Studies on the Microbiological Degradation of Steroid Ring A*

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SUMMARY

The synthesis of 2-oxo-cis-4-hexenoic acid from DL-2-amino-cis-4-hexenoic acid is described. The α-oxo acid appears to exist at neutral pH largely in the dienolic form. Ultracentrifuged extracts of steroid-induced Pseudomonas testosteroni rapidly converted 2-oxo-cis-4-hexenoic acid-1-14C to 14CO2. The same enzyme preparations, when supplemented with NAD and a NADPH-generating system, have been previously shown to convert Δ4-androstene-3,17-dione-4-14C to 14CO2 and to accumulate L-2-amino-cis-4-hexenoic acid-1-14C and DL-alanine-1-14C in the presence of ethylene-diaminotetraacetate. It has now been shown that, under similar conditions, 2-oxo-cis-4-hexenoic acid is efficiently converted to 2-amino-cis-4-hexenoic acid and alanine.

The α-oxo acid also undergoes stereospecific enzymatic hydration to 2-oxo-4-hydroxyhexanoic acid, which is lactonized in the presence of acid to give a product that has been identified as one of the optically active isomers of 2-oxo-4-ethylbutyrolactone of unestablished configuration. These findings suggest that 2-oxo-cis-4-hexenoic acid is a key intermediate in the degradation of steroid ring A by microbial enzymes. The enzyme preparations also contain an enzyme which reduces the double bond of the α-oxo acid but does not appear to be involved in the degradative pathway of the steroids.

A number of microorganisms that grow on steroids as the only source of organic carbon can effect the complete oxidative degradation of the steroid skeleton by a series of induced enzymes (1, 2). The complex pathways involved in these degradations have been partially elucidated, and the structures of a number of intermediates are now known. The first clues as to the initial site of microbiological attack on the steroid skeleton were provided by Dodson and Muir (3, 4) who demonstrated the conversion of Δ4-androstene-3,17-dione (I, Scheme 1) to 3-hydroxy-9,10-seco-

Δ1,3,5(10)-androstatriene-9,17-dione (IV) by a species of Pseudomonas. These workers provided evidence that this transformation resulted from the participation of two enzymatic reactions, 9α-hydroxylation and Δ-dehydrogenation, and involved the intermediate formation of the hypothetical β-hydroxydienone III which would be expected to undergo facile reverse aldol condensation to form the seco-phenol IV. Further work from several laboratories resulted in the identification of 3α,4β,5,6,7,7α-hexahydro-7αβ-methyl-1,5-dioxo-4-indanpropionic acid (V) and related compounds in which Rings B and C of the steroid contribute to the carbon skeleton of the perhydroindane structure, and the propionic acid group is derived from C-5, C-6, and C-7 of the steroid (5, 8).

Information on the fate of the remainder of the A ring was provided by the work of Gibson et al. (9), who isolated 2-oxo-4-ethylbutyrolactone (VIII) presumably as a consequence of the nonenzymatic lactonization of 2-oxo-4-hydroxyhexanoic acid (VII). In a series of elegant experiments by Sih et al. (10), evidence was submitted that the cleavage of Ring A resulted from an hydroxylation of the steroid at C-4 and the subsequent cleavage of the C-4 to C-5 bond by a metapyrocatechase type reaction.

Earlier studies from our laboratory have shown that suitably supplemented cell-free extracts of steroid-induced Pseudomonas testosteroni rapidly converted Δ4-androstene-3,17-dione-1-14C (I) to 14CO2, and that, under specific conditions, L-2-amino-cis-4-hexenoic acid-1-14C (IX) and DL-alanine-1-14C (X) accumulate (2). We suggested that these amino acids are formed by amination of the corresponding α-oxo acids, and that 2-oxo-cis-4-hexenoic acid (VI) and pyruvate were the actual intermediates in the steroid Ring A degradation. Gibson et al. (9) have further shown that 2 oxo 4 hydroxyhexanoic acid (VII) (isolated as the lactone, VIII) undergoes aldol cleavage to propionaldehyde and pyruvate. The latter may yield alanine by well known amination reactions, and the isolation of DL-alanine, rather than an optically active isomer, is accounted for by the presence in these enzyme preparations of an extremely active alanine racemase (2).

