Cytochrome P-450 from Bovine Adrenocortical Mitochondria: an Enzyme for the Side Chain Cleavage of Cholesterol

I. PURIFICATION AND PROPERTIES

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SUMMARY

One cytochrome P-450 has been purified from bovine adrenocortical mitochondria to near homogeneity. The enzyme catalyzes the conversion of cholesterol and 20α-hydroxycholesterol to pregnenolone (side chain cleavage) and shows traces of 11β-hydroxylase activity. The conversion of cholesterol to pregnenolone occurs without demonstrable accumulation of biosynthetic intermediates, i.e., hydroxylated sterols. The kinetic constants for side chain cleavage are as follows: cholesterol: \( K_m = 0.19 \text{ mM} \); and 20α-hydroxycholesterol: \( K_m = 0.012 \text{ mM} \).

The P-450 described here shows the following properties: molecular weight, 850,000; \( s_{20, w} = 22 \times 10^{-12} \text{ cm}^2 \text{g}^{-1} \text{s}^{-1} \); partial specific volume, 0.765. The enzyme is isolated in a form which is approximately 50% high spin and 50% low spin; it contains eight heme groups per molecule and is stable in 50% glycerol.

Cytochrome P-450 is required for a number of steroid hydroxylation reactions occurring in adrenocortical mitochondria including 11β-hydroxylation (1), 18-hydroxylation (2), and hydroxylation reactions concerned with the side chain cleavage of cholesterol (3, 4) and cholesterol sulfate (5, 6). P-450 serves as the terminal oxygenase for a system of electron carriers which conveys electrons from TPNH to oxygen according to the following scheme (1):

\[
\text{TPNH} \rightarrow \text{diaphorase} \rightarrow \text{adrenodoxin} \rightarrow \text{cytochrome P-450} \rightarrow \text{oxygen}
\]

The questions of whether more than one P-450 is necessary for and how the cytochrome catalyzes these reactions have been approached by recent investigations using P-450 from bovine adrenocortical mitochondria. Kinetic analysis of side chain cleavage of cholesterol and cholesterol sulfate suggested the presence of more than one P-450 (6), and this suggestion was supported by the results obtained from ammonium sulfate fractionation of cytochrome P-450 (7, 8). Moreover, soluble preparations of cytochrome P-450 from the same source have revealed specificity in the hydroxylation reactions catalyzed by the different cytochromes P-450 (9). The present studies describe the purification and certain properties of one cytochrome P-450 from bovine adrenocortical mitochondria. P-450 is believed to cause hydroxylation of cholesterol and the products of this hydroxylation, including 20α-hydroxycholesterol, are presumed to be intermediates in the conversion of cholesterol to pregnenolone (10, 11).

EXPERIMENTAL PROCEDURE

Purification of Cytochrome P-450

Cholic Acid Extraction—Mitochondria were prepared from fresh bovine adrenal cortex (300 to 420 g) as described by Omura et al. (1). The mitochondria were washed in potassium phosphate buffer (800 ml, 100 mM, pH 7.6) and resuspended before being resuspended in water (1). All subsequent procedures were performed at 4°C. The mitochondrial suspension (6,400 to 7,800 mg of protein, 210 to 250 ml) was sonicated at 20 kev for 10 min at the full power setting of a 75-watt Branson Sonifier in 40- to 50-ml portions. Sodium cholate (pH 7.0, 0.45 mg per mg of protein) was added to the sonicate; the mixture was stirred for 60 min and then centrifuged at 105,000 \( \times g \) for 90 min. The preparation at this stage consists of a fluffy layer, an orange-yellow supernatant layer which makes up the bulk of the material, one lightly packed layer, and a pellet.

Ammonium Sulfate Precipitation—The orange-yellow layer was carefully removed (total volume, 300 to 350 ml; 3,200 to 4,100 mg of protein) and treated with ammonium sulfate to 60% saturation; the pH was maintained at 7.0. The resulting precipitate was dialyzed against potassium phosphate buffer (50 mM, pH 7.6) containing cysteine (10 mM) and EDTA (1 mM) for 3 hours and then centrifuged at 105,000 \( \times g \) for 60 min. The supernatant layer (80 to 110 ml, 2,100 to 2,500 mg of protein) was dialyzed for 16 hours against potassium phosphate buffer

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1 The abbreviation used is: pregnenolone, 3β-hydroxy-5-pregnene-20-one.
precipitate was formed during ultrafiltration and subsequently removed by centrifugation (800 × g for 10 min).

