Mechanisms of Steroid Oxidation by Microorganisms

IX. ON THE MECHANISM OF RING A CLEAVAGE IN THE DEGRADATION OF 9,10-SECO STEROIDS BY MICROORGANISMS*


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SUMMARY

Cell extracts, prepared from Nocardia restrictus, rapidly oxidize 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione with the consumption of 1 mole of oxygen per mole of substrate; no carbon dioxide is evolved. The reaction is stimulated by the presence of ferrous ions.

The first product of ring fission has been identified as 4(5),9(10)-diseco-3-hydroxyandrosta-1(10),2-diene-5,9,17-trion-4-oic acid. The enzyme catalyzing the formation of this compound has been tentatively termed 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-dioxygenase. The possible role of a quinone intermediate in the formation of the ring fission compound has been eliminated.

Further metabolism of the ring fission compound results in the formation of 2-oxo-4-hydroxycaproic acid (isolated as its y-lactone) and 3aα-H-4a-[3'-propionic acid]-7αβ-methylhexahydro-1,5-indanedione. Whole cells in the presence of 0.001 M arsenite cleave 2-oxo-4-hydroxycaproic acid to pyruvate and propionaldehyde.

A reaction sequence has been proposed for the metabolism of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by N. restrictus.

The microbial degradation of the steroid nucleus was first reported by Turfitt (1), who showed the formation of Winands' keto acid (5-oxo-A-nor-3,5-secocholestan-3-oic acid) as a product of cholesterol-4-en-3-one metabolism by Pseudoomonas erythropolis. Although this result has not been confirmed, the rapid loss of carbon atom 4, observed when 4-14C-cholesterol was incubated with a Mycobacterium species (2), would seem to indicate that the degradation by Nocardia restrictus results in the formation of 3aα-H-4a-[3'-propionic acid]-7αβ-methylhexahydro-1,5-indanedione. We now wish to present evidence for the intermediates in the conversion of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione into 3aα-H-4a-[3'-propionic acid]-7αβ-methylhexahydro-1,5-indanedione. A preliminary report of the work described herein has been published (8).

EXPERIMENTAL PROCEDURE

Materials—3,4 Dihydroxy 9,10 secoandrosta-1,3,5(10) triene-9,17-dione was synthesized by the procedure of Sih et al. (7), 3aα-H-4a-[3'-Propionic acid]-7αβ-methylhexahydro-1,5-indanedione was prepared according to Sih and Wang (6). 2-Oxo-4-ethylbutyroacetone was obtained by the method of Rossi and Shinh (9). 2-Oxo-4-hydroxyacryl acid was prepared by mild hydrolysis of its y-lactone with 1 eq of alkali at room temperature for 4 hours. Relactonization of the hydroxy acid was achieved by heating in 0.8 N H2SO4 on a boiling water bath for 3 min. 9,10-secoster-1,5(10)-dione-3,4,9,17-tetraene was prepared by oxidation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione with AgO according to the method of Willstatter and Muller (10). Diazomethane was prepared from Diazald according to the method of Moore and Reed (1). All of the other catechol compounds were from Gallard-Schlesinger Chemical Manufacturing Corporation, Garden City, New York. Sodium pyruvate and propionaldehyde were from Matheson, Coleman, and Bell. Ethylene-1,2-diamino dihydrochloride was from New England Nuclear Corporation. Mushroom tyrosinase was from Sigma. Silica Gel HF for thin layer chromatography was purchased from Brinkmann Instruments, Inc. Silica gel (Malinekrodt 2847) was used for column chromatography. Standard grade cellulose powder was purchased from H. Reeve Angel and Company. Diazald (N-methyl-N-nitro-p-toluenesulfonamide) was a product of Aldrich Chemicals Company. Sephadex G-25 (100 to 250 mesh) was purchased from Pharmacia Fine Chemicals.
Oxidation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione by cell extract. The main compartments of Warburg flasks contained, in a final volume of 3.0 ml, 20 μmoles of KHzPO4, pH 8.0, and cell extract (18 mg of protein). 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (5 μmoles in 0.1 ml of a solution containing 1 part of dimethylformamide to 4 parts of water) was added from the sidearm. Results are corrected for endogenous oxygen utilization. ••••, FeSO4 absent; O—O, FeSO4 (3.3 X 10^-4 M). and neutral alumina (Woelm) was obtained from Alupharm Chemicals. All of the other reagents were of the highest purity commercially available.

