BIOLOGICAL CONVERSION OF 17α-HYDROPEROXYPROGESTERONE TO 11α,17α-DIHYDROXYPROGESTERONE*

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SUMMARY

The metabolism of 17α-hydroperoxyprogesterone and of its 17α-hydroxy analogue by aerated vegetative cell cultures of Aspergillus ochraceus NRRL 405, was investigated using the steroid substrates as the sole source of carbon. The two differently substituted progesterone derivatives gave rise to a single and identical metabolite. The structure of this unknown compound was established by chemical degradation, gas chromatography, infrared-, and mass spectroscopy as 11α,17α-dihydroxyprogesterone. The implications of this finding in terms of the concept of hydroperoxides as possible biological intermediates in steroid hydroxylations, are discussed.

Hydroperoxy derivatives of cholesterol have very recently been advanced as possible intermediates in the biodegradation of the C_{27} sterols to the C_{24} bile acids and the C_{21} pregnenes (1,2). Earlier, 20-, 24-, and 25-hydroperoxycholesterol have been isolated from aged cholesterol samples of animal tissue origin. At this stage however, the status of hydroperoxysterols as true enzymic intermediates, or as simple autoxidative artifacts, remains to be clarified. Of the equally interesting 17α-hydroperoxy derivatives of the pregnene series, even less information is available concerning their potential role as intermediates in the biosynthesis of the androstene hormones.

We have been interested for some time in the 20-ketosteroid C-17(20)-lyase, which we were able to induce in vegetative cell cultures of A.ochraceus NRRL 405. This enzyme system cleaved the acetyl side-chain of progesterone

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with concomitant hydroxylation to yield 11α-hydroxytestosterone. The latter C_{19} metabolite has been demonstrated both in incubations with intact mycelia (3), and with cell-free crude enzyme preparations (4). In concert with other authors (5-9), we have postulated for the cleavage reaction effected by A.ochraceus a Baeyer-Villiger oxidative mechanism, initiated by a putative peroxy radical attack at the C-20 ketone function, followed by a Wagner-Meerwein type 1,2-shift to yield, via the 17β-acetate ester, the free 17β-alcohol. However, with our microorganism, we have never been able to detect during the course of the incubations by gas chromatographic analysis the formation of any 17β-acetate ester as an obligatory intermediate.

In this study, we report on the metabolism of 17α-hydroperoxyprogesterone and of 17α-hydroxyprogesterone by vegetative cells of A.ochraceus in an attempt to discover, whether an alternative mechanism for the progesterone side-chain scission by this fungus may be operative.

MATERIALS AND METHODS

Progesterone was purchased from Organon, Oss, The Netherlands; 17α-hydroxyprogesterone from Sigma Chemical Company, St. Louis, Mo. The A.ochraceus NRRL 405 strain was obtained through the courtesy of Dr. C.W.Hesseltine, Peoria, Ill. All reagents were analytical grade. Solvents were predistilled before use. Determination of melting points, infrared spectroscopy, thin-layer and gas chromatography were performed as described earlier (10). Mass spectra were obtained with a Hitachi RMU-6E instrument via the direct introduction probe under the following parameters: ion source temperature 210°, electron energy 70 eV, target current 20 mA, accelerating voltage 1 kV.

Preparation of 17α-Hydroperoxyprogesterone

17α-Hydroperoxyprogesterone was prepared via the 3-ethyl-3,5-dienolether by a combination of the procedures, as described by Barton, et al. (11) and van Rheenen, et al. (12). The uptake of molecular oxygen was followed in a slightly modified hydrogenation apparatus. After hydrolysis with acetic acid, precipitation in ice-water, filtration, and recrystallization from aqueous

dioxane, the colourless needles of 17α-hydroperoxyprogesterone obtained had a melting point of 186-190°; ν_{max}^{KBr}: 3190 and 871 (-OOH), 1710 (20-ketone), and 1660, 1610 (Δ^4-3-ketone) cm^{-1}. TLC with $CHCl_3$/ CH_3OH (9:1) as a solvent system gave a single spot, R_f 0.57. As expected, the compound underwent thermal decomposition in the gaseous state and at 230° on a 3% OV-1 column, the progesterone-17α-hydroperoxide can be characterized by its "gas fragmentogram" consisting of a major peak with RRT* 1.16 and satellite peaks with RRT 0.32, 0.42, 0.50, 0.67, 0.76 and 0.82.