In continuation of our earlier studies, we wish now to report the synthesis of 2-oxo-cis-4-hexenoic acid-1-14C (VI) and to present evidence for its participation as an intermediate in the degradation of steroid Ring A. Our findings suggest that this α-oxo acid undergoes a stereospecific hydration to form 2-oxo-4-
hydroxyhexanoic acid (VII). A preliminary report on this subject has appeared (11).

EXPERIMENTAL PROCEDURE

Materials—Δ4-Androstene-3,17-dione-4-14C (New England Nuclear, specific activity 58.0 mC per mmole) was diluted with purified unlabeled steroid to a specific activity of 23.8 mC per mmole at a concentration of 10.49 mM in methanol.

The ultracentrifuged cell-free extracts of steroid-induced P. testosteroni (ATCC 11996) were prepared by the method of Shaw, Borkenhagen, and Talalay (2) from lyophilized cells grown under specified conditions.

Racemic 2-oxo-4-ethylbutyrolactone was synthesized according to the procedure of Rossi and Schinz (12). 1-Chloro-2-butyne was prepared by the method of Ettlinger and Hodgkins (13). 2-Oxohexanoic acid was prepared from n-leucine according to the method of Woygand, Stogieh, and Tanner (14). Purified yeast glucose 6-phosphate dehydrogenase was obtained from C. F. Boehringer and Soes (Mannheim, Germany), and was assayed and diluted according to Shaw et al. (2). NAD and NADP were obtained from P-L Biochemicals. Disodium glucose 6-phosphate was supplied by Sigma. Crystalline bovine plasma albumin was obtained from Armour and Company. Protein concentrations were measured by the biuret reaction (15), with bovine serum albumin as a standard. The purified hog kidney ω-amino acid oxidase was generously donated by Dr. D. S. Coffey (Johns Hopkins University School of Medicine). Samples of synthetic cis- and trans-DL-2-amino-4-hexenoic acid were gifts of Dr. William Shive.

All of the solvents were the best commercially available analytical reagents and were distilled prior to use. The inorganic chemicals were reagent grade.

Methods—Melting points were determined on the Kofler hot stage. Infrared spectra were recorded on a Perkin-Elmer 137 spectrophotometer. Ultraviolet spectra were obtained on a Cary model 15 recording spectrophotometer. Nuclear magnetic resonance spectra were determined on Varian model A 60 or HR 100 spectrometers, in deuterated chloroform with tetramethylsilane as an internal standard, or in deuterium oxide with tetramethylsilane as an external standard. The optical rotatory dispersion measurements were obtained with the use of a Cary model 60 recording spectropolarimeter. Elemental analyses were performed by A. Bernhardt (Mulheim, Germany). Assays of radioactivity were carried out as described (2). The method used
malonate with 1-chloro-2-butyne to give ethyl 2-acetamido-2-carbethoxy-4-hexynoic acid (XI), which is hydrogenated catalytically and then hydrolyzed and decarboxylated to give the desired product IX. All intermediates were obtained as pure entities and characterized. Yields were in excess of 75% at each step.

Ethyl 2-Acetamido-2-carbethoxy-4-hexynoic Acid (XI)—To a solution of 3.91 g of sodium in 100 ml of absolute ethanol were added 37.0 g (0.17 mole) of diethyl acetamidomalonate, and the mixture was stirred for 30 min at room temperature. The resulting yellow solution received, by dropwise addition with stirring, 15.7 g (0.177 mole) of 1-chloro-2-butyne. Stirring was continued for 2 hours at room temperature and for 6 hours at reflux temperature. After cooling and standing for 14 hours, the ethanol was removed under reduced pressure, and the resulting oil was extracted with three 100-ml portions of ether. The combined ether extracts were washed successively with water, saturated sodium bicarbonate solution, and water, and then were dried over sodium sulfate. Upon evaporation of the ether to a small volume and addition of hexane, crystallization began. The sodium bicarbonate solution, and water, and then were dried over sodium sulfate. Upon evaporation of the ether to a small volume and addition of hexane, crystallization began. The resulting oil was collected and dried to yield 6.04 g (80%) of DL-2-acetamido-cis-4-hexenoic acid, melting at 114–116°. Yield, 77% based on XII. Found: C 57.61, H 8.02. 

