Mechanisms of Steroid Oxidation by Microorganisms

XI. ENZYMATIC CLEAVAGE OF THE PREGNANE SIDE CHAIN*

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SUMMARY

Two enzymes are responsible for the cleavage of pregnane side chains. A steroid-inducible oxygenase, in the presence of reduced nicotinamide adenine dinucleotide and molecular oxygen, catalyzes the conversion of progesterone into testosterone acetate. The latter ester is then hydrolyzed by an esterase to yield testosterone. The oxygenase has been partially purified and separated from the esterase.

Evidence is herein presented to show that these two enzymes are also responsible for the degradation of pregnane side chains in 17α-hydroxypregn-4-ene-3,20-dione, deoxycorticosterone, pregna-4,16-diene-3,20-dione, and 16α,17α-oxidopregn-4-ene-3,20-dione.

Various fungi are capable of oxidizing side chains of steroids. Vischer and Wettstein (1) first noted that a Fusarium sp. was capable of cleaving the methyl ketone or the hydroxymethyl ketone but not the dihydroxymethyl ketone side chain of several 5α- and 5β-pregnanes. Since then extensive reports on the scope of side chain cleavage reactions by species of Penicillium, Aspergillus, Mucor, Fusarium, Gliocladium, and Cylindrocarpon have appeared (2, 3). As a rule, the final products after exposure of progesterone to these organisms are usually androst-4-ene-3,17-dione, testosterone, 17α-oxa-androst-4-ene-3,17-dione, or their 1-dehydro analogues. However, different microorganisms appeared to have altered specificity with respect to the steroid side chain; some organisms are unable to attack steroid side chains bearing substituents at C-16 or C-17, whereas other organisms are unaffected by substituents at these positions.

Cylindrocarpon radicicola is an organism which degrades progesterone via the sequence (4): progesterone \( \rightarrow \) pregna-1,4-diene-3,20-dione \( \rightarrow \) 17β-hydroxyandrost-4-ene-3,17-dione \( \rightarrow \) androsta-1,4-diene-3,17-dione \( \rightarrow \) 17α-oxa-androst-4-ene-3,17-dione. More recently, Fonken et al. (5) isolated the intermediate, testosterone acetate, after exposure of progesterone to Cladosporium resinae. Prázirč and Talalay (6) succeeded in obtaining an enzyme preparation from Penicillium lilacinum capable of converting androst-4-ene-3,17-dione into 17α-oxa-androst-4-ene-3,17-dione. From these results, the conversion of progesterone into 17α-oxa-androst-4-ene-3,17-dione by microorganisms may be ostensibly represented as follows.

![Chemical diagram]

The side chain cleavage enzyme system of C. radicicola appears to be nonspecific since the side chains of 17α-hydroxyprogren-4-ene-3,20-dione, pregna-4,16-diene-3,20-dione, and 16α,17α-oxidopregn-4-ene-3,20-dione were also readily attacked by this organism (7, 8). In all instances, the presumed ester intermediate, testosterone acetate, or its corresponding analogue has yet to be detected in intact cell experiments.

This study was initiated to decipher the mechanism of pregnane side chain cleavage with the use of partially purified preparations of C. radicicola and to establish whether a single enzyme system or multiple enzyme systems is involved in cleaving pregnane side chains bearing substituents at C-16 or C-17.

EXPERIMENTAL PROCEDURE

Materials—All solvents and inorganic chemicals were reagent grade. DEAE-cellulose (0.91 meq per g) was a product of Bio-Rad. NADPH and EDTA were purchased from Calbiochem. p-Hydroxymercuribenzoate and NADH were obtained from Sigma. All the radioactive steroids used in this work were products of the New England Nuclear Corporation. These include progesterone-4-14C (46 mCi per mmole), 17α-hydroxyprogren-4-ene-3,20-dione-4-14C (55 mCi per mmole), and 21-hydroxyprogren-4-ene-3,20-dione-4-14C (35 mCi per mmole). Nonradioactive steroids such as progesterone, testosterone, pregna-4,16-diene-3,20-dione, and 17α-hydroxyprogren-4-ene-3,20-dione were purchased from G. D. Searle and Co. 16α,17α-Oxidopregn-4-ene-3,20-dione was obtained from Nutritional Biochemical.
For routine experiments, the following procedure was followed. Stock cultures of *C. radicicola* (ATCC 11011) were maintained on Difco nutrient agar slants, supplemented with 1 % glucose and 1 % yeast extract. This organism was grown in 250-ml Erlenmeyer flasks containing 50 ml of the following medium: 0.6 % corn steep liquor, 0.3 % NH₄H₂PO₄, 0.35 % CaCO₃, 0.22 % soybean oil, 0.25 % yeast extract, and 1 % glucose. After incubation at 25° on a rotary shaker (250 rpm, 1 inch stroke) for 24 hours, 25 mg of progesterone in 0.4 ml of dimethylformamide were added to each flask to act as an inducer of the oxygenase, and the incubation was continued for an additional 10 hours. The cells were then harvested by filtration through cheesecloth and were washed with demineralized water, followed by 0.05 M Tris buffer, pH 7.6, containing 5 mM EDTA. The cell cake was stored in a deep freeze.

