Bacterial Oxidation of Steroids

I. RING A DEHYDROGENATIONS BY INTACT CELLS

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Since the original observations of Söhngen in 1913, it has been known that a variety of soil microorganisms oxidize and utilize cholesterol and other steroids for their growth (reviewed in (1)). Pseudomonas testosteroni was isolated from the soil by enrichment culture for testosterone (2). This microorganism grows rapidly on certain C₁₉ and C₂₁ steroids† as its sole carbon source and under suitable conditions degrades such steroids completely to CO₂ and water by a series of enzymes, at least some of which are known to be steroid-induced (adaptive). Evidence has been obtained that the terminal oxidations of these steroids proceed through the tricarboxylic acid cycle (2), but many of the undoubtedly complex intermediate steps involved in the cleavage of the steroid nucleus remain unknown.

Three adaptive enzymes concerned with steroid metabolism have been isolated from P. testosteroni. Two diphosphopyridine nucleotide-linked hydroxysteroid dehydrogenases which interconverst reversibly specific hydroxyl and ketone functions of steroids have been characterized. One of these, 3α- and 17β-hydroxy-steroid dehydrogenase (3, 4) interconverts 3α-hydroxyl and 3α-ketone functions in C₁₉ and C₂₁ steroids and also catalyzes the reversible oxidation of the 17β-hydroxy groups in steroids of the estrane, androstane, and etiocholane series. A 3α-hydroxy steroid dehydrogenase (3, 4) catalyzes the reversible oxidation of 3α-hydroxy steroids of the C₁₉, C₂₀, and C₂₁ series. In addition, a steroid isomerase has been described which converts certain Δ₅ to Δ₃-3-ketosteroids (5).

The present studies were directed toward elucidating further steps in the degradation of steroids by P. testosteroni. This paper describes the introduction of unsaturation into ring A of steroids (6) principally by intact bacterial cells. These transformations appear to prepare the steroid nucleus for further oxidative cleavage. Experiments on the enzymatic mechanism of these dehy- drogenations are reported separately (7). Ring A dehydrogenations are carried out by a wide variety of microorganisms (1, 8, 9), including a species of Pseudomonas (10). These transformations of steroids are also of considerable interest because of the interesting physiological properties of certain 1-dehydro analogues of adrenal cortical steroids (see review (11)). The possible importance of these reactions in the aromatization of ring A and hence the biosynthesis of phenolic estrogens has also been pointed out (1).

EXPERIMENTAL

Pseudomonas testosteroni (ATCC 11996) was grown in a liquid medium, similar to that previously described (4), containing sodium lactate (added as a neutralized 50 per cent solution) in place of yeast extract. The final concentration of lactate generally was 0.5 per cent. The pH was adjusted to 7.0 with NaOH. Culture densities were determined by turbidimetric measurement of appropriately diluted cell suspensions at 650 μm in the Beckman spectrophotometer.

Reagents—All organic solvents used were redistilled. n-Hexane and n-hexane were obtained from the Phillips Petroleum Company and purified by exhaustive treatment with concentrated sulfuric acid followed by alkaline potassium permanganate, dried over sodium metal, and distilled on an efficient fractionating column. Dioxane was thoroughly treated with ferrous sulfate, dried over sodium metal and distilled on a fractionating column.

Steroids were commercial preparations, purified when necessary by crystallization and sublimation. The steroids were added to the growth flasks in acetone solutions. For manometric experiments, 10 mg. of steroid were dissolved in 1.0 ml. of dioxane, and the solution added to 19.0 ml. of a 0.5 per cent aqueous solution of high viscosity carboxymethyl cellulose (Hercules Powder Company). All melting points are corrected. Infrared spectra were determined as KBr disks on a Perkin-Elmer model 21 spectrophotometer.

Growth and Induction—A starter culture was prepared by inoculating 100 to 200 ml. of lactate and minerals medium with cells from a slant. It was grown at 30°, with shaking, for 18 to 24 hours, in small culture flasks. Larger culture flasks (Corning No. 4422), usually containing 500 or 1000 ml. of medium, were inoculated with 2.5 or 5 ml., respectively, of the starter culture. The cells were allowed to grow for 16 to 24 hours at 30° on a platform shaker. At this time the culture had usually attained a concentration of 1 to 2 mg., dry weight, of cells per ml. An acetone solution of the steroid (25 mg. per ml.) was then added to give a final concentration of 100 mg. per l., and the incubations were continued for specified times up to 72 hours.

