual spectral characteristics, i.e. strong Soret absorption and accompanying \( \alpha \) - and \( \beta \)-bands. A large amount of protein is also removed during Step II, especially in the first extraction (Table II).

The extraction with 0.2% Triton N-101 in phosphate buffer (Step III) yields material with a ratio of P-450: (P-450 + P-420) \( \approx 0.98 \), as shown in Table II. Such high ratios have been consistently observed in the course of numerous enzyme preparations over a period of more than 3 years; the lowest ratio found was 0.85. The extract is conveniently concentrated (without further purification) by precipitation with ammonium sulfate to half-saturation and dialysis against phosphate buffer, with added dithiothreitol for increased stability (Step IV). No significant loss of P-450 or conversion to P-420 are found associated with this step.

The preparative yield of Steps III + IV (cf. Table II) is very low in comparison with the P-450 content of the starting material. This fact makes further extraction advisable; a considerable increase in the yield of P-450 can be achieved by the treatment outlined in Step VI, with comparatively little loss of either P-450 purity or steroid hydroxylase activity.

Removal of P-420 material is possible by carrying the preparation through the chromatography described in Step V. The higher purity of the heme protein achieved in this step is, however, often outweighed by substantial losses and a generally lower stability of the resulting preparation. Therefore, this step is frequently omitted.

Chemical assays indicate that practically no other heme proteins are present in the better preparations. In an example typical for material carried through Steps I to V, a P-450 concentration of 7.5 \( \mu \)M was determined from the difference spectrum of P-450-CO, while a direct determination of the protoheme IX content by the pyridine hemochrome formation gave a value of 7.4 \( \mu \)M. The protoheme IX moiety remains unaltered or is very little altered during the preparation procedure when compared with P-450 observed in tissue slices, homogenates, isolated adrenal mitochondria, and a number of submitochondrial preparations. This is demonstrated by a comparison of its optical (26) and chemical (18, 25, 26) properties, its activity in the reconstituted hydroxylase system (10), and, most conclusively, by the near identity of the EPR spectra of the low spin \( (S = \frac{1}{2}) \) P-450 (Fe\(^{3+}\)) (19).

The resulting P-450 preparation is active in steroid 11\( \beta \)-hydroxylation (turnover numbers 5 to 10 min\(^{-1}\) at 25°; cf. also (10) and Table III for quantitative data of the various steps) and, in addition, active in side chain cleavage of cholesterol (turnover numbers \( \geq 2 \) min\(^{-1}\) at 23°) and the metabolism of hydroxylated cholesterol derivatives (29, 30).  

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 \( \alpha \) - and \( \beta \)-bands (see Reference 32 for details).

Fig. 1 (left). Difference spectrum of P-450-CO. Cary spectrophotometer, model 14; optical path length 1.0 cm in anaerobic cuvettes. P-450 preparation (Steps I to IV, 5.7 \( \mu \)M) in phosphate buffer (50 mM, pH 7.0). Preparation contains some P-420 type material (0.38 \( \mu \)M); ratio of P-450: (P-450 + P-420) = 0.94. The difference spectrum (reduced + CO) minus (reduced) was recorded after chemical reduction with sodium dithionite in \( \text{N}_2 \) atmosphere and equilibration of the sample cuvette with \( \text{O}_2 \)-free carbon monoxide.

Fig. 2 (right). Absorption spectra of P-450 (Fe\(^{3+}\)) and P-450 (Fe\(^{2+}\)). P-450 preparation (Steps I to IV) in phosphate buffer (50 mM, pH 7.0). Final concentrations were: P-450 (5.7 \( \mu \)M), P-420 type material (0.38 \( \mu \)M), and protein (3.8 mg per ml). Ratio of P-450: (P-450 + P-420) = 0.94; ratio of P-450:protein = 1.5 nmoles per mg of protein. Instrumental conditions were as in Fig. 1. ---, P-450 (Fe\(^{3+}\)) as isolated from adrenal cortex; - - - , P-450 (Fe\(^{2+}\)) after reduction with solid sodium dithionite in \( \text{N}_2 \)-free atmosphere.
The optical absorption spectrum of the isolated preparation, P-450 (Fe3+), 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 wavelength (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 (Fe3+) (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 (Fe3+) 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 (E°3 = -400 + 10 mV), for the Fe3+/Fe2+ 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 (Fe3+) 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 (Fe3+) 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 (Fe3+) 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 turnover number, from 9.0 to 5.6 for the first 15 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 Fe3+/Fe2+ oxidation reduction couple (26, 27) and associated kinetic parameters (18), its interaction (in the Fe3+ form) with ligands (32), with steroids and other "substrate" molecules (31), and its reactivity (in the Fe2+ 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 multi-enzyme 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 n-caprool 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 hydroxylase 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 isooctane as solvent. P-450 was extracted with sodium cholate in the presence of glycercol 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-450CAM, the soluble heme protein component of a 5-exomethylene hydrolase 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 mercaptosuccinate 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 = ½) form of P-450CAM 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 nmols 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 hydrolase 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 from P. putida and R. japonicum. Of these, P-450CAM 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 nmols 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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