**Enzyme Assay**—A thin layer chromatographic assay was used to determine the quantity of this oxygenase. Although the product, testosterone acetate, could be separated by careful chromatography from the substrate, progesterone, an excess amount of esterase was added to the reaction mixture to facilitate the assay. The series of reactions involved can be visualized as follows.

\[
\text{Progesterone + O}_2 + \text{NADPH} \xrightarrow{\text{oxygenase}} \text{testosterone acetate} + \text{NADP}^+ + \text{H}_2\text{O} \tag{1}
\]

\[
\text{Testosterone acetate} + \text{H}_2\text{O} \xrightarrow{\text{esterase}} \text{testosterone} + \text{acetic acid} \tag{2}
\]

\[
\text{Sum: Progesterone + O}_2 + \text{NADPH} \rightarrow \text{testosterone + acetic acid + NADP}^+ \tag{3}
\]

By including an excess of esterase in the assay system, the rate-limiting reaction is the oxygenation step.

The routine assay system contained progesterone-4-¹⁴C (10,000 cpm), 0.26 µmole of NADPH, and a suitable amount of oxygenase in a total volume of 2.0 ml of 0.05 M phosphate buffer, pH 7.5, containing 2 mM EDTA. In crude enzyme preparations, the esterase is already present in excess amounts, but in purified preparations esterase must be added to the assay mixture. This mixture was incubated for 2 min at 25° with air as the gas phase. The reaction was terminated by the addition of 2 N HCl; extraction of the reaction mixture was performed with 3 ml of chloroform.

Two milliliters of the solvent layer were carefully transferred to a separate tube and evaporated to dryness. The residue was dissolved in 0.05 ml of chloroform, and 0.03 ml was applied onto a thin layer of silica gel HF plate. The plate was developed with a solvent of benzene-ethyl acetate-methanol (66:33:1). The relative mobility of the substrate progesterone is 0.45, and the relative mobility of the product testosterone is 0.25. The radioactivity on the thin layer plates was quantitatively assayed on a Vanguard Autoscaner model 880 ADS with the automatic data system. Under these conditions, the rate of formation of testosterone was proportional to the amount of added enzyme (Fig. 2). One unit of enzyme is defined as that amount of protein which catalyzed the formation of 5 × 10⁻⁶ moles of product per min at 25° under the specified reaction conditions.

**Purification Procedure: Step 1**—Frozen cells, 180 g, were thawed and diluted with 200 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 5 mM EDTA. The suspended cells were divided into
four equal portions for mechanical disintegration. The cells were ruptured by grinding with twice their weight of acid-washed sand for 15 min with a mortar and pestle. The mixture was diluted with buffer, and the cell debris was removed by centrifugation in a Servall SS-1 centrifuge for 20 min at 20,000 × g.

The supernatant was filtered through Whatman No. 1 filter paper, and 140 ml of the crude extract, containing approximately 20 mg of protein per ml, were obtained (Step 1). Disruption of cells by means of a Waring Blender or a magnetostriuctive oscillator did not yield as active extracts.

**Step 2: Ammonium Sulfate Precipitation**—To 135 ml of the crude extract (Step 1), 44 g of solid ammonium sulfate were slowly added with stirring to 60% saturation. The solution was kept at pH 7.5 by the constant addition of 1 N NH₄OH. After standing for 1 hour at 4°C, the precipitate was collected by centrifugation at 10,000 × g for 10 min, and the supernatant was discarded. The precipitate was dissolved in 24 ml of 0.05 M Tris buffer, pH 7.5, and was dialyzed for 90 min against three successive changes of 0.01 M Tris buffer, pH 7.55, containing 2 mM EDTA.

**Step 3: Protamine Sulfate Treatment**—The dialyzed enzyme solution was centrifuged at 5,000 × g for 5 min to remove protein precipitate. To 25 ml of the clear supernatant, 167 mg of protamine sulfate (1.0 mg of protamine sulfate for every 5 mg of protein) in 4.0 ml of 0.05 M Tris buffer, pH 7.5, were added dropwise with stirring. After standing for 5 min at 4°C, the precipitate was removed by centrifugation at 15,000 × g for 5 min. The supernatant was dialyzed against three changes of 800 ml of 0.01 M Tris buffer, pH 7.5, for 3 hours. The inactive protein precipitate was removed by centrifugation at 10,000 × g for 5 min. The volume of supernatant was 32 ml at this stage.