The steroids were extracted from the culture, or from aliquots thereof, with ethyl acetate. The extracts were dried with anhydrous sodium sulfate, filtered, and evaporated to dryness on a rotary evaporator maintaining the temperature below 50°.
residue were dissolved in small volumes of methanol and aliquots were chromatographed on filter paper. Products from the incubation of C-19 steroids were spotted on Whatman No. 43 paper which had been impregnated with a stationary phase consisting of equal volumes of formamide and methanol (12). The papers were developed by descending chromatography with a mixture of equal volumes of hexane and benzene, saturated with formamide. The α,β-unsaturated ketosteroids were detected on the paper with the aid of an ultraviolet scanner. Ketosteroids were visualized by spraying with 2,4-dinitrophenylhydrazine (13). Products from the incubation of 19-nor-testosterone were also chromatographed on paper with another type of system (14). Whatman No. 3 MM paper was washed for several days by extracting with a mixture of equal parts of benzene and methanol. Phenolic steroids were detected as dark blue spots by spraying the papers with a freshly prepared mixture of equal volumes of 1 per cent ferric chloride and 1 per cent potassium ferricyanide (15), followed by washing of the papers with HCl and water.

Isolation of steroids was accomplished by chromatography on silicic acid columns by gradient elution (16). The steroids were further purified by crystallization, sublimed in vacuo, and characterized as described below.

RESULTS

When testosterone (I, Scheme I) was added to a growing culture of P. testosteroni and aliquots were examined at various time intervals by paper chromatography, the first product was found to be 4-androstene-3,17-dione (II). Considerable quantities of this product were present in the incubation mixture within 6 hours after addition of testosterone. This oxidation of the 17β-hydroxyl group is catalyzed by β-hydroxysteroid dehydrogenase, large amounts of which are present in adapted cells (4). Longer incubations resulted in the formation of two other ultraviolet-absorbing compounds, more polar than 4-androstene-3,17-dione, identified as 1,4-androstadiene-3,17-dione (III) and 1,4-androstadien-17β-ol 3 one (IV).

When 4-androstene-3,17-dione was used as a substrate for the incubation, III and small amounts of I and IV were detected in the medium. In one experiment, 45 mg. of 4-androstene-3,17-dione were added to 450 ml of a 24-hour growth of the microorganism. At 72 hours the contents of the culture flask were extracted with ethyl acetate and chromatographed on silicic acid. They yielded a crystalline product which, upon recrystallization from hexane and acetone and sublimation (90-115°, <0.001 mm. of Hg), gave 18.7 mg of a product which was characterized as 1,4-androstadiene-3,17-dione by the following criteria (17, 18): m.p. 140.5-141° (no depression upon admixture with an authentic sample); [α]D +123° (c, 1.04, CHCl3); λD 243 μg (ε 16,200); the infrared spectrum showed λmax 5.77 μ (17-ketone) and 6.04, 6.17, and 6.25 μ (Δ14-3-ketone), and was identical with an authentic sample; sulfuric acid chromogens (19, 20) were identical with an authentic sample (peaks at 265, 305, and 390, and minima at 288 and 350 μm); analysis

C19H28O2

Calculated: C 80.24, H 8.51

Found: C 80.08, H 8.46

Measurement of the 17-ketone group with β-hydroxysteroid dehydrogenase (16) gave 104 per cent of theory. The product and authentic III could not be separated by paper chromatography.

When androstan-3,17-dione (V) or 1-androsten-3,17-dione (VI) were incubated with growing cultures of P. testosteroni under similar conditions, 1,4-androsten-3,17-dione was isolated and identified as the principal product in both cases. Small amounts of IV accompanied the main product. The experimental details and yields of product in these incubations were similar to those of the incubation with 4-androstene-3,17-dione, except that the extractions were performed 22 hours after the addition of steroid to the culture. It was noted that when cells were incubated with androstan-3,17-dione (V) the time sequence of appearance of products was always V → VI → III, and that no II appeared in the incubation mixture. This conversion sequence does not necessarily reflect the relative activities of the enzymes introducing double bonds at the 1-2 and the 4-5 positions. In suitably prepared cell-free preparations, the conversion sequence V → II → III has been demonstrated.

Cultures of P. testosteroni converted 17α-methyltestosterone to 1-dehydro-17α-methyltestosterone in good yield. This latter steroid was not further metabolized and accumulated in the medium. The product obtained after 72 hours of incubation was isolated and purified in the usual manner. It had the following characteristics (21): m.p. 164-166°; the infrared spectrum showed λmax 6.02, 6.18, and 6.26 μ (Δ14-3-ketone) and 2.92 μ (OH group); [α]D 2.6° (c, 1.15, CHCl3); it migrated on paper with the same velocity as authentic 1-dehydro-17α-methyltestosterone.