Chemical Analysis—Pyruvic acid was determined by the method of Friedemann and Haugen (12). When 2-oxo-4-hydroxyacrylic acid and pyruvic acid were present in a reaction mixture, the solution was acidified and heated on a boiling water bath for 3 min. Under these conditions, the hydroxy acid lactonized to give 2-oxo-4-ethylbutyrolactone which did not interfere in the estimation.

Chromatography—Whatman No. 1 paper (Reeve Angel and Company) was used for paper chromatography. Paper chromatography of neutral compounds was carried out in a toluene-propylene glycol system (13). Acidic compounds were chromatographed in butanol saturated with 1.5 N NH4OH (14). Acidic 2,4-dinitrophenylhydrazones were chromatographed on thin layers of Silica Gel HF (Brinkmann Instruments) prepared as described by Stahl (15). The solvent systems used were benzene-ethyl acetate-acetic acid (90:5:5) and benzene-ethyl acetate-propionic acid (90:5:5). Neutral 2,4-dinitrophenylhydrazones were identified by reverse phase chromatography (16). Whatman No. 1 paper was pretreated with a 1:1 mixture of dimethylformamide and ethyl ether. The chromatogram was developed with decahydronaphthalene, saturated with dimethylformamide-ether (1:1).

Other Methods—Melting points, determined on a Thomas-Hoover melting point apparatus, are corrected. Ultraviolet absorption spectra were determined on a Cary model 11 M3 recording spectrophotometer. Infrared spectra were recorded on a Beckman model IR 5A double beam infrared recording spectrophotometer. All of the nuclear magnetic resonance spectra were determined on a Varian Associates recording spectrometer (A 60) at 60 Mc in either deuterated chloroform or deuterated acetone. Chemical shifts are reported in r values (parts per million) (17). Microanalyses were carried out by Mr. J. Alicino of Metuchen, New Jersey. Mass spectra of all of the samples were taken on a CEC-103C mass spectrometer operating at an ionization voltage of 70 volts and employing a heated glass inlet system at 250°.

Preparation of Enzyme Extract— Cultures of Nocardia restrictus (ATCC 14887) were grown in 2-liter Erlenmeyer flasks with 400 ml of Difco nutrient broth and 0.05% progesterone as an inducer of enzymes. The flasks were incubated at 25° on a rotary shaker (250 rpm, 1-in. stroke). After 48 hours of growth, the cells were harvested in a Servall (SS-1) continuous flow centrifuge and then washed with 0.03 m phosphate buffer, pH 7.0. The washed cells were suspended in 0.02 m potassium phosphate buffer, pH 7.2 (2 ml of buffer being used for each gram of cell paste), and placed in a sonic field of Raytheon magnetostriective oscillator (10 Kc) for 15 min. After clarification of the extracts by centrifuging at 27,000 X g for 45 min, protein concentrations were determined by the method of Gornall, Bardawill, and David (18). In some instances the extracts were applied to a column of Sephadex G-25 (100 to 250 mesh).
and were eluted with 0.02 M potassium phosphate buffer, pH 7.6.

**RESULTS**

Oxidation of 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by Cell Extracts—Cell extracts of *N. restrictus* oxidized 1 mole of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione with the uptake of 1 mole of oxygen, but no carbon dioxide was evolved. During the reaction, a transient yellow color was produced in the reaction mixture. When ferrous ions were preincubated with the cell extract prior to the addition of the substrate, there was a significant increase in the rate of oxygen utilization, although the over-all stoichiometry of the reaction remained the same (Fig. 1). The specificity of this cell extract on a variety of catechols is shown in Fig. 2. Of the following compounds, 3-methylcatechol, 4-methylcatechol, catechol, 4-isopropylcatechol, 3-methyl-5-tertiary octylcatechol, 4-tertiary octylcatechol, 4-hydroxyestradiol, 3-isopropylcatechol, and 3-tertiary butyl-5-methylcatechol, only the last two compounds were significantly metabolized.