Dl-8-Acetamido-cis-4-hexenoic Acid (XIII) Twenty millimoles (5.42 g) of ethyl 2-acetamido-2-carbethoxy-cis-4-hexenoic acid (XII) were refluxed for 4 hours in 40 ml of 2.5 N sodium hydroxide, cooled, and acidified to pH 2 with 6 N hydrochloric acid. The dicarboxylic acid which slowly precipitated was collected, dissolved in 75 ml of water, and the solution was refluxed for 4 hours to effect decarboxylation. The solution was filtered through a layer of charcoal and evaporated to dryness under reduced pressure. The product was crystallized from ethyl acetate-hexane to afford 2.62 g of the acid melting at 114–116°. Yield, 77% based on XII. Found: C 57.61, H 8.02.

Dl-8-Amino-cis-4-hexenoic Acid (IX) Ten grams (58.5 mmole) of XIII in 200 ml of 2.5 N sodium hydroxide were refluxed for 4 hours. The solution was cooled, acidified to pH 5 with 6 N hydrochloric acid, and desalted by adsorption onto a column of Dowex 50-X8 (H+). After thorough washing of the column with water, the amino acid was eluted with 1 M ammonium hydroxide. The eluate was evaporated to dryness under reduced pressure, and the product was dissolved in hot water, filtered through a layer of charcoal, and adjusted to pH 6. Upon the addition of absolute ethanol, crystallization began. The product was collected and dried to yield 6.04 g (90%) of Dl-2-amino-cis-4-hexenoic acid. An additional 0.48 g of product was obtained by concentrating the mother liquor. When analyzed with the automatic amino acid analyzer, less than 2% of the trans isomer and 1% of norleucine were detected. Catalytic hydrogenation of a sample at 25°C and atmospheric pressure in the presence of 10% palladized charcoal resulted in complete conversion to norleucine when analyzed with the amino acid analyzer. The infrared (Fig. 1) and NMR spectra (2) of DL-2-amino-cis-4-hexenoic acid were identical with those of a sample prepared by an alternate method (17). NMR (D2O) signals at τ 8.39 (3H, doublet), 7.38 (2H, triplet), 6.22 (1H, triplet), 3.8 to 4.9 (2H, multiplet) (2).

Synthesis of Dl-2-Oxo-cis-4-hexenoic Acid

Many α-amino acids are conveniently prepared from their corresponding α-amino acids by the procedure of Weygand et al. (14), which involves treatment of the amino acid with an ex-
cess of trifluoroacetic anhydride and hydrolysis of the resultant oxazolone. However, treatment of the 2-amino-cis-4-hexenoic acid with trifluoroacetic anhydride under the prescribed conditions (14) resulted in the formation of an intractable brown oil presumably because of the addition of the trifluoroacetyl group to the double bond. By modifying the reaction conditions it was possible to obtain the N-trifluoroacetyl derivative in good yield and to effect cyclization to the oxazolone with N,N'-dicyclohexylcarbodiimide (18).

**N-Trifluoroacetyl-2-amino-cis-4-hexenoic Acid (XIV)**—To a suspension of 129 mg (1 mmole) of [2-amino-cis-4-hexenoic acid in 3 ml of dry benzene was added 0.14 ml of trifluoroacetic anhydride, and the reaction mixture was slowly brought to reflux. After 1 hour the solvent was removed under vacuum, and the residue was dissolved in ether and extracted five times with 10 ml of a cold saturated solution of sodium bicarbonate. The aqueous extracts were combined, acidified with dilute hydrochloric acid, and extracted with ether. Evaporation of the ether after drying over sodium sulfate afforded 236 mg of a crystalline product. Crystallization from benzene-hexane gave 163 mg (73% yield), m.p., 89-91°, 3.00, 3.70 to 4.20, 5.78, 5.84, 5.99 (shoulder), 6.39, 7.06, and 14.13 μ.

\[
\text{C}_{14}H_{12}NO_{3}F_{3} (225.18)
\]
Calculated: C 42.67, H 4.48, N 6.22
Found: C 42.88, H 4.72, N 6.24