The oxidation at C-4,5 is not limited to steroids in which the A:B ring fusion is trans oriented. Thus, etiocholan-17β-ol-3-one (VII, A:B cis) was converted to testosterone, 4-androstene-3,17-dione, 1,4-androsten-3,17-dione, and 1,4-androsten-17β-ol-3-one.
ol-3-one by a cell-free extract prepared from cultures grown on 0.5 per cent lactate and 0.01 per cent testosterone (6, 7).

In another incubation, conducted like the one with 4-androstene-3,17-dione, 19-nor-testosterone (VIII, Scheme II) was converted to estrone (X), which was accompanied by a small quantity of estradiol-17β (XI). This reaction probably proceeds preferentially via 4-estrene-3,17-dione (IX), which is a better substrate than 19-nor-testosterone preparations (7). The conversion of the 17β-hydroxy group to the 17-ketone group is again consistent with the action of β-hydroxysteroid dehydrogenase. Since estrone is not oxidized further by P. testosteroni, incubation for 72 hours resulted in a culture medium containing estrone as the principal steroid. Estrone could be crystallized directly from the extracts in 67 per cent yield. The product was sublimed at 135-150° (<0.0005 mm. of Hg) and characterized by the following criteria: m.p. 253-255°; [α]D +154° (c, 0.644, dioxane); λmax 282 mp (ε 1960); upon addition of NaOH, λmax 245 mp (ε 8000) and 300 mp (ε 2000); the infrared spectrum showed λmax 5.83 μ (17-ketone), 6.32, 6.18 μ (aromatic –C=C–), and 3.06 μ (OH group), identical with an authentic sample. On paper the product traveled at the same rate as authentic estrone and gave the characteristic blue color of phenols with Turnbull’s reagent (15).

The tendency of Pseudomonas cells to introduce the 1–2 double bond before the 4–5 double bond also obtained in the C19 series. This permitted the preparation of 1-estrene-3,17-dione (XIII), a compound which has not to our knowledge been heretofore described, from 5α-estr-17β-ol-3-one (XII) (22). Direct addition of XII to a growing culture of P. testosteroni resulted in only negligible conversion to XIII. Since the enzymes responsible for the introduction of double bonds are steroid-induced (adaptive) (7), the possibility existed that XII was ineffective as an inducer of the enzyme. Support for this view was obtained with the demonstration that testosterone-adapted, washed cells (or extracts thereof) readily transformed XII to XIII.

Thus, 45 ml. of a thick, washed cell suspension, representing approximately 1500 ml. of the original, adapted culture, were disrupted by sonic oscillation in a Raytheon 9 kc. sonic oscillator. An equal volume of 0.1 M tris(hydroxymethyl)aminomethane, pH 9.2, and 51 mg. of 5α-estr-17β-ol-3-one, dissolved in 2 ml. acetone, were added. The mixture was incubated for 3 hours at 30°, in air, with gentle agitation, and then extracted with ethyl acetate. The extract was evaporated to dryness and dissolved in a small volume of methanol. The methanol solution was applied along the origin of four sheets of washed Whatman No. 3 MM paper (16 cm. wide) and chromatographed for 9 hours in the heptane-methanol system. The ultraviolet-absorbing areas corresponding to the new compound were eluted with methanol. The methanol solution was dried and the residue crystallized twice from a mixture of hexane and acetone, yielding 17.3 mg. of a product, m.p. 188-189°; [α]D +221° (c, 0.954, CHCl3); λmax 231 mp (ε 11,350); infrared analysis showed λmax 5.82 μ (17-ketone) and 6.01 μ (conjugated 3-ketone); sulfuric acid chromogens (19, 20) showed λmax 297 mp, λmin 225 mp.

The identification of this compound as 1-estrene-3,17-dione is supported by the following facts. Its formation from XII is consistent with the previously established reaction pattern in the C19 series of steroids. On paper it migrates at a rate 1.3 times as fast as 4-estrene-3,17-dione, which is consistent with the relative migration rates of 1-estrene-3,17-dione and 4-androstene-3,17-dione in the same system. The absorption peak at 231 mp and εmax of 11,350 are characteristic for a Δ1-3-ketosteroid, being at a shorter wave length and of lower intensity than those of Δ3-ketosteroids (22). Sulfuric acid chromogens for Δ3-keto- and Δ3-3-ketosteroids are very similar (20) but are different from related steroids, and this fact, taken in conjunction with the ultraviolet absorption and the distinctly different melting point from 4-estrene-3,17-dione, strongly support the identity of XII as 1-estrene-3,17-dione. Finally, incubation of XIII with a purified double bond-introducing enzyme system (7) gave rise to the expected product, estrone, which was identified by its migration on paper chromatograms and its reaction with Turnbull’s reagent.