The enzyme was taken to a final concentration of 2 to 3 mg of protein per ml by membrane ultrafiltration. The concentrated sample (5 ml) was further purified by ascending chromatography on a column of Bio-Gel A-15m (2 × 90 cm). The column was eluted at a constant flow rate of 34 ml per hour with a gradient of potassium phosphate buffer (10 and 500 mM, 200 ml each; pH 7.6 together with dithioerythritol and EDTA, 0.1 mM each). Fractions containing P-450 eluted by phosphate buffer (150 to 300 mM) were pooled and concentrated by membrane ultrafiltration (Diaflo XM-100A) under nitrogen (15 p.s.i.). A small amount of precipitate was formed during ultrafiltration and subsequently removed by centrifugation (800 × g for 10 min).

Chromatography on Bio-Gel—The concentrated sample (5 ml) was further purified by descending chromatography on a column of Bio-Gel A-15m (2 × 90 cm). The column was eluted at a constant flow rate of 34 ml per hour with a gradient of potassium phosphate buffer (100 mM, pH 7.0) containing dithioerythritol and EDTA (0.1 mM each); hereafter this buffer mixture will be referred to as standard buffer. The cytochrome P-450 eluted between 1.4 and 2.0 void volumes was cycled a second time through the same column. Unless otherwise stated, it is this material upon which all subsequent studies reported in this paper were performed. The enzyme was taken to a final concentration of 2 to 3 mg of protein per ml by membrane ultrafiltration. The concentrated solution was mixed with equal parts of glycerol and stored at −20°C.

**Analytical Methods**

**Enzymatic Activity of P-450**—The capacity of P-450 to catalyze the side chain cleavage of cholesterol, 11β-hydroxylation, and 18-hydroxylation was measured by incubating P-450 (0.1 nmole) in 2 ml of potassium phosphate buffer (100 mM, pH 7.0) containing cysteine (10 μmoles), EDTA (2 μmoles), TPNH (200 μmole-), adrenodoxin reductase (diaphorase) (eight 2.6 di chloromethylpheno1 units), and adrenodoxin (2 μmoles) together with cholesterol or [4-14C]deoxycorticosterone (20 nmole added in 50 μl of N,N-dimethylformamide). The reaction was started by addition of TPNH and continued for 20 min at 37°C.

Following incubation the medium was extracted with methylene dichloride three times. Pregnenolone (1 to 10 nmole) was measured by modification of the method of Kriisa and Mounts (13). The dried sample was dissolved in dioxane (50 μl) and mixed with 1.5 ml of 100 mM sodium pyrophosphate buffer (pH 8.9) containing DPN+ (300 nmole) and a microsomal enzyme preparation with 3β-hydroxysteroid dehydrogenase and Δ5-3-ketosteroid isomerase activities (14). The DPNH produced was measured in an Eppendorf fluorometer (15).

In a series of experiments, [4-14C]cholesterol was used to place of cholesterol. The methylene chloride extract was applied to thin layer chromatography in the system petroleum ether-diethyl ether-glacial acetic acid (64:35:1; v/v), and [14C]pregnenolone was isolated and measured by liquid scintillation spectrometry as described previously (16). The identity of [14C]pregnenolone was established by re-crystallization following addition of authentic pregnenolone. Values for pregnenolone determined by the radioactive assay and by the fluorometric method were in agreement within 5%, when purified P-450 was used.

Assay of 11β- and 18-hydroxylation activities was performed with [14C]Corticosterone as substrate; 18-hydroxy[14C]Cortico- terone and 18-hydroxy[14C]Deoxycorticosterone were purified by thin layer chromatography as described elsewhere (17). In no case were detectable amounts of radioactive 18-hydroxylated steroid observed. The identity of [14C]Cortico-terone was determined by re-crystallization with authentic corticosterone.

**Measurement of Cholesterol**—P-450 preparations (5 to 10 mg of protein) were extracted with methylene dichloride after addition of a known amount of [4-14C]cholesterol. Following purification by thin layer chromatography, cholesterol (0.1 to 1.0 μg) was measured by means of a gas chromatograph (Hewlett-Packard model 402) as described elsewhere (18). The column (SE-30) was 3 × 1200 mm, and under the conditions used, the retention time of cholesterol was 38 min.