Formation of Yellow Intermediate—The oxidation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione was followed spectrophotometrically as adjudged by the decrease in absorption at 250 μm (Fig. 3). Cell extracts which were eluted from a Sephadex column accumulated a yellow compound with absorption maxima at 325 and 393 μm. The yellow color rose to maximum over a period of 30 min and then gradually disappeared (Fig. 4). Such extracts were used to isolate small quantities of the yellow intermediate for further studies. 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione was incubated with Sephadex-treated cell extract (5.0 mg of protein) in 4 ml of 0.03 M potassium phosphate buffer, pH 7.6, for 1 hour at 25°C. The yellow reaction mixture was then extracted three times with 1.0-ml portions of chloroform in order to remove unreacted substrate. The aqueous phase was then acidified to pH 3.0 with acetic acid, and the chloroform extraction was repeated. The combined chloroform extracts were evaporated to dryness under a stream of nitrogen. When the yellow intermediate, prepared in this manner, was incubated with the crude cell extract, it was further metabolized (Fig. 5). Chromatography of the products formed in this reaction revealed the presence of 3αα-H-4α-[3'-propionic acid]-7αβ-methylhexahydro-1,5-indandione.

The absorption spectrum of the yellow intermediate varied with pH (Fig. 6). At neutral pH, peaks were observed at 330 and 393 μm. In acid solution, the peak at 393 μm shifted to 315 μm while the peak at 330 μm disappeared. In alkaline solution, the peak at 393 μm increased in absorption. The extinction in alkali was approximately 3 times as great as that observed in acid. Such changes in absorption spectra are indicative of keto-enol tautomerism and are similar to the spectra given by the products formed from catechol (19), 2,3-dihydroxy-β-phenylpropionic acid (20), and 3,4-dihydroxyphenylacetic acid (21) by asymmetrical oxygenases.
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Fig. 5. Metabolism of the yellow intermediate by cell extract. The cuvettes contained, in a final volume of 3.0 ml, 270 μmoles of KH₂PO₄, pH 7.6, the yellow intermediate obtained from the reaction of 1.58 μmoles of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione with Sephadex-treated extract, and crude cell extract (3.0 mg of protein). A reference cuvette contained all of the components except for the omission of the yellow intermediate.

Fig. 6. Absorption spectrum of the yellow intermediate. The cuvettes contained the yellow intermediate, formed after reaction of 0.39 μmoles of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione with Sephadex-treated cell extract in 3.0 ml of 0.1 N HCl; Curve A; 0.1 N NaOH, Curve B; 0.3 M KH₂PO₄ buffer, pH 5.5. Curve C; same buffer at pH 7.5, Curve D. The content of Curve B was diluted 4 times with 0.1 N NaOH.

Scheme 1. Possible sites of ring fission

Scheme 1 illustrates the possible sites of ring cleavage in 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione by N. restrictus. Fission A would produce a muconic acid with no associated keto-enol tautomerism, and thus this reaction may be eliminated from consideration. The properties of the yellow intermediate would be consistent with structures postulated in Fission B (between C-2 and C-3) and in Fission C (between C-4 and C-5). In order to establish the correct mode of ring fission, it appeared necessary to isolate this yellow intermediate and characterize its properties further.

Isolation of Yellow Intermediate as Its Methyl Ester—Owing to the extreme instability of this yellow compound, it was isolated as its methyl ester and also its pyridine derivative.

The reaction mixture contained, in a final volume of 2.4 liters, 57 mmole of KH₂PO₄, pH 7.6, and cell extract (3.0 g of protein). 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-trien-9,17-dione (350 mg in 25 ml of acetone) was added to start the reaction. Incubation was carried out at 25° and aeration was provided by means of a magnetic stirrer. After 1 hour, the yellow reaction mixture was extracted with two 600-ml portions of chloroform, acidified to pH 3.0 with acetic acid, and filtered through Celite. The filtrate was then extracted three times with 600-ml portions of chloroform. Excess diazomethane in ether was added to the combined chloroform extracts and the resulting mixture was allowed to stand at room temperature for 3 hours. At this time the solution was warmed to 40° and the solvent was then removed by means of a stream of nitrogen gas. The oily residue (150 mg) obtained from the chloroform extract was chromatographed on a cellulose powder column (1.8 x 38 cm), with propylene glycol as the stationary phase; 5-ml fractions were collected. Elution of the column with isooctane-cyclohexane (9:1) saturated with propylene glycol afforded 44 mg (Fractions 5 to 100) of the methyl ester of the yellow compound. Many attempts to crystallize this compound were unsuccessful. The nuclear magnetic resonance spectrum of this derivative was very complex due to the presence of a mixture of keto-enol forms. However, the spectrum showed a single peak at 6.16 τ (3 protons) which is characteristic for COOCH₃. This result shows that only 1 carboxyl group is present in the yellow intermediate.