**Step 4: First DEAE-Cellulose Chromatography**—A column (1.2 × 35 cm) was packed with 4.5 g of diethylaminoethyl cellulose (prepared as described in "Experimental Procedure") and was equilibrated for 2 days with 0.03 M Tris buffer, pH 7.5, containing 2 mM EDTA. A 30-ml portion of the enzyme preparation from Step 3 was applied to the top of the column. The column was eluted according to a linear gradient elution technique in which the mixing vessel contained 200 ml of 0.03 M Tris buffer, pH 7.5, and the reservoir vessel contained 200 ml of 1 M NaCl in 0.03 M Tris buffer, pH 7.5. Fractions of 5.5 ml of eluates were collected in each test tube, and the flow rate was 1 ml per min. The protein concentration was followed spectrophotometrically by determining the absorbance at 280 μM. The esterase activity resided in the first protein peak (Fractions 6 through 20). The second protein peak (Fractions 29 to 39) contained the oxygenase activity; it was eluted when the NaCl concentration was 0.1 to 0.13 M. The elution profile is shown in Fig. 3. Fractions 29 to 39 were pooled, and the enzyme solution was brought to 70% saturation with ammonium sulfate. Simultaneously, the pH was adjusted to 7.5 by the addition of 1 N NH₄OH. After the solution had stood for 20 min, the precipitate was collected by centrifugation at 10,000 × g for 5 min. The precipitate was dissolved in 6.0 ml of 0.05 M Tris buffer, pH 7.5, and the solution was recentrifuged at 10,000 × g for 5 min to remove insoluble precipitates. The supernatant solution was dialyzed for 2 hours against 0.01 M Tris buffer, pH 7.5, containing 2 mM EDTA.

**Step 5: Second-Cellulose Column**—A column (1 × 16 cm) is prepared and equilibrated as described for Step 4. The supernatant solution (6 ml) from Step 4, containing 48 mg of protein, was added to the top of the column. The column was eluted with the same linear gradient system as described in the previous step. The flow rate was 1 ml per min and 3.5-ml fractions were collected. The oxygenase activity (Fractions 17 to 24) was pooled. This fraction represented approximately a 15-fold purification. Because of the instability of this oxygenase, the entire purification procedure was conducted as rapidly as possible. Usually the entire procedure requires continuous work of about 18 hours. A summary of the purification procedure is given in Table I.

**Enzymatic Conversion of Progesterone into Testosterone**—When the crude extract (Step 1) was incubated with progesterone, a product was formed having a mobility (0.25) upon paper chromatography (toluene-propylene glycol system) similar to that of testosterone. The structure of the end product was unequivocally established by the following experiment. The reaction mixture consisted of 50 mg of progesterone in 1.0 ml of dimethylformamide, 18 mg of NADP, 52 mg of glucose 6-phosphate, 10 K units of glucose 6-phosphate dehydrogenase, and 175 ml of enzyme (750 mg of protein), prepared by centrifuging the crude extract (Step 1) for 3 hours at 144,000 × g in a Spinco model L ultracentrifuge. The total volume was brought to 400 ml with 0.03 M phosphate buffer, pH 7.5, in a 2-liter Erlenmeyer flask. After incubation for 13 hours at 25°C, 20 ml of 2 N HCl were added. The protein precipitate was removed by filtration, and the filtrate was extracted with three 150-ml portions of chloroform. The chloroform extract was dried over sodium sulfate and concentrated to dryness to give a viscous oil. The oil was dissolved in 1.5 ml of chloroform and applied on three sheets (20 × 145 cm) of Whatman No. 1 paper. The chromatogram was developed for 8 hours in a toluene-propylene glycol system (12). The slower moving, ultraviolet-absorbing band was cut out and extracted with three 100-ml portions of chloroform-methanol (1:1) mixture. The combined extracts were evaporated to dryness under reduced pressure. The residue was dissolved in 50 ml of chloroform and was washed with 2 volumes of water. The solvent layer was dried over sodium sulfate and evaporated to dryness.