**Measurement of Phospholipid**—Extraction of phospholipid from P-450 (5 to 10 mg of protein) was performed as described by Folch (19). Extracts were digested by conventional acid hydrolysis.

**Ultracentrifugation**—Cytochrome P-450 was examined in the Beckman model E ultracentrifuge. The cytochrome was prepared for sedimentation equilibrium by dialyzing an aliquot against potassium phosphate in D2O (50 mM, pH 7.6) for 60 hours at 4°C. Following dialysis, D2O phosphate buffer was diluted to 10 mM by addition of further D2O. Sedimentation was examined simultaneously in H2O and D2O (0.5 mg of protein per ml). The fluorescent FC-43 was used as base fluid. Centrifugation was continued for 3 days at 20°C.

**Sedimentation Velocity**—The measurement in phosphate buffer (75 mM; pH 7.6) was examined as a function of protein concentration. Sucrose gradient centrifugation was performed in an SW28 rotor as described by Martin and Ames (20). Fractions were collected by a density gradient fractionator (Isof model 640).

**Spectral Studies**—CO-difference spectra were recorded in potassium phosphate buffer (pH 7.0, 100 mM) containing cysteine (5 mM) and EDTA (1 mM). P-450 was reduced with sodium dithionite (2.5 mg per ml) before treatment with carbon monoxide. The P-450 content of the enzyme has been calculated on the basis of an extinction coefficient of 91 nm−1 cm−1 (21); the reason for this method of calculation is given under “Discussion.” Pyridine hemochrome was prepared and measured as described by Falk (22); millimolar difference extinction coefficients of 20.7 (Reference 23) and 32.4 (Reference 21) were used to determine the content of heme (see “Results”). These studies were performed with a Perkin-Elmer spectrophotometer (model 356).

**Miscellaneous**—Protein determinations were performed by the method of Lowry et al. (24), using crystalline bovine serum albumin as standard. Phosphate was determined by the method of Allen (25). Absorbance at 280 and 415 nm was measured using a Gilford recording spectrophotometer. Absorbance of column effluents was monitored at 280 nm by means of an ultraviolet flow analyzer (Ise model 224). Liquid scintillation spectrometry was performed by published methods (17) using a Packard Tri-Carb model 3375 Ultrafiltration membranes (Diaflo XM-100A) were purchased from Amicon, Lexington, Massachusetts.
Hemin was purchased from Sigma Chemical Co. and was recrystallized twice as described by Falk (22). Deuterium-water (D₂O) was obtained from Bio-Rad, Richmond, Virginia (99.86 moles %, biological grade). The following chromatographic materials were used: DEAE-cellulose, Brown Co., DeLin, New Hampshire; Bio-Gel A-15m (200- to 400-mesh), Bio-Rad; hydroxylapatite (Hyapatite C), Clarkscn Chemical Co., Inc., Williamsport, Pennsylvania; and silica gel thin layer chromatogram sheet (Eastman 6060). Sodium cholate (Sigma Chemical Co.) was dissolved in water and then brought to pH 7.0 to 7.3 with HCl. Methylene dichloride (analytical reagent grade, Mallinckrodt Chemical Works) was redistilled before use. Petroleum ether (boiling point 30-60°) was obtained from Mallineckrot Chemical Works and used without purification. Authentic steroids were purchased from Ikapharma (Ramat-Gan, Israel). [4-i4C]Cholesterol (Lot No. 302-159, 0.05 mCi/0.318 mg) and [4-14C] deoxycorticosterone (Lot No. 546.088, 5 mCi/30.5 mg) were obtained from New England Nuclear Corp. These authentic steroids were purchased from Ikapharm (Ramat-Gan, Israel). [4-i4C] Cholesterol (Lot No. 302-159, 0.05 mCi/0.318 mg) and [4-14C] deoxycorticosterone were obtained from New England Nuclear Corp. These radioactive steroids were diluted with cold carrier to a final specific activity of 4200 dpm per nmole (cholesterol) and 740 dpm per nmole (deoxycorticosterone) and purified by thin layer chromatography.