Isolation of Yellow Intermediate as Its Purine Derivative—The ring fission product (α-hydroxy-γ-carboxymuconic semial-
3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (200 mg) was oxidized by cell extracts of N. restrictus under conditions similar to those in the previous experiment. The combined chloroform extracts were dried over sodium sulfate and the solvent was removed to give 71 mg of an oily residue. This oil was chromatographed over a cellulose powder column (1.8 x 34 cm) with propylene glycol as the stationary phase; 8-ml fractions were collected. Benzene saturated with propylene glycol was used to elute the column. Fractions 15 to 28 gave 30.5 mg of the desired product. By rechromatographing this 30.5 mg of oily material on a second cellulose powder column (1 x 32 cm), 14.5 mg of colorless material were obtained after elution with chloroform. The residue was dissolved in a mixture of acetone-petroleum ether (b.p. 30-70). After evaporation of the solvent, an amorphous material was obtained, m.p. 94-97°. The ultraviolet spectrum λ max (EtOH) 273 mµ (ε 4700) is characteristic of a pyridine acid. The infrared spectrum in KBr shows peaks at 5.78 and 5.86 μ which is characteristic of 5-membered and 6-membered ring ketones, respectively. The nuclear magnetic resonance spectrum (Fig. 7) showed bands at 8.78 τ (3H, one tertiary methyl group) and 7.52 τ (3H, one methyl group on pyridine ring). The peak at 3.65 τ is probably due to impurities in the sample. The two aromatic protons on the pyridine carboxylic acid should show a characteristic AB type of spin coupling. But due to the small amount of sample, resulting in a high noise background, the quartet is not clearly defined. However, two definite peaks can be seen at 2.74 and 2.16 τ (2H, aromatic protons). These results are consistent with the ring fission structure proposed under Scheme 1, Fission C. Further evidence for the proposed structure was provided by the mass spectrum (Fig. 8). Although no parent ion peak at m/e 329 was noted, there was a prominent (M-CO₂) peak at m/e 285. It was assumed that CO₂ was lost in the inlet of the apparatus which was maintained at 250°. The base peak, m/e 107, was assigned the structure shown. If cleavage had occurred as in Fission B of Scheme 1, the resulting pyridine derivative would not undergo this type of rearrangement. Instead, a prominent fragment corresponding to m/e 106 would be expected to form (22, 23).

It is evident that 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione undergoes oxidative cleavage in a reaction similar to those described for other catechol compounds. The proposed structure of the first product of ring fission is 4(5),9(10)-diseco-3-hydroxyandrosta-1(10),2-diene-5,9,17-trion-4-oic acid (Fission C of Scheme 1). This compound is formed by a new enzyme which will be tentatively identified as 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione 4,5-oxygenase.

Although the exact mechanism of this oxidative cleavage reaction has not been elucidated, the participation of a quinone intermediate was eliminated by the following experiments.
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556

80.

60. HOOC

40. 63 120

85

265

259

x10

60 80 160 240 260 280

FIG. 8. The mass spectrum of pyridine derivative of the yellow intermediate.

FIG. 9. Absence of quinone formation during the oxidation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by cell extract of N. restrictus. A, mushroom tyrosinase (2.0 mg of protein) incubated with the steroid catechol in the presence of ethylene-1,2-14C-diamine (0.8 pmole, 1.68 μCi). B, N. restrictus cell extract (4.5 mg of protein) incubated with the steroid catechol in the presence of ethylene-1,2-14C-diamine (0.8 μmol, 1.68 μCi). After incubation for 1 hour, the reaction mixtures were extracted with chloroform, chromatographed on Whatman No. 1 paper, and developed for 3 hours in a toluene-propylene glycol system. The radioactivity on the paper chromatograms were measured with the Vanguard Autoscanner, model 880 ADS.