![Fig. 2. Linearity of the progesterone oxygenase assay. The enzyme fraction used was that of Step 3. The conditions are described in the text.](http://www.chemistry.org/figures/fig2.png)
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Enzymatic Conversion of 17α-Hydroxypregn-4-ene-3,20-dione into Androst-4-ene-3,17-dione—To 50 mg of 17α-hydroxypregn-4-ene-3,20-dione in 1 ml of dimethylformamide were added 18 mg of NADP, 50 mg of glucose 6-phosphate, 15 K units of glucose 6-phosphate dehydrogenase, and 190 ml of crude enzyme (Step 1, centrifuged at 140,000 × g for 1 hour). The final volume was adjusted to 200 ml with 0.05 M phosphate buffer, pH 7.5. The reaction mixture was incubated for 16 hours at 25°. The product, androst-4-ene-3,17-dione, was isolated by chromatographing the chloroform extract on a silica gel G plate (1 mm thick, 20 × 20 cm) and developed in chloroform-acetone (98:2). The faster moving, ultraviolet-absorbing band was eluted with a mixture of chloroform-methanol (1:1).

Enzymatic Transformation of Pregna-4,16-diene-3,20-dione into Androst-4-ene-3,17-dione—To 200 ml of crude enzyme (Step 1, centrifuged at 140,000 × g for 1 hour) were added 50 mg of pregna-4,16-diene-3,20-dione in 1 ml of dimethylformamide, 50 mg of glucose 6-phosphate, and 15 K units of glucose 6-phosphate dehydrogenase, and the reaction mixture was allowed to stand at 25° for 16 hours. The product, androst-4-ene-3,17-dione, was isolated by streaking the chloroform extract residue across a sheet of Whatman No. 1 paper (20 × 45 cm) and developed for 3 hours in an isooctane-propylene glycol system (13). The slower moving, ultraviolet-absorbing band was eluted with chloroform-methanol (1:1). A total of 7 mg of androst-4-ene-3,17-dione was obtained, m.p. 152–169°.

Enzymatic Transformation of Deoxycorticosterone into Testosterone—To 150 mg of deoxycorticosterone, dissolved in 2 ml of dimethylformamide, were added 54 mg of NADP, 155 mg of glucose 6-phosphate, 50 K units of glucose 6-phosphate dehydrogenase, and 686 mg of oxygenase protein (Step 1, centrifuged at 38,000 × g for 90 min) in a total volume of 300 ml of 0.05 M Tris buffer, pH 7.5. The reaction mixture was allowed to react at 25° for 9 hours. The product, testosterone, was isolated in a manner similar to that in the progesterone experiment. This time, 8 mg of testosterone, m.p. 152–154°, were obtained.

Enzymatic Transformation of 16α,17α-oxidopregn-4-ene-3,20-dione into 16α-Hydroxyandrost-4-ene-3,17-dione—To 50 mg of 16α,17α-oxidopregn-4-ene-3,20-dione in 1 ml of dimethylformamide were added 18 mg of NADP, 50 mg of glucose 6-phosphate, 20 K units of glucose 6-phosphate dehydrogenase, 100 ml of oxygenase (specific activity 15, 950 units), and 50 ml of esterase (70 mg of protein obtained from first DEAE-cellulose crystallizations from acetone-petroleum ether afforded 9.5 mg of androst-4-ene-3,17-dione, m.p. 168–169°.
reaction was terminated by the addition of 2 N HCl, and the incubated at 25 ° for 4 hours with occasional shaking. The mixture was allowed to react for 10 hours at 25 °. After removing the protein precipitate by filtration, the filtrate was combined chloroform extract was evaporated to dryness to give 56 mg of residue. 16a-Hydroxyandrost4-ene-3,17-dione was then extracted with three 200-ml portions of chloroform. The combined chloroform extract was evaporated to dryness to give 56 mg of residue. 16a-Hydroxyandrost4-ene-3,17-dione was isolated by chromatographing the residue on a silica gel G plate in a total volume of 2 ml of 0.05 M Tris buffer, pH 7.5. The reaction mixture was incubated for 3 min at 25 °.

<table>
<thead>
<tr>
<th>Substrate added</th>
<th>Substrate remaining</th>
<th>Coenzyme added</th>
<th>Product formed</th>
</tr>
</thead>
<tbody>
<tr>
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<td>cpm</td>
<td>cpm</td>
<td>cpm</td>
</tr>
<tr>
<td>9368</td>
<td>9221</td>
<td>None</td>
<td>54</td>
</tr>
<tr>
<td>9968</td>
<td>2510</td>
<td>NADP</td>
<td>7462</td>
</tr>
<tr>
<td>8833</td>
<td>8780</td>
<td>NADH</td>
<td>148</td>
</tr>
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</tr>
<tr>
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<td>8000</td>
<td>FAD</td>
<td>132</td>
</tr>
<tr>
<td>8250</td>
<td>8100</td>
<td>-</td>
<td>21</td>
</tr>
</tbody>
</table>

Table II

Cofactor Requirement

The assay system contained 06.6 μg of progesterone oxygenase (Step 5) and an excess of esterase (first DEAE-cellulose column), with or without 0.25 μmole of reduced pyridine nucleotides and progesterone-4-14C, in a total volume of 2 ml of 0.05 M phosphate buffer, pH 7.5. The reaction mixture was incubated for 3 min at 25 °.