**4-(2-cis-Butenyl)-2-trifluoromethyl-2-oxazolin-5-one (XV)**—To a stirred solution of 1.51 g (6.7 mmoles) of N-trifluoroacetyl-2-amino-cis-4-hexenoic acid in 20 ml of methylene chloride were added 1.38 g (6.7 mmoles) of N,N'-dicyclohexylcarbodiimide in 2 ml of methylene chloride. A copious precipitate of dicyclohexylurea formed after several seconds, but stirring was continued for 24 hours. The precipitate was removed by filtration and washed with three 15-ml portions of methylene chloride. A colorless liquid was distilled at 88° (bath temperature) and 0.25 mm of mercury to yield 800 mg (67% yield) of a colorless product. The product was redistilled and a center fraction taken for analysis: λ_{max} 5.52, 6.05, 11.50, and 14.5 μ; NMR (CDCl3) signals at τ 8.4 (doublet), 8.15 (doublet), 6.35 (doublet), 4.2 (multiplet), 3.6 (triplet), and 3.35 (doublet); λ_{max} 274 με (ε 10,000); λ_{max} 281 με (ε 10,400); λ_{max} 307 με (ε 7,700) (Fig. 2). The product was stable for several days when stored under nitrogen at −20°; however, a decrease in the absorbance at 274 με was noted with time, even under these conditions. The absorption peak at 307 με observed in base disappeared rapidly, presumably because of decarboxylation of the α-oxo acid.

**Enzymatic Synthesis of 2-Oxo-cis-4-hexenoic Acid (VI)**—In an alternative procedure, [2-amino-cis-4-hexenoic acid was oxidized with hog kidney ω-amino acid oxidase directly to the α-oxo acid (VI), according to the general procedure of Meister (20). The complete incubation system contained, in a final volume of 2.5 ml, 200 μmoles of Tris-HCl buffer of pH 8.2, 20.4 μmoles of [2-amino-cis-4-hexenoic acid, 42 μg of crystalline bovine liver catalase, and 300 μg of purified hog kidney ω-amino acid oxidase. The oxidation was carried out for 40 min at 38°. When the ultraviolet absorption spectrum of the reaction mixture was examined against a reference cuvette containing all components except the amino acid, a maximum was observed at 274 με. Upon addition

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**Fig. 1.** Infrared spectrum of dl-2-amino-cis-4-hexenoic acid (KBr pellet)
SYNTHETIC 2-0XO-CIS-4 HEXENOIC ACID

FIG. 2. Ultraviolet absorption spectra of chemically synthesized 2-oxo-cis-4-hexenoic acid in water, 0.39 N HCl, and 0.32 N NaOH. The concentration was 121 μM. Light path, 1.0 cm.

FIG. 3. Oxidation of L-2-amino-cis-4-hexenoic acid to 2-oxo-cis-4-hexenoic acid by L-amino acid oxidase. The system contained, in a final volume of 10.0 ml, 800 μmoles of Tris-HCl buffer (pH 8.2), 200 μmoles of L-2-amino-cis-4-hexenoic acid, 3 mg of crystalline bovine liver catalase, 2.5 mg of purified hog kidney L-amino acid oxidase, and 0.1 μmole of FAD. Temperature, 38°. Aliquots (0.05-0.10 ml) of the reaction mixture were removed at indicated intervals and mixed with 30% trichloracetic acid to give a volume of 1.0 ml. Then 0.2 ml of a saturated 2,4-dinitrophenylhydrazine solution in 1.0 N HCl was added, and after standing for 15 min at room temperature, 3.0 ml of 2 N NaOH were added, and the absorbance was determined at 525 μm in cuvettes with a 1.0-cm light path (21). The values shown are corrected for 0.1-ml aliquots of the oxidation system.

FIG. 4. Ultraviolet absorption spectra of synthetic (---) and enzymatic (—) 2-oxo-4-ethylbutyrolactone in water and in 0.033 N NaOH. The concentration of the synthetic sample was 120 μM. Light path, 1.0 cm.
in the presence of 5 mg of 10% palladium on calcium sulfate. A volume of 1.46 ml (55% of theory) of hydrogen was consumed. The catalyst was removed by centrifugation and the solvent was evaporated with a stream of nitrogen to give a clear oil. The product was characterized as its quinoxaline derivative by treating the oil with α-phenylenediamine dibydrochloride in 2 x hydrochloric acid. The derivative, 2-butyl-3-hydroxyquinoline, melted at 153-156° (lit. 153.5-154°) (22) after crystallization from ethanol-water, and its infrared spectrum was identical with that of an authentic sample prepared from 2-oxohexanoic acid.

**Synthesis of Δ^2-oxo-cis-4-hexenoic Acid-l-^14C**—The radioactive α-oxo acid was prepared from dl-2-amino-cis-4-hexenoic acid (1X) (2.62 x 10^7 cpm per μmole) by the procedure described above for the nonradioactive compound.