Incubation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione with mushroom tyrosinase afforded a quinone (24) which readily condensed with ethylene-1,2-14C-diamine to give a radioactive dihydropyrazine compound. When cell extracts of N. restrictus were substituted for mushroom tyrosinase in this reaction, no dihydropyrazine derivative was formed (Fig. 9). In a separate experiment, 9,10-secoandrosta-1,5(10)-diene-3,4,9,17-tetraone was insignificantly metabolized by N. restrictus extracts as compared to the parent catechol (Fig. 10).

Identification of 6-Carbon Fragment—The identification of the first product of ring fission and the demonstration that this intermediate is further metabolized by cell extracts to 3αα-4αα-[3'-propionic acid]-1αβ-methylhexahydro-1,5-indanediene left a missing 6-carbon compound or smaller fragments to be identified. The identity of this missing fragment was established in the following experiment.

Cell extract (4.4 g of protein) in 400 ml of 0.02 M potassium phosphate buffer, pH 7.5, was brought to 104 M with respect to ferric sulfate. 3,4-Dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione (350 μg in 10 ml of acetone) was added to the reaction mixture over a 15-min period. Aeration was provided by means of a magnetic stirrer and the reaction was performed at 25°C. After 2 hours, the reaction mixture was extracted with two 100-ml portions of chloroform. The resulting emulsion was broken by filtration through Celite. The aqueous phase was acidified by the addition of 70 ml of 5 N HCl, heated on the steam bath for 15-min, and then centrifuged to remove the precipitated protein. The clear supernatant solution was continuously extracted with ethyl ether for 36 hours. Removal of the ether under a vacuum left an oily residue which was chromatographed over a silicic acid-Celite (9:1) column (2.5 × 26 cm); 5-ml fractions were collected. Elution of the column with benzene-ethyl acetate-acetic acid (90:5:5) afforded 35.4 mg (Fractions 28 to 31) of a compound whose properties were consistent with those of 2-oxo-4-ethylbutyrolactone. Its ultraviolet spectrum in acid showed an absorption peak at 228 μm which disappeared on the addition of alkali with the appearance of a new peak at 202 μm. Its infrared spectrum in KBr showed bands at 3.02, 5.75, 5.82, and 6.04 μ. The nuclear magnetic resonance spectrum of this compound (Fig. 11) revealed peaks at 8.97 τ (3H, triplet, J, 8 cps; CH3-CH2); 8.22 and 8.37 τ (2H, quartets, J’s 6.5 and 2 cps; CH3-CH=CH); 5.13 τ (1H, sextet, J’s 7 and 2.5 cps; CH=CHOH-CH); 3.83 τ (1H, doublet, J, 2 cps; vinyl proton coupled with adjacent H); and 2.80 τ (1H, enolic OH). The structure of this compound was conclusively established.

Fig. 10. Kinetics of the metabolism of 9,10-secoandrosta-1,5-
(10)-diene-3,4,9,17-tetraone by cell extract. The cuvettes contained, in a final volume of 3.0 ml, 105 pmol of KH2PO4, pH 7.6, cell extract (4.5 mg of protein), and substrate (1.58 pmol in 0.1 ml of dimethylformamide). Reference cuvettes contained all of the components except the substrate. ○—○, 9,10-secoandrosta-1,5(10)-diene-3,4,9,17-tetraone plus boiled extract; □—□, 9,10-secoandrosta-1,5(10)-diene-3,4,9,17-tetraone plus active extract; □—□, 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione plus active extract.
as 2-oxo-4-ethylbutyrolactone since its infrared and nuclear magnetic resonance spectra were superimposable upon those given by a synthetic sample.

In a similar experiment, 100 mg of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione were incubated with cell extract (2.0 g of protein) in 200 ml of 0.02 M potassium phosphate buffer, pH 7.5, for 90 min at 25°. The reaction mixture was then acidified with 55 ml of 5 N HCl and the protein precipitate was removed by centrifugation. The acid solution was allowed to stand at room temperature for 12 hours and 125 mg of 2,4-dinitrophenylhydrazine, in 50 ml of 5 N HCl, were then added. The mixture was allowed to stand at 25° for an additional 24 hours. The mixture was then extracted with ethyl acetate and the organic layer was dried over sodium sulfate. Removal of the solvent in a vacuum left 156 mg of an orange residue. The 2,4-dinitrophenylhydrazone mixture was chromatographed over a silicic acid-Celite (9:1) column (2.5 X 26 cm); 10-ml fractions were collected. Elution of the column with benzene-ethyl acetate (95:5) gave 25 mg of a 2,4-dinitrophenylhydrazone which was recrystallized from dichloromethane, m.p. 168-171°.