Isolation and Characterization of Testosterone Acetate—The reaction mixture consisted of 50 mg of progesterone, dissolved in 0.5 ml of dimethylformamide, 20 μmoles of NADP+ in 2 ml of 0.05 M Tris buffer (pH 7.5), 10 K units of glucose 6-phosphate dehydrogenase, 50 μg of glucose 6-phosphate, and 12 mg of purified oxygenase (Step 5, specific activity 15) in a total volume of 100 ml of 0.05 M Tris buffer (pH 7.5). The mixture was incubated at 25 ° for 4 hours with occasional shaking. The reaction was terminated by the addition of 2 N HCl, and the protein precipitate was removed by filtration. The filtrate was extracted five times with five 100-ml portions of chloroform.

RESULTS

Stability—Preparations of the oxygenase enzyme were highly unstable; with crude extracts, 85% of the enzyme activity was lost in 24 hours when stored at 0–6 °, whereas 80% of the enzyme activity was lost in 7 days when kept frozen. The most highly purified preparation (Step 5) appears to be even more unstable; the half-life was 12 hours when kept frozen. The oxygenase was found to be most stable between pH 7 and 8, and it was irreversibly inactivated below pH 5.4.

In an attempt to stabilize the enzyme, a variety of reducing agents were added at 10⁻⁴ and 10⁻³ M concentrations to the enzyme, but no protection of enzyme activity was observed. These included glutathione, cysteine, mercaptoethanol, sodium hydrosulfite, 2,3-dimercaptopropanol, mercaptoacetic acid, and dithiothreitol. Albumin at a concentration of 1 mg per ml stabilized the crude enzyme extract for a month when kept frozen. EDTA (5 × 10⁻³ M) afforded some protection, especially among crude extracts. Preincubation of the inactivated enzyme (due to prolonged storage) with 0.1% NaBH₄, for 20 min at 0 ° restored about 40% of its original activity. Similar reactivation has been noted with other enzymes (14). Attempts to store enzyme preparations under an atmosphere of nitrogen gas afforded no protection.
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5.0 6.0 7.0 8.0 9.0 10.0
pH of Incubation Mixture

Fig. 5. Effect of pH on enzyme activity. The reaction mixture contained 0.65 mg of enzyme protein (Step 4), 0.38 mmole of NADPH, and progesterone-4-¹⁴C or 17α-hydroxypregn-4-ene-3,20-dione or deoxycorticosterone (10,000 cpm), in a total volume of 2.0 ml of 0.05 M Tris buffer at the pH values indicated.

Effect of pH on enzyme activity. The reaction mixture contained 0.65 mg of enzyme protein (Step 4), 0.38 mmole of NADPH, and progesterone-4-¹⁴C or 17α-hydroxypregn-4-ene-3,20-dione or deoxycorticosterone (10,000 cpm), in a total volume of 2.0 ml of 0.05 M Tris buffer at the pH values indicated.

TABLE III
Effect of inhibitors on progesterone oxygenase activity

Progesterone oxygenase (80 µg, Step 4) was preincubated with various inhibitors for 5 min at 25°C at the indicated concentrations. The reaction was initiated by the addition of progesterone-4-¹⁴C and 0.36 mmole of NADPH in a total volume of 2.0 ml of 0.05 M phosphate buffer, pH 7.5. The reaction was terminated after 15 min. The product, testosterone acetate-4-¹⁴C, was assayed quantitatively on a Vanguard Autoscanner model 880 ADS with the automatic data system. The paper chromatogram had been developed in a isooctane-propylene glycol system (13). In this system, progesterone has a relative mobility of 0.9 of testosterone acetate.