The authors are grateful to Dr. D. G. Schmidlin of Ciba-Geigy Laboratories, Basel, Switzerland for a gift of 18-hydroxycorticosterone and to Dr. Roger L. Bergstrom of G. D. Searle and Co., Chicago, Illinois, for generously providing 18-hydroxydeoxycorticosterone.

**Proteins—**An enzyme preparation containing 3β-hydroxysteroid dehydrogenase and Δ⁶-3-ketosteroid isomerase activities was prepared from *Pseudomonas testosteroni* by ammonium sulfate fractionation (14). Diaphorase and adrenodoxin were prepared from bovine adrenocortical mitochondria according to Kimura and Suzuki (26). It was found that both the diaphorase and the adrenodoxin preparations used in these studies each contained less than 0.05 nmole of cholesterol per mg of protein.

**RESULTS**

**Purification of Cytochrome P-450—**The cholic acid extract of sonicated adrenal mitochondria can be resolved into two overlapping protein components containing P-450 with side chain cleavage and 11β-hydroxylase activities by chromatography on hydroxylapatite (Fig. 1). The studies reported here are concerned with the second of these components to be eluted from the column. This component was further purified by chromatography on Bio-Gel (Fig. 2). In addition to the peak shown in Fig. 2 (void volume, 1.0 to 1.2), two other protein fractions are separated from the major P-450 component during exclusion chromatography (void volumes, 1.0 to 1.2 and 2.1 to 2.5); one of these compounds contains P-450 (void volume, 1.0 to 1.2). At this stage the major P-450 component appears to be highly purified according to the following criteria: (a) enzymatic activities (side chain cleavage and 11β-hydroxylase); (b) absorbance at 280 and 415 nm; (c) P-450 content; and (d) phosphate concentration. (a), A₂₅₀; (b), A₄₁₅; O, side chain cleavage; Δ, 11β-hydroxylase; ■, P-450 content; ○, phosphate concentration.

![Fig. 1. Chromatography of cytochrome P-450 on hydroxylapatite.](image)

**Fig. 1.** Chromatography of cytochrome P-450 on hydroxylapatite. P-450 was applied to hydroxylapatite following chromatography on DEAE-cellulose. Fractions (11.5 ml each) were collected from the column. Aliquots from selected fractions were subjected to the following determinations: (a) enzymatic activities (side chain cleavage and 11β-hydroxylase); (b) absorbance at 280 and 415 nm; (c) P-450 content; and (d) phosphate concentration. (a), A₂₅₀; (b), A₄₁₅; O, side chain cleavage; Δ, 11β-hydroxylase; ■, P-450 content; ○, phosphate concentration.

**Fig. 2.** Chromatography of P-450 on Bio-Gel A-15m. Fractions were pooled from chromatography on hydroxylapatite (Fig. 1), and the pooled material was concentrated before being applied to Bio-Gel (void volume, 124 ml). The column was washed with phosphate buffer by reversed flow and fractions were collected (0.8 ml each) after the eluate between 1.4 and 2.0 void volumes was recycled through the same column. Aliquots from selected fractions were examined as follows: (a) enzymatic activities (side chain cleavage, 11β-hydroxylation); (b) absorbance at 280 and 415 nm; and (c) P-450 content. (a), A₂₅₀; (b), A₄₁₅; O, side chain cleavage; Δ, 11β-hydroxylase; ■, P-450 content.
Evidently there is no loss of heme relative to protein. The content of endogenous cholesterol decreases to negligible levels (<0.002 nmole per nmole of P-450) as the enzyme is purified. On the other hand, side chain cleavage activity increases during purification (Table I), although a number of factors to be discussed make the interpretation of enzyme activity complicated (see "Discussion"). The purified enzyme contains phospholipid in an amount of 0.08 to 0.09 nmole of phosphorous per mg of protein.

The enzyme shows 11β-hydroxylase activity which decreases during purification. The amount of this enzyme activity is too small for accurate determination of specific activity and kinetic constants, so that the difference between the last two values shown in Table I (0.14 and 0.15) is probably within the limits of experimental error. Moreover 11β-hydroxylase activity was not increased by changing either the absolute or the relative amounts of diaphorase and/or adrenodoxin through a 10-fold range of concentrations above and below those routinely used in the assay system (see "Experimental Procedure").