The infrared, mass spectrum, and chromatographic behavior of this compound were identical with those given by a synthetic derivative prepared from synthetic 2-oxo-4-ethylbutyrolactone.

**Metabolism of 2-Oxo-4-hydroxyacaproic Acid**—Although 2-oxo-4-ethylbutyrolactone was isolated from a reaction mixture, its role as a true intermediate in the enzymatic degradation of 3,4-dihydroxy-9,10-secoandrost-1,3,5(10)-triene-9,17-dione could not be unequivocally established. Since under the conditions used in the isolation procedure 2-oxo-4-hydroxyacaproic acid would lactonize to give 2-oxo-4-ethylbutyrolactone, an experiment was devised to determine whether the lactone per se or its hydroxy acid is the true intermediate.

Table I shows that 2-oxo-4-hydroxyacaproic acid was metabolized by cell extracts of *N. restrictus* to give pyruvic acid as one reaction product. No gas exchange was observed during the reaction. On the other hand, 2-oxo-4-ethylbutyrolactone was not attacked. Cell extracts and whole cells of *N. restrictus* showed no activity toward 2-oxo-4-hydroxyacaproic acid if they were frozen before use. Even freshly prepared cell extracts were not very active when compared to the level of enzyme catalyzing ring fission.

**Isolation and Characterization of Products from 2-oxo-4-hydroxyacaproic Acid**—Freshly grown *N. restrictus* cells (100 g) were suspended in 300 ml of 0.1 M potassium phosphate buffer, pH 8.0. Sodium arsenite was added to a concentration of 10⁻⁴ M. Aeration was provided by means of a magnetic stirrer and the cells were preincubated with this inhibitor for 30 min prior to the addition of 2-oxo-4-hydroxyacaproic acid (512 mg). The

![Fig. 11. The nuclear magnetic resonance spectrum of 2-oxo-4-ethylbutyrolactone in carbon tetrachloride](http://www.jbc.org/)

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**Table I**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pyruvate</th>
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<tr>
<td>2-Oxo-4-ethylbutyrolactone</td>
<td>0.06</td>
</tr>
<tr>
<td>2-Oxo-4-hydroxyacaproic acid</td>
<td>1.58</td>
</tr>
<tr>
<td>2-Oxo-4-hydroxyacaproic acid plus boiled cell extract</td>
<td>0.08</td>
</tr>
<tr>
<td>Pyruvic acid</td>
<td>5.00</td>
</tr>
<tr>
<td>Pyruvic acid plus boiled cell extract</td>
<td>5.06</td>
</tr>
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reaction was terminated after 120 min at 25°, by the addition of 50 ml of 6% (v/v) perchloric acid. The protein precipitate was removed by centrifugation and 450 ml of 0.016 M 2,4-dinitrophenylhydrazine (in 2 N HCl) was added to the clear supernatant solution. After 24 hours at room temperature, the resulting orange precipitate was collected and was dissolved in 500 ml of ethyl acetate. The ethyl acetate solution was extracted with three 100 ml portions of 6% (w/v) sodium bicarbonate and was then dried over sodium sulfate. Removal of the solvent in a vacuum gave 172 mg of an orange residue, which was chromatographed over a column (2 x 28 cm) of neutral alumina. Elution of the column with chloroform gave 63 mg of a compound which, after recrystallization from ethanol, m.p. 152-153°, gave an infrared spectrum identical with that given by a synthetic sample of propionaldehyde 2,4-dinitrophenylhydrazone. A mixed melting point of the isolated product and synthetic propionaldehyde 2,4-dinitrophenylhydrazone showed no depression.