Microbiological incubations

A flask containing 250 ml of sterilized nutrient medium (1% glucose, 1% yeast extract) was inoculated with spores of A.ochraceus (14 months old), conserved on agar in a refrigerator. After 48 hours of growth at 29° on a gyrotory shaker, a few cells were transferred to a 5L fermentation flask (3,4), containing 3L of the same nutrient medium. Progesterone (400mg) and 17α-ethinyl-17β-hydroxy-4-estren-3-one (200mg) were then added as inducers. After a further 48 hours of propagation, the cells were harvested, washed thoroughly free from steroids (verified by extraction and GLC analysis of an aliquot) with a 0.5% solution of NaCl, and blotted on filter paper. A portion of the wet cells (75g) was resuspended in 900ml of a sterile, inorganic phosphate buffer of pH 7.0. The suspension was then divided in 6 equal parts.
A solution of 17α-hydroperoxyprogesterone in dioxane was injected into a 150ml portion of the inorganic cell suspension to give a final concentration of 150μg/ml. As a control, the same amount of the hydroperoxy steroid was injected into a flask, containing only the phosphate buffer solution. Simultaneously and in the same manner, an incubation using 17α-hydroxyprogesterone as a substrate was carried out in the presence of the vegetative cells. After shaking at 29° for 36 hours, the incubations were stopped. Extractions with methylisobutylketone and purifications of the reaction product by repetetive preparative TLC were performed as described previously (3,4).

* Retention time, relative to 5α-cholestane

RESULTS AND DISCUSSION

From the two separate incubations with 17α-hydroperoxyprogesterone and 17α-hydroxyprogesterone as substrates, a single and identical metabolite was obtained as demonstrated by GLC analysis on different liquid phases, superimposable infrared spectra and a mixed melting point determination. On 3% OV-1 at 230°, the common metabolite had a RRT of 2.09. Upon further purification by preparative TLC, a crystalline compound could be isolated. Recrystallization from ether gave a m.p. of 215-220°. Its infrared spectrum (KBr) showed strong hydroxyl absorption at 3400-3460 and 1025-1050 cm^{-1} (disappearance of the hydroperoxy band at 3190 cm^{-1}), Δ^4-3-ketone absorption at 1655 and 1606 cm^{-1}, whereas the presence of the C-20 ketone was indicated by a shoulder at 1690 cm^{-1}. Because the latter band was not clearly resolved, and formation of a C_{19} androstene metabolite by side-chain cleavage could thus not be ruled out, it was decided to submit the unknown compound to electron-impact induced fragmentation. Its mass spectrum is shown in Fig. 1.

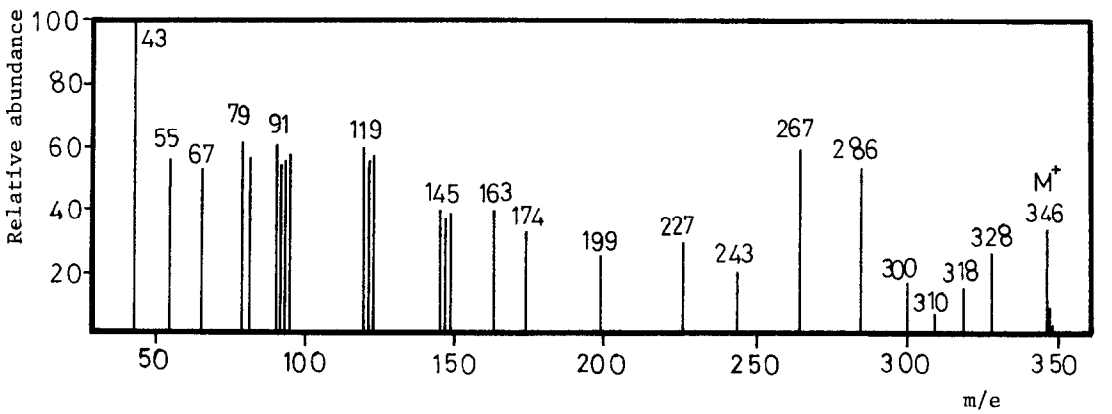