**Molecular Weight and Sedimentation Properties**—The sedimentation coefficient of P-450, as determined by sucrose gradient (Fig. 5), was found to be $22 \times 10^{-13}$. This value is consistent with that determined by sedimentation velocity ($s_{20,w} = 21 \times 10^{-13}$), as already reported from this laboratory (12). In addition, the following data were calculated from sedimentation equilibrium (27) (Fig. 4): partial specific volume, 0.765; molecular weight, 850,000. This estimate of molecular weight is in agreement with the value (800,000) obtained from exclusion chromatography (Fig. 2 and Reference 12).

**Spectral Properties**—Fig. 6 shows certain important spectral properties of cytochrome P-450 from bovine adrenocortical mitochondria. The absolute spectrum shows five peaks (395, 412, 520, 565, and 650 nm), together with a small shoulder at 360 nm. The spectrum shows a trough at 312 nm. The carbon monoxide difference spectrum shows a peak at 447 nm and a small shoulder at 420 nm. The characteristics of these spectra, together with spectra of chemically reduced P-450 and the pyridine hemochrome, are summarized in Table II. When the millimolar extinction coefficient of 20.7 for $\Delta A_{557..538}$ nm (Reference 23) was used, heme content was calculated to be 7.4 moles of heme per mole of P-450. When the millimolar extinction coefficient of 32.4 (Reference 21), the heme content of P-450 was calculated to be 8.4 moles per mole of P-450.

Since cytochrome P-450 was normally kept in glycerol (see "Experimental Procedure"), it was decided to examine the influence of glycerol upon the spectral properties of P-450. Between the concentrations of 10 and 50%, glycerol increased $A_{138}$ by 14 to 66% and decreased $A_{450}$ by 11 to 56%. These changes are reversible and linear with respect to the concentration of glycerol increased 1 to 2%.
FIG. 5. Sedimentation of P-450 in sucrose density gradient. Peak fractions from chromatography on Bio-Gel (Fig. 2) were pooled and concentrated by membrane ultrafiltration. An aliquot (0.5 ml) of the concentrated sample was layered onto a gradient (30 ml) of sucrose (5 to 20%) in phosphate buffer (0.1 M, pH 7.6). The gradient was centrifuged at 28,000 rpm for 16 hours. Fractions (0.6 ml each) were collected from the gradient and analyzed as follows: (a) enzymatic activities (side chain cleavage, 11β-hydroxylation, catalase); (b) absorption at 280 nm; and (c) P-450 content. The arrow shows the position of the peak of fractions containing the internal standard (catalase, \(e_{403} = 11.3\)). ●, A₄₃₃₃; ○, side chain cleavage; △, 11β-hydroxylase; ■, P-450 content.

glycerol, and isosbestic points were observed at 408, 552, and 565 nm.

Enzymatic Activities—The adrenal P-450 which forms the subject of this report shows side chain cleavage activity with both cholesterol and 20α-hydroxycholesterol as substrates. The following kinetic constants were calculated for cholesterol: \(K_m = 0.19\) mM; \(V_{max} = 20.6\) nmoles per min per mg of protein; and for 20α-hydroxycholesterol: \(K_m = 0.012\) mM; \(V_{max} = 20.6\) nmoles per min per mg of protein.

Conversion of both substrates to pregnenolone was linear with respect to time (0 to 30 min) and to concentration of P-450 (0.05 to 0.5 n mole). The enzyme reaction is absolutely dependent on the electron transport system; removal of TPNH or adrenodoxin or adrenodoxin reductase from the reaction mixture completely abolished the production of pregnenolone from cholesterol. Addition of either hemin (1 to 4 nmoles) or MgSO₄ (1 to 20 nmoles) or bovine serum albumin (100 μg) to the assay solution (2 ml) had no effect on the side chain cleavage of cholesterol. On the other hand, addition of glycerol (5 to 20%) increased the enzyme activity by 20 to 50%.

It is also of interest to notice that with [4-¹⁴C]cholesterol as substrate, no intermediates (20α-hydroxycholesterol and 20α,

FIG. 6. Absorption spectroscopy of cytochrome P-450. P-450 was examined at room temperature both as the native protein and as the carbon monoxide complex (fixed) as described under “Experimental Procedure.” The concentrations used were as follows: absolute spectrum, 1.60 mg of protein per ml; CO-difference spectrum, 0.12 mg of protein per ml. Full scale for absorbance with the absolute spectrum is 1.0, while that for CO-difference spectrum is 0.1.