Manometric Experiments—Washed, resting cell suspensions of P. testosteroni grown in the absence of steroids, will adapt to oxidize certain of these compounds, as evidenced by increased oxygen consumption. The duration of the adaptive lag is highly variable (from about 3 hours to more than 48 hours) and depends among other factors on the composition of the growth medium and the duration of growth. Dr. Jay Y. Roshal of this laboratory has undertaken a detailed study of the conditions influencing adaptation of cells grown on a medium containing sodium.
androstadiene-3,17-dione is a key intermediate in the oxidation of testosterone and related steroids. When 1,4-androstadiene-3,17-dione was added to a growing culture of \( P. \) testosteroni, and ethyl acetate extracts of the culture periodically examined by paper chromatography, this steroid was found to be utilized and converted to unidentified compounds which do not absorb ultraviolet light.

Thus, as might be expected on chemical grounds, the 1,4-diene-3-one structure appears to render the A ring especially susceptible to further oxidation and constitutes a region of early attack on the steroid. This suggestion is consistent with the observations of Turff and Krog (24) that the microbiological oxidation of cholesterol yields small amounts of Windaus' keto acid in which ring cleavage has occurred between C-3 and C-5, and C-4 has been oxidized to \( CO_2 \). Stadtman et al. (25) have reported similar results in another microbial system. Unpublished experiments of Dr. J. Y. Rosenthal have shown that the release of Carbon dioxide from testosterone-\( 4-C^{14} \) incubated with \( P. \) testosteroni occurred early in the oxidation of this steroid, indicating that ring A undergoes early attack.

The presence of the 1,4-diene-3-one structure in a steroid, however, is not in itself sufficient to assure the further oxidative cleavage of the compound, and other structural features may be overriding in this respect. Thus, although 17a-methyltestosterone is readily converted to 1-dehydro-17a-methyltestosterone, the latter compound is not further metabolized, as evidenced by its persistence in growing cultures and by its lack of oxidation by testosterone-adapted cells. The presence of a 17a-methyl group prevents complete oxidation of the steroid. Similarly, the presence of substituents at other points on the steroid skeleton also affects the oxidation of these compounds, e.g. 11b-hydroxyl groups or 11-ketone groups render steroids incapable of utilization by this microorganism. It is shown in the accompanying paper (7) that such 11-oxygenated steroids are poor substrates for the introduction of unsaturation into ring A.

Table I gives a summary of the specificity of steroid oxidation by \( P. \) testosteroni. It appears from these studies that the structural requirements for complete oxidation of steroids include appropriate molecular features in ring A, as well as at positions 17 and 11.

Table I

<table>
<thead>
<tr>
<th>Steroid specificity of Pseudomonas testosteroni*</th>
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<tr>
<td>Steroids oxidized</td>
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<tr>
<td>Testosterone</td>
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<tr>
<td>4-Androstene-3,17-dione</td>
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<tr>
<td>Androstan-33-ol-17-one (epiandrosterone)</td>
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<tr>
<td>5-Androsten-33-ol-17-one (dehydroleponandrosterone)</td>
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<tr>
<td>4-Androsten-17a-ol-3-one (17-epiandrostosterone)</td>
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<td>Androstane-3,17-dione</td>
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<td>Androstan-3,17-dione</td>
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<tr>
<td>Progesterone</td>
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<td>17α-Hydroxyprogesterone</td>
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* From (2) and unpublished experiments.
in animal tissues the hydrogenation of double bonds at C-1, 2 and C-4, 5 of steroids is a widespread and common metabolic occurrence, whereas the introduction of unsaturation has been less frequently recognized (1). Thus, Tomkins (26) has partially purified an enzyme from rat liver which reduces the C-4, 5 double bond of cortisone, apparently irreversibly. Many microorganisms also reduce double bonds of steroids, especially under fermentative conditions (1). At present it is not known whether similar enzymatic mechanisms are involved in the addition and removal of hydrogen atoms to and from adjacent carbon atoms of steroids.

The experiments reported here demonstrate that P. testosteroni, like a number of other microorganisms, can convert the saturated ring A of 3-ketosteroids to the 1, 4-diene-3-one group. The introduction of unsaturations also occurs in 19-nor-steroids, which then undergo facile and favorable tautomerization that leads to the formation of the aromatic A ring. The last reaction has also been reported by Kushinsky (27) in Corynebacterium simplex and by Peterson in Septomyxa affinis 3. In contrast to the results of Kushinsky, we have found no hydroxylated intermediates or side products in the reaction catalyzed by P. testosteroni. The conversion of 19-nor-testosterone to estrone is of interest in connection with the mechanism of aromatization reactions. Meyer (23) showed that 19-hydroxy-4-androstene-3,17-dione was converted to estrone in mammalian tissue preparations, but cannot oxidize 14-nor-testosterone beyond estrone.

S.A.

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REFERENCES