The L-2-amino-cis-4-hexenoic acid-l-^14C was prepared enzymatically from Δ^2-androstene-3,17-dione-4-^14C by the procedure of Shaw et al. (2). The reaction system contained, in a final volume of 8.0 ml, 400 μmoles of potassium phosphate (pH 7.4), 4 μmoles of NAD, 4 μmoles of NADP, 40 μmoles of sodium glucose 6-phosphate, 40 μmoles of EDTA, 16,000 units of purified yeast glucose 6-phosphate dehydrogenase, 5.65 μmoles of Δ^2-androstene-3,17-dione-4-^14C, and 2.0 ml (90 mg of protein) of extract of steroid-induced P. testosteroni. The incubation was carried out for 60 min at 30°, and the mixture was processed as described previously (2). The fractions containing L-2-amino-cis-4-hexenoic acid were purified by chromatography on the amino acid analyzer column, and 5 mg of synthetic dl-2-amino-cis-4-hexenoic acid were added. The pooled crude material was chromatographed a second time on the amino acid analyzer column, and 50 μmoles of dl-2-amino-cis-4-hexenoic acid, with a specific activity of 2.6 x 10^8 cpm per μmole, were obtained.

A portion of the radioactive dl-2-amino-cis-4-hexenoic acid (25 μmoles) was diluted with 390 μmoles of the unlabeled synthetic amino acid (calculated specific activity, 1.88 x 10^9 cpm per μmole). This material was converted to XV-5-^14C by the procedure described above. The oxazoline thus obtained was purified with a microsublimation apparatus and hydrolyzed in 2 ml of citrate-phosphate buffer at pH 6.8 to 2-oxo-cis-4-hexenoic acid-l-^14C. The oxo acid (203 μmoles, specific activity, 11,200 cpm per μmole), was dissolved in 2.5 ml of 0.02 M potassium phosphate buffer at pH 7.1 and used in the subsequent radioactive experiments.

**RESULTS**

**Conversion of 2-Oxo-cis-4-hexenoic Acid-l-^14C to ^14CO_2 by Cell-free Enzyme Preparations**—When 2-oxo-cis-4-hexenoic acid-l-^14C was added to ultracentrifuged extracts of steroid-induced P. testosteroni, a rapid liberation of ^14CO_2 was observed (Table I), and the rate of formation of ^14CO_2 increased with the quantity of enzyme protein added (Table I, Experiment A). Even in the absence of the enzyme, a small conversion of the substrate to ^14CO_2 was observed. The rate of this nonenzymatic decarboxylation was decreased in the presence of EDTA and in the zero time control, which was acidified at the time of addition of the substrate rather than being incubated at pH 7.4 for 1 hour at 30° before acidification (Table I, Experiment A). These findings are consonant with the greater stability of α-oxo acids in acid solution and in the absence of metal ions. The addition of 10 μmoles of EDTA to the enzymatic reaction system inhibited the formation of ^14CO_2, but this effect was less pronounced when the enzyme concentration was increased. The presence of 0.5 mM NAD stimulated the enzymatic liberation of ^14CO_2 (Table I, Experiment A), whereas the further addition of a NADPH-generating system (Table I, Experiment C) appears to be slightly inhibitory. The inhibitory effect of EDTA on the formation of ^14CO_2 from Δ^2-androstene-3,17-dione-4-^14C has been previously described (2), and it would appear that the inhibitory effect of the chelating agent is exerted at least partly beyond the stage of 2-oxo-cis-4-hexenoic acid. The presence of both NAD and a NADPH-generating system enhances the oxidation of the steroid, whereas NAD alone is stimulatory to the degradation of the α-oxo acid.