TABLE II
Spectral properties of purified cytochrome P-450 of adrenal mitochondria

Purified cytochrome P-450 was subjected to absorption spectroscopy as the native protein, after reduction by sodium dithionite, as carbon monoxide complex and as pyridine-hemochrome (see “Experimental Procedure”). Values for millimolar extinction coefficient are based on the protein concentration and the molecular weight (850,000) of P-450; these values represent means of determinations on three different preparations of P-450.

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<tr>
<th>Protuberance</th>
<th>Spectral peak</th>
<th>Extinction coefficient</th>
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* \(A_{414}-A_{403}\).*
22β-dihydroxycholesterol) were observed. No radioactive compound other than [4C]pregnenolone was isolated by thin layer chromatography. Moreover, it was shown that the specific radioactivity of [4C]pregnenolone remained constant through four recrystallizations from different solvents, within the limits of experimental error. The disappearance of [4C]cholesterol could be accounted for by the amount of [4C]pregnenolone formed.

Enzyme activity (side chain cleavage) of the purified cytochrome P-450 is stable for at least 3 months, if stored at -20° in 50 mm potassium phosphate buffer (pH 7.0) containing 50% glycerol. When stored at 5° without glycerol, activity decreased by 21 to 45% within 30 days and was completely lost in 4 months.

The optimal pH for the conversion of both cholesterol and 20α-hydroxycholesterol to pregnenolone in 75 mm potassium phosphate buffer was 6.8. The enzyme activity decreased by more than 50% at pH >7.5 and <6.0. The pH optimum for the 11β-hydroxylase activity was also found to be about 6.8. In addition, the magnitude of the CO-difference spectrum was greatest when the P-450 was dissolved in phosphate buffer at pH 6.5 to 7.0 (before addition of sodium dithionite).

**DISCUSSION**

The present observations deal with only one of the cytochromes P-450 extracted from bovine adrenal mitochondria. The yield of P-450 from the initial sonicate to final purification is approximately 7%. The enzymatic activities and other properties of the P-450 lost during purification have not been studied in detail. The method of isolating P-450 reported here is based upon cholic acid extraction of mitochondria as described by Mitani and Horie (28).

The purified cytochrome P-450 appears to be almost homogeneous according to three criteria: sedimentation velocity, sedimentation equilibrium in D2O and H2O, and chromatography on Bio-Gel. However the extent of purification effected by the methods used here is difficult to compute because certain changes, occurring in the enzyme during purification, make interpretation of enzyme assays difficult. For example, cholic acid is removed from the enzyme during purification, and since this steroid induces a difference spectrum with P-450, it may be that cholic acid influences enzymatic activities. Secondly, the concentration of adenodoxin and adenodoxin reductase (diphosphorase) required to secure maximal activity varies during purification. Although the activity of the purified enzyme is measured under optimal conditions, available supplies of adrenal tissue make it unrealistic to perform sufficient measurements to determine optimal conditions at every step in the purification of the cytochrome. Again P-450 contains bound (endogenous) cholesterol which is removed during purification. It is not known whether endogenous and exogenous cholesterol have equal access to the enzyme. Moreover the electron carriers and P-450 are presumably lipid-bound within the mitochondrion. During purification, lipid is removed from the enzyme, and it has been shown that addition of adrenal mitochondrial lipid accelerates the activity of P-450 (29). It may be then, that the activity of the enzyme is not optimal in aqueous solution and hence the comparison of crude and purified samples of P-450 may be misleading.

Purified P-450 shows, in addition to side chain cleavage activity, 11β-hydroxylase activity but no 18-hydroxylase activity. The capacity of the enzyme to support some 11β-hydroxylation relative to side chain cleavage activity decreases during purification to a point at which 11β-hydroxylase activity can be measured with only limited accuracy. This observation would be compatible with the presence of small amounts of 11β-hydroxylase as a contaminant, although it is not possible to exclude some inherent 11β-hydroxylase activity in the side chain cleavage enzyme. Evidently 18-hydroxylase activity requires a different enzyme system.