<table>
<thead>
<tr>
<th>Inhibitors</th>
<th>Concentration</th>
<th>Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Hydroxymercuribenzoate</td>
<td>5 × 10⁻³</td>
<td>81</td>
</tr>
<tr>
<td>α-Iodoacetamide</td>
<td>5 × 10⁻³</td>
<td>0</td>
</tr>
<tr>
<td>KCN</td>
<td>1 × 10⁻²</td>
<td>55</td>
</tr>
<tr>
<td>α,α'-Dipyridyl</td>
<td>5 × 10⁻³</td>
<td>0</td>
</tr>
<tr>
<td>α,α'-Diphenanthroline</td>
<td>5 × 10⁻³</td>
<td>0</td>
</tr>
<tr>
<td>Phenazine methylsulfate</td>
<td>5 × 10⁻²</td>
<td>95</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>1 × 10⁻²</td>
<td>35</td>
</tr>
<tr>
<td>Hydroxylysinamide</td>
<td>1 × 10⁻²</td>
<td>0</td>
</tr>
<tr>
<td>8-Hydroxyquinoline</td>
<td>1 × 10⁻³</td>
<td>0</td>
</tr>
</tbody>
</table>

Cofactor Requirement—The oxygenase showed an absolute requirement for NADPH (Table II). FMN, FAD and 2-amino-6,7-dimethyl-4-hydroxy-5, 6, 7, 8-tetrahydropteridine, and NADH could not replace NADPH as the hydrogen donor.

When the purified enzyme preparation (Step 5) was dialyzed against 0.01 M Tris buffer, pH 7.5, containing 0.005 M EDTA for 10 hours, 50% of the enzyme activity was lost. The activity was not restored by the addition of Fe⁺⁺, Cu⁺⁺, Co⁺⁺, Mn⁺⁺, Zn⁺⁺, Ni⁺⁺, and Mg⁺⁺ ions at 10⁻³ M concentrations.

Aerobic Nature of Reaction—A Thunberg tube (1.5 × 15 cm) contained a mixture of progesterone-4-¹⁴C (10,000 cpm), 0.25 mmole of NADPH, and 0.32 mg of esterase protein (first DEAE column) in a total volume of 1.8 ml of 0.05 M phosphate buffer (pH 7.5). Oxygenase (0.22 mg in 0.2 ml from Step 5) was added to the side arm. The tube was repeatedly evacuated with a

Table IV
Comparison of oxygenase activity on progesterone-4-¹⁴C, deoxycorticosterone-4-¹⁴C, and 17α-hydroxypregn-4-ene-3,20-dione-4-¹⁴C during all stages of purification

The assay system is described in the text.

<table>
<thead>
<tr>
<th>Steps</th>
<th>17α-HPD:PD</th>
<th>21α-HPD:PD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ ppt.</td>
<td>0.90</td>
<td>0.91</td>
</tr>
<tr>
<td>Protamine treatment</td>
<td>1.20</td>
<td>1.20</td>
</tr>
<tr>
<td>First DEAE-cellulose effluents</td>
<td>1.10</td>
<td>0.77</td>
</tr>
<tr>
<td>Second DEAE-cellulose effluents</td>
<td>0.80</td>
<td>0.91</td>
</tr>
</tbody>
</table>

The abbreviations used are: Pd, progesterone-4-¹⁴C; 21α-HPD, deoxycorticosterone-4-¹⁴C; and 17α-HPD, 17α-hydroxypregn-4-ene-3,20-dione-4-¹⁴C.

* Ratios of activity in the crude extract have been arbitrarily set at 1.00.
A (left), competitive inhibition of progesterone oxygenase activity by 17α-hydroxyprogren-4-ene-3,20-dione. •—•, 3 mmoles; O—O, 6 mmoles; △—△, 9 mmoles. The condition of assay is described in the text, except the substrate (progesterone) concentrations were varied as indicated. B (right), competitive inhibition of progesterone oxygenase activity by pregna-4,16-diene-3,20-dione. ▲—▲, no pregna-4,16-diene-3,20-dione; □—□, 3 mmoles; ■—■, 6 mmoles; △—△, 9 mmoles. The condition of assay is described in the text except the substrate (progesterone) concentrations were varied as indicated.

water aspirator and then filled with an atmosphere of N₂ and CO₂ gas (95:5). The oxygenase was then allowed to mix with the reaction mixture, and the mixture was then incubated for 3 min at 25°C. A control tube was run simultaneously under identical conditions except that the tube was left open to air. No significant amount of product formation was observed under anaerobic conditions. Although the direct incorporation of oxygen has not yet been shown with ¹⁸O, the oxygenation reaction apparently requires O₂ as well as NADPH, and may be classified as a mono-oxygenase or mixed function oxidase.

Effect of pH on Enzyme Activity—The activity of C. radicicola oxygenase was tested over the pH range 6 to 9 in Tris-HCl and phosphate buffers. Optimal enzyme activity was observed around pH 7.5 for progesterone, deoxycorticosterone, and 17α-hydroxyprogren-4-ene-3,20-dione (Fig. 5). Both Tris-HCl and phosphate buffer had the same effect on enzyme activity.