**Table I**

<table>
<thead>
<tr>
<th>Composition of reaction system</th>
<th>Quantity of ^14CO_2 formed in mpmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment A</strong></td>
<td><strong>The quantity of enzyme protein</strong></td>
</tr>
<tr>
<td>Enzyme only</td>
<td>22.5</td>
</tr>
<tr>
<td>Plus 10 μmoles of EDTA</td>
<td>390</td>
</tr>
<tr>
<td>Plus 1.0 μmole of NAD</td>
<td>580</td>
</tr>
<tr>
<td>Plus 10 μmoles of EDTA + 1.0 μmole of NAD</td>
<td>40</td>
</tr>
<tr>
<td>Omit enzyme</td>
<td>70</td>
</tr>
<tr>
<td>Omit enzyme; add 10 μmoles of EDTA</td>
<td>40</td>
</tr>
<tr>
<td>Zero time control</td>
<td>10</td>
</tr>
<tr>
<td><strong>Experiment B</strong></td>
<td><strong>all vessels also contained 1.0 μmole of NAD, 1.0 μmole of NADP, 10 μmoles of sodium glucose 6-phosphate, and 3400 units of purified yeast glucose 6-phosphate dehydrogenase. The quantity of enzyme protein was varied</strong></td>
</tr>
<tr>
<td>Enzyme, 0 mg</td>
<td>70</td>
</tr>
<tr>
<td>Enzyme, 4.5 mg</td>
<td>160</td>
</tr>
<tr>
<td>Enzyme, 9.0 mg</td>
<td>380</td>
</tr>
<tr>
<td>Enzyme, 15.0 mg</td>
<td>1150</td>
</tr>
<tr>
<td>Zero time control (45.0 mg of enzyme)</td>
<td>10</td>
</tr>
<tr>
<td><strong>Experiment C</strong></td>
<td><strong>experimental conditions as described for Experiment B. The quantity of enzyme protein was 45.0 mg</strong></td>
</tr>
<tr>
<td>Complete system</td>
<td>1290</td>
</tr>
<tr>
<td>Plus 10 μmoles of EDTA</td>
<td>730</td>
</tr>
<tr>
<td>Omit glucose-6-P and glucose-6-P dehydrogenase</td>
<td>1540</td>
</tr>
<tr>
<td>Omit glucose-6-P and glucose-6-P dehydrogenase, add 10 μmoles of EDTA</td>
<td>390</td>
</tr>
<tr>
<td>Zero time control</td>
<td>120</td>
</tr>
</tbody>
</table>
produced 193 and 49.5 μmoles of 14CO₂ in the absence and presence of 10 μmoles of EDTA, respectively. These findings suggest strongly that synthetic 2-oxo-cis-4-hexenoic acid-1-14C is capable of being converted to 14CO₂ by the enzyme preparations which carry out the oxidation of the steroid, and that the reaction conditions and oxidation rates are compatible with the participation of the α-oxo acid as an intermediate in the oxidation of the steroid.

Enzymatic Conversion of 2-Oxo-cis-4-hexenoic Acid to 2-Amino-cis-4-hexenoic Acid, Alanine, and Norleucine—Earlier experiments from our laboratory indicated that cell-free extracts of steroid-induced P. testosteroni converted Δ⁴-androstene-3,17-dione-4-14C to 14CO₂, and that under specified conditions L-2-amino-cis-4-hexenoic acid-1-14C and DL-alanine-1-14C accumulated (2). Since 2-oxo-cis-4-hexenoic acid appeared to be an intermediate in the steroid degradation, we examined the question of whether the α-oxo acid could also serve as a precursor of the amino acids.

The reaction system was similar to that used for Experiment B of Table I, and contained, in a final volume of 2.0 ml, 100 μmoles of potassium phosphate (pH 7.4), 1.0 μmole of NAD, 1.0 μmole of NADP, 10 μmoles of sodium glucose 6-phosphate, 3400 units of purified yeast glucose 6-phosphate dehydrogenase, 4.0 μmoles of synthetic 2-oxo-cis-4-hexenoic acid-1-14C (11,200 cpm per μmole), and ultracentrifuged extract of P. testosteroni equivalent of 45 mg of protein. After incubation for 60 min at 30°, the reaction was arrested by addition of 0.2 ml of 10 % sulfuric acid, and the mixture was extracted with ethyl acetate. The aqueous phase was passed over a Dowex 50-X8 column (1.0 x 7.0 cm), and the unadsorbed material was then applied to a Dowex 50-X8 (H⁺) column (1.0 x 7.0 cm). Most of the radioactivity was retained on the column, and was then eluted with 1 M ammonium hydroxide. The dried eluate was dissolved in citrate buffer at pH 2.88, and was applied to the column of the Technicon amino acid analyzer and chromatographed in the usual manner without the use of the optical system. The eluate was collected in fractions (10 min or 5.8 ml), and aliquots were examined for radioactivity. Only three radioactive peaks were detected and these corresponded precisely to the elution patterns of alanine, 2-amino-cis-4-hexenoic acid, and norleucine (XVII, Scheme 3). The eluates corresponding to the 2-amino-cis-4-hexenoic acid were combined and desalted by passage over ion exchange resins as described, and an aliquot was chromato-

![Scheme 3](image)