The bicarbonate extract was acidified with 5 N HzSO4 and was extracted with two 250-ml portions of chloroform. The organic layer was dried over sodium sulfate and the solvent was removed in a vacuum to leave 210 mg of a yellow residue. Chromatography of the residue over a silicic acid-Celite (9:1) column (2.5 x 24 cm) with benzene-ethylacetate (90:10) as the mobile phase afforded 27 mg of pyruvic acid 2,4-dinitrophenylhydrazone. After recrystallization from acetic acid, the isolated compound, m.p. 217.5-218°, showed no depression of the melting point when mixed with a synthetic sample. The infrared spectrum was also superimposable upon that of a synthetic sample of pyruvic acid 2,4-dinitrophenylhydrazone.

DISCUSSION

The rupture of steroid ring A has been shown by Santer and Ajl (25) as evidenced by the appearance of radioactive 14CO2 when 4-14C-testosterone was exposed to a soil Pseudomonas. However, the intermediates and reaction sequence involved in this A ring cleavage are not well defined. In the previous paper (7), evidence was provided for the role of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-3,17-dione as an intermediate in the degradation of androst-4-ene-3,17-dione by N. restrictus. In this paper, we have shown the oxidation of this 3,4-catechol, by cell extracts of this organism to 3α-H-4α-[3'-propionic acid]-7αβ-methylhexahydro-1,5-indandione and 2-oxo-4-hydroxyhexanoic acid.

Since the first product of ring fission has been identified as 4(5),9(10)-dihydroxyandrosta-1(10),2-diene-5,9,17-trione 4-ol acid, it is apparent that cleavage of this aromatic ring occurs in a manner analogous to the reactions involved in the ring fission of 2,3-dihydroxy-β-phenylpropionic acid (20), 7,8-dihydroxykynurenine acid (26), 1,2-dihydroxyanthranilic acid (27), and 2,3-dihydroxyphenylactic acid (21). The role of oxygenases in the microbial metabolism of various aromatic and alicyclic compounds has been discussed by Hayaishi (28), who proposed the terms monoxygenases and dioxygenases for enzymes catalyzing the incorporation of 1 or 2 atoms of molecular oxygen, respectively, into a molecule of organic substrate. The field of steroid metabolism has numerous examples of monoxygenases and these have been reviewed by Peterson (29), but to our knowledge, the oxidation of 3,4-dihydroxy-9,10-secoandrosta-1,3,5(10)-triene-9,17-dione by cell extracts of N. restrictus constitutes the first positive demonstration of a dioxygenase in the microbial metabolism of steroids. Recently, Laskin et al. (30) have shown that Glomerella fusaroides catalyzes the fission of ring A of eburic acid with the formation of 3,4-seco-Δ5,14(28)-eburicadien-4-ol-3,20-dioic acid. This reaction is envisaged as an oxidation to the ketone, followed by oxygenation of the A ring (a reaction analogous to the Baeyer-Villiger reaction), which after hydrolysis afforded the seco acid. This mechanism of ring fission is probably utilized by microorganisms when they encounter a saturated alicyclic system and has been amply shown in the metabolism of ring D of steroids (31, 32) and also in the metabolism of camphor (33).

The further metabolism of 4(5),9(10)-dihydroxyandrosta-1(10),2-dien-5,9,17-trion-4-ol acid is in accord with the general scheme for the degradation of catechols which has been proposed by Dagley et al. (34). 2-Oxo-4-hydroxyhexanoic acid could be formed by the addition of 1 mole of water to the ethylenic bond after hydrolytic cleavage between carbon atom

![Scheme 2. Mode of a ring cleavage by N. restrictus](http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1065839/fig2.png)
5 and 10 or alternatively water could be added to the ethylenic bond prior to hydrolytic cleavage (Scheme 2). According to this reaction sequence, carbon atom 4 of the steroid forms the bond prior to hydrolytic cleavage (Scheme 2). According to 111, 6 (1950), carbonyl group of pyruvic acid, which could then enter the triarboxylic acid cycle and readily explain its rapid appearance as $^{14}$CO$_2$. 12.

Although 2-oxo-4-ethylbutyrolactone was isolated from a reaction mixture, the failure of cell extracts to metabolize this compound suggests that 2-oxo-4-hydroxycaproic acid is the true intermediate. This is substantiated by the enzymatic reaction mixture, the failure of cell extracts to metabolize this compound (1957).

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Mechanisms of Steroid Oxidation by Microorganisms: IX. ON THE MECHANISM OF RING A CLEAVAGE IN THE DEGRADATION OF 9,10-SECO STEROIDS BY MICROORGANISMS


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