Effect of Inhibitors—The following compounds showed no inhibition when they were incubated with the purified oxygenase (Step 5) at a concentration of 10⁻³ M: iodoacetamide, α,α',dipyridyl, o-phenanthroline, hydroxylamine, and 8-hydroxyquinoline. p-Hydroxymercuribenzoate at 5 X 10⁻³ M afforded 81% inhibition of oxygenase activity, and this inhibition was completely reversed by the addition of 5 x 10⁻³ M mercaptoethanol. This result implicates the presence of a reactive sulfhydryl group in the enzyme. KCN at 1 x 10⁻² M gave 55% inhibition. Phenazine methosulfate and methylene blue were also inhibitory to this oxygenase, presumably by the nonenzymatic oxidation of NADPH (Table III).

Substrate Specificity—The relative activity of the oxygenase on a number of steroids was compared as shown in Table IV. As the end products of different steroidal substrates are different, the comparison of rates was based on the disappearance of substrates. Several conclusions can be drawn with respect to chemical structure and enzymatic activity. The presence of electron-withdrawing or electron-rich substituents at C-16 or C-17, or both, did not inhibit but appeared to stimulate the rate of metabolism, for 17α-hydroxyprogren-4-ene-3,20-dione, 16α,17α-oxidopregren-4-ene-3,20-dione, and pregna-4,16-diene-3,20-dione were all metabolized at rates faster than that of progesterone. However, it appeared that electron-donating groups may interfere with the oxygenase, for 17α-methylpregn-4-ene-3,20-dione was metabolized at a very slow rate. (On a thin layer plate, a compound with a mobility of 17α-methyltestosterone was noted.) The presence of a 20-carbonyl group appears to be essential for activity; 20α-hydroxyprogren-4-ene-3-one and 20α-hydroxy-16α,17α-oxidopregren-4-ene-3,20-dione were not metabolized. The presence of a hydroxyl function at C-21 did not affect the rate appreciably as deoxycorticosterone was metabolized at a rate of 65% of progesterone. On the other hand, no activity was observed when cortisol was incubated with the enzyme, which suggests that 11β-hydroxy compounds do not serve as substrates for the enzyme.

Since the end products from the reaction of oxygenase with different steroids are different, a series of large scale incubations were carried out to isolate and identify the reaction products. For convenience, crude enzyme preparations which contained both oxygenase and esterase were used. However, the end products in each case were the same (checked by mobilities on thin layer and paper chromatography) as those obtained by the use of purified oxygenase and esterase, obtained from DEAE-cellulose chromatography. Under these conditions, progesterone and deoxycorticosterone were transformed into testosterone, whereas 17α-hydroxyprogren-4-ene-3,20-dione and pregna-4,16-diene-3,20-dione were converted into androst-4-ene-3,17-dione. 16α,17α-Oxidopregren-4-ene-3,20-dione was converted into 16α-hydroxyandroster-4-ene-3,17-dione.

From the work of Fonken et al. (5) with Cladosporium resinae, it appeared that C. radicicola may also cleave the side chain of...
progesterone via oxygenation of the Baeyer-Villiger type, resulting in the formation of testosterone acetate. However, due to the broad substrate specificity of this side chain cleavage system and the fact that 17α-hydroxyprogesterone was metabolized at a faster rate than progesterone, an alternate mode of side chain cleavage cannot be excluded at this stage. Thus, it appeared imperative to establish the intermediate in the conversion of progesterone into testosterone. As intact cells and crude cell extracts of *C. radicicola* contain a high level of esterase, it is necessary to prepare oxygenase preparation free from esteratic activity. This can be conveniently achieved by DEAE-cellulose chromatography (Fig. 3). When progesterone was incubated with this purified enzyme preparation, free from esterase, testosterone acetate was indeed the end product. This experiment establishes the mechanism of side chain degradation by *C. radicicola*.

The next step is to establish whether the same oxygenase or different oxygenases are responsible for the breakdown of pregnane side chains, bearing different substituents at C-16 and C-17. An experiment was devised to assay the relative ratio of oxygenase activity of 17α-hydroxyprogesterone and pregnenolone as well as deoxycorticosterone and progesterone. If different enzymes were responsible for the degradation of these side chains, one would expect that the ratio of relative enzyme activities would vary markedly during the purification procedure. However, the results in Table V show no significant variation in relative ratios of enzyme activity; in fact, they remained fairly constant as the enzyme extract undergoes purification. These data are consistent with the fact that a single enzyme is responsible for the cleavage of all these different steroids. This view is further substantiated by the fact that 17α-hydroxyprogesterone and pregnenolone competitively inhibited the conversion of progesterone into testosterone (Fig. 6, A and B).