FIG. 5. Amino acid analyzer tracings showing the conversion of 2-oxo-cis-4-hexenoic acid to 2-amino-cis-4-hexenoic acid and norleucine. The incubation systems contained, in a final volume of 2.0 ml, 100 μmoles of potassium phosphate (pH 7.4), 1 μmole of NAD, 1 μmole of NADP, 10 μmoles of sodium glucose 6-phosphate, 3900 units of purified glucose 6-phosphate dehydrogenase, 4 μmoles of synthetic 2-oxo-cis-4-hexenoic acid-1-14C, and 10 μmoles of EDTA. The quantity of enzyme (ultracentrifuged extract of P. testosteroni) added to the complete system (A) was 0.5 ml (22.5 mg of protein) and to the zero time control (B) was 1.0 ml (45 mg of protein). The incubations were carried out for 60 min at 30°. Reactions were arrested by addition of 0.2 ml of 10 % H₂SO₄, and the reaction mixtures were processed as described in the text. The aliquots of the aqueous phase finally applied to the amino acid analyzer column were equivalent to 0.2 ml of enzyme solution for A, and 0.5 ml of enzyme solution for B. Panel C shows a standard mixture of 0.1 μmole each of L-valine, L-cystine, L-methionine, L-isoleucine, L-leucine, L-norleucine, L-tyrosine, and L-phenylalanine, and 0.2 μmole of DL-2-amino-cis-4-hexenoic acid.
detected. In the aqueous phase obtained from the incubation vessel which contained synthetic 2-oxo-cis-4-hexenoic acid (Fig. 5, tracing A), 123 mpmoles of 2-amino-cis-4-hexenoic acid and 150 mpmoles of norleucine (XVII, Scheme 3) were present, but only a trace (9 mpmoles) of material was eluted in the region just beyond the cis isomer, where 2-amino-trans-4-hexenoic acid is found (2).

Since incubation of Δ4-androstene-3,17-dione with these enzyme preparations did not lead to the formation of norleucine, possible reasons for the relatively efficient formation of norleucine from synthetic 2-oxo-cis-4-hexenoic acid were examined. The addition of 4 μmoles of 2-amino-cis-4-hexenoic acid to the incubation system, followed by examination of the products of the aqueous phase in the amino acid analyzer, established that this amino acid is not converted to any detectable extent to norleucine or other amino acids. In earlier experiments, incubation of 2-amino-cis-4-hexenoic acid-1-14C with the same enzymatic system failed to produce 14CO2 (2). In contrast to this finding is the observation that the addition of 4 μmoles of 2-oxohexenoic acid (XVI) to the system gave rise to 770 mpmoles of norleucine (XVII) under similar conditions of incubation. In the presence of 10 μmoles of EDTA 740 mpmoles of norleucine were formed from the saturated α-oxo acid. Zero time controls in these cases also confirmed the almost complete absence of any ninhydrin-positive material with the chromatographic properties of norleucine.

In sum, these observations indicate that the enzyme preparations contain a novel enzymatic system which reduces the double bond of 2-oxo-cis-4-hexenoic acid (VI) but is not capable of reducing the double bond of 2-amino-cis-4-hexenoic acid (IX).
The saturated and unsaturated α,ω-oxo acids both undergo rapid amiation to the corresponding amino acids. The rapid formation of norleucine (XVII) from synthetic 2-oxo-cis-4-hexenoic acid (VI) must therefore proceed by way of a reduction of the double bond to form 2-oxo-hexanoic acid, followed by the amiation of the oxo group to an amino group, rather than by the reverse sequence of events (Scheme 3).

**Enzymatic Conversion of 2-Oxo-cis-4-Hexenoic Acid to 2-Oxo-4-hydroxyhexanoic Acid and Isolation of Its Lactone—**Five milliliters of cell-free extract (295 mg of protein) of *P. testosteroni* were added to 270 ml of 0.03 M potassium phosphate buffer (pH 7.4), followed by 25 ml of an aqueous solution containing 89 mg of freshly prepared 2-oxo-cis-4-hexenoic acid, and the mixture was incubated at 25° for 45 min. During the incubation the absorption at 274 μm decreased from 9.1 to 0.7 (Fig. 6). Then 10 ml of concentrated sulfuric acid were added and the solution was heated for 20 min on the steam bath. Upon cooling, the precipitated protein was removed by centrifugation and the solution was extracted with ether. The ether extract was dried over sodium sulfate and evaporated under nitrogen to yield 67 mg of a residue which was chromatographed on a silicic acid-Celite (9:1) column (1 x 15 cm). Elution of the column with benzene-acetone-acetic acid (90:5:5) (9) afforded 43 mg of light yellow residue, which upon sublimation at 25° and 0.001 mm of mercury yielded 5.8 mg of pure optically active 2-oxo-4-ethylbutyrolactone. The infrared spectrum (CHCl₃) (Fig. 7) and the ultraviolet spectrum (Fig. 4) of this product were identical in deuterium oxide. The disappearance of this signal in D₂O presumably results from exchange of the C-3 methyl protons of VIb. Since no signal in the NMR spectrum can be assigned to an ethyl group, the conjugated dienolic (cis and trans) structures in which the compound may exist have not been carried out, it seems plausible to assign an isomeric structure to the acid product.