The cytochrome P-450 which is not extracted by cholic acid under the conditions reported is relatively rich in 11β-hydroxylase activity as compared to side chain cleavage (30). It seems probable that the P-450 described here is a major, if not the major, side chain cleavage enzyme.

The conversion of cholesterol to pregnenolone by the side chain cleavage enzyme occurs without demonstrable accumulation of the postulated intermediates 20α-hydroxycholesterol and 20α,22β-dihydroxycholesterol (10, 11), in keeping with findings using less purified enzyme (31, 32). If such intermediates do occur, they must be present in very small amounts.

The spectral properties of cytochrome P-450 indicate that it fulfills the requirements for classification as a cytochrome of the b type since the pyridine hemochromogen reveals the presence of protoporphyrin (33). In addition the pyridine hemochromogen reveals the presence of eight heme groups per molecule of P-450.

The molar extinction coefficient of the carbon monoxide complex (Table II) requires comment. The table shows the coefficient for 1 mole of P-450, so that the value per heme group would be 23, which is considerably lower than values observed with other cytochromes P-450 (21, 34). Moreover a variety of conditions including regassing, variations in the amount and state of dithionite added, etc., have all failed to increase the value. At this time we are unable to resolve this paradox which may result from failure of some heme groups to react with carbon monoxide or from an inherently low extinction for the heme groups of this P-450. In view of the findings with other cytochromes P-450, this last explanation seems less likely. It should be added that examination of the absolute spectrum of the carbon monoxide complex has failed to resolve these possibilities. For this reason, values for the amounts of P-450 presented in this paper are based upon a millimolar extinction coefficient of 91 in accordance with current practice; until this problem can be resolved, this value might be regarded as the contribution from active heme groups. The carbon monoxide difference spectrum also shows that this side chain cleavage P-450 contains no P-420 (i.e. less than 1%, Fig 6). It is interesting that this feature of this P-450 distinguishes it from most other cytochromes P-450 in which large amounts of P-420 are usually observed. Moreover, no more than minute amounts of P-420 are observed at any stage of the preparation. The amounts of P-420 are always too low to measure accurately in the presence of P-450. Ando and Horie (34) have also succeeded in preparing a cytochrome P-450 from adrenal mitochondria with very little P-420.

The absolute absorption spectrum of P-450 at room temperature shows a peak of approximately equal intensity at 412 and 395 nm. Absorbance at 412 nm (low spin ferriprotoheme) indicates the existence of four such heme groups per molecule of P-450. The peak at 395 nm reveals the presence of four high spin ferrisporphyrin groups per molecule, suggesting that under the conditions of isolation, P-450 is present as an approximately equal mixture of two forms in which the iron is present in high spin and low spin states, respectively. This conclusion is supported by spectral changes resulting from addition of glycerol. It has been found that glycerol converts the high spin state
(peaks at 650, 520, and 395 nm) to the low spin state (peaks at 565, 532, 417, and 356 nm).

The observations presented here are of interest in relation to those reported by Schleyer et al. (35) for a cytochrome P-450 from the same source as that from which the P-450 reported in the present paper was prepared. The enzyme isolated by Schleyer et al. catalyzes 11β-hydroxylation, shows little side chain cleavage activity, and is isolated in low spin state (35). Further comparison between these two cytochromes must await a detailed report of the physical properties of this 11β-hydroxylase cytochrome.

The cytochrome P-450 discussed here is of high molecular weight (850,000), contains eight heme groups per molecule, and shows an unusually high partial specific volume (0.765), suggesting that the molecule is rather loosely organized or packed. It is therefore not surprising to learn that P-450 is composed of subunits. The subunit structure of the adrenocortical side chain cleavage cytochrome P-450, forms the subject of the accompanying paper (36).

Acknowledgments—The authors are grateful to Dr. John C. Sutherland of the Department of Physiology and Dr. G. Wesley Hatfield of the Department of Microbiology, University of California, Irvine, School of Medicine for valuable discussions. The assistance of Mr. Vladimir Sturm with analytical ultracentrifugation and the excellent technical assistance of Miss Jennifer Lee Lewis are also gratefully acknowledged.

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Cytochrome P-450 from Bovine Adrenocortical Mitochondria: an Enzyme for the Side Chain Cleavage of Cholesterol: I. PURIFICATION AND PROPERTIES
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