**Discussion**

The results herein presented clearly show that two enzymes are involved in the degradation of the progesterone side chain. A steroid-inducible oxygenase, in the presence of molecular oxygen and NADPH, is capable of inserting an oxygen atom between C-17 and C-20 of progesterone, resulting in the formation of testosterone acetate. An esterase hydrolyzes the latter ester into testosterone and acetic acid. The failure to detect the presence of testosterone acetate with intact cells or crude cell extracts of *C. radicicola* can be attributed to the high level of esterase present, which readily hydrolyzed the ester intermediate.

According to the terminology of Hayaishi (15), this oxygenase belongs to the class of mono-oxygenases, or in the terminology of Mason (16), mixed function oxidases. The conversion of progesterone into testosterone acetate parallels the mechanism of the chemical Baeyer-Villiger reaction in which a peracid attacks the 20-carbonyl to give an addition compound which then rearranges to yield testosterone acetate.

Previous work in our laboratory (7, 8) has shown that when 16α,17α-oxidopregnen-4-ene-3,20-dione was exposed to *C. radicicola*, four products were obtained. These have been identified as 20α-hydroxy-16α,17α-oxidopregnen-4-ene-3-one, 20α-hydroxy-16α,17α-oxidopregnen-4-ene-3-one, 16α-hydroxy-17α-oxandrosta-1,4-diene-3,17-dione, and 16α,17β-dihydroxy-17α-oxandrosta-1,4-diene-3-one. Although 16α-hydroxyandrosta-1,4-diene-3,17-dione has been shown to be converted into 16α-hydroxy-17α-oxandrosta-1,4-diene-3,17-dione, it had never been detected in the fermentation medium. The failure of cell extracts to metabolize 20α-hydroxy-16α,17α-oxidopregnen-4-ene-3-one, coupled with the isolation of this missing intermediate, 16α-hydroxy-17α-oxandrosta-1,4-diene-3,17-dione, which was formed by reaction of 16α,17α-oxidopregnen-4-ene-3,20-dione with purified oxygenase and esterase, a reasonable mechanism for epoxide opening can now be formulated.

It was shown previously that 17α-hydroxyprogren-4-ene-3,20-dione and pregna-4,16-diene-3,20-dione were converted by intact cells of *C. radicicola* in a series of reactions to 17α-oxandrosta-1,4-diene-3,17-dione as the end product. With the isolation of androsta-4-ene-3,17-dione after incubation of these substrates with the cell extracts of *C. radicicola*, a reasonable scheme for the loss of their pregnane side chains can be visualized as above (lower scheme). The mode of cleavage of the side chain in 17α-hydroxyprogren-4-ene-3,20-dione could proceed via two routes. Oxygenation of 17α-hydroxyprogren-4-ene-3,20-dione yields 17α-hydroxytestosterone acetate; the latter ester could then undergo spontaneous nonenzymic rearrangement with the loss of acetate and the formation of androsta-4-ene-3,17-dione. Alternatively, this ester may be hydrolyzed by the esterase to yield the unstable intermediate, 17α-hydroxytestosterone; elimination of water results in androsta-4-ene-3,17-dione. The loss of the side chain in pregnen-4,16-diene-3,20-dione may proceed via oxygenation to afford an enol acetate which could
then be hydrolyzed by an esterase to give androst-4-ene-3,17-dione.

The 17-keto steroids, thus formed, could then be oxygenated by the lactonizing enzyme (6) to yield their respective lactones.

Several lines of evidence support the view that a single oxygenase, possessing broad substrate specificity, is involved in attacking pregnane side chains possessing different substituents at C-16 and C-17. It was shown that the pH profile for progesterone, deoxycorticosterone, and 17α-hydroxypregn-4-ene-3,20-dione had a similar behavior. The ratio of enzymatic activities on these different substrates did not change during purification. Also, 17α-hydroxypregn-4-ene-3,20-dione and pregn-4,16-diene-3,20-dione competitively inhibited the conversion of progesterone into testosterone. In addition, the end products, obtained after the enzymatic incubation with these different substrates, are all in accord with the proposed mechanisms.

This type of oxygenase appears to be widely distributed in nature, for example, the lactonization of camphor (17), of androst-4-ene-3,17-dione (6), and that of eburicoic acid (18). From our cofactor studies, this oxygenase appears to differ from that of camphor-lactonizing enzyme, where NADH, FMN, and Fe++ have been shown to be required for the reaction. Our enzyme has an absolute requirement for NADPH, tetrahydropteridine, FMN, FAD, and Fe++ ions had no apparent effect on this oxygenase. These properties are similar to the enzyme catalyzing the lactonization of androst-4-ene-3,17-dione (6).

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