The 2-oxo-cis-4-hexenoic acid appears to exist in aqueous solution predominantly as the dienol (VIb). The reaction with ferric chloride and the strong absorbance at 274 μm and its shift to 307 μm in base (presumably due to the enolate ion) support this contention. The enolate anion is quite unstable in base, as might be expected for an α,ω-oxo acid. Rapid acidification of the basic solution does not re-establish the 274 μm band of the original compound but results in a peak of approximately the same intensity at 281 μm. Although detailed studies of the several dienolic (cis and trans) structures in which the compound may exist have not been carried out, it seems plausible to assign an isomeric structure to the acid product.

The NMRR spectrum of VI was complicated by the simultaneous presence of both VIa and VIb. The doublet at 6.35 τ in deuterated chloroform was absent when the NMR spectrum was determined in deuterium oxide. The disappearance of this signal in D₂O presumably results from exchange of the C-3 methyl ene protons of VIa. The doublet at 8.4 τ probably arises from the methyl protons of VIa whereas the doublet at 8.15 τ may be attributed to the methyl protons of VIb. Since no signal in the NMR spectrum can be assigned to an ethyl group, the conjugated α,ω-oxo acid structure is excluded.

**Comments on Degradation Pathway of Steroids—**In earlier experiments, Shaw et al. (9) provided evidence that cell-free ultracentrifuged extracts of steroid-induced *P. testosteroni* converted Δ⁴-androstene-3,17-dione-1⁴C (I) to l-2-amino-cis-4-hexenoic acid-1⁴C (IX) and l-α-alanine-1⁴C (X) and suggested that 2-oxo-cis-4-hexenoic acid (VI) was a key intermediate in these transformations. The enzyme preparations did not convert the amino acids further to CO₂. The isolation of 2-oxo-4-ethylbutyrolactone (VIII) by Gibson et al. (9) led these authors to postulate that 2-oxo-4-hydroxyhexanoic acid (VII) (which was further converted enzymatically to pyruvate and propionaldehyde) served as an intermediate in the degradative pathway of steroids. However, the question remained unresolved as to the origin of the 2-oxo-4-hydroxyhexanoic acid and whether it was in fact formed from a 1-hydroxy seco-steroid, or whether the hydroxyl group was introduced after cleavage of the C₅ fragment.
derived from Ring A. Moreover, no information on the optical activity of the lactone or its hydroxy acid has been available. We undertook the synthesis of 2-oxo-cis-4-hexenoic acid-1-14C and were able to show that this compound was rapidly converted to 14CO2 by enzyme preparations which oxidized Δ4-androstene-3,17-dione-4-14C to 14CO2. Our experiments also indicate that synthetic 2-oxo-cis-4-hexenoic acid is converted to 2-amino-cis-4-hexenoic acid and alanine. This finding is compatible with the proposal that the two amino acids originate from the α-oxo acid. Finally, it has been shown that if 2-oxo-cis-4-hexenoic acid is added to the steroid-oxidizing enzyme preparations under suitable conditions, the ultraviolet absorption of the enolic form of the α-oxo acid disappears, and, upon heating the reaction mixture with acid, an optically active form of 2-oxo-4-ethyl-3-butyrolactone may be isolated. The latter finding provides critical evidence for the asymmetric hydration of the synthetic 2-oxo-4-hexenoic acid to give 2-oxo-4-hydroxyhexanoic acid of established optical configuration. As might be expected, the direct conversion of 2-oxo-cis-4-hexenoic acid to 2-oxo-4-ethylbutyrolactone by hot acid leads to an optically inactive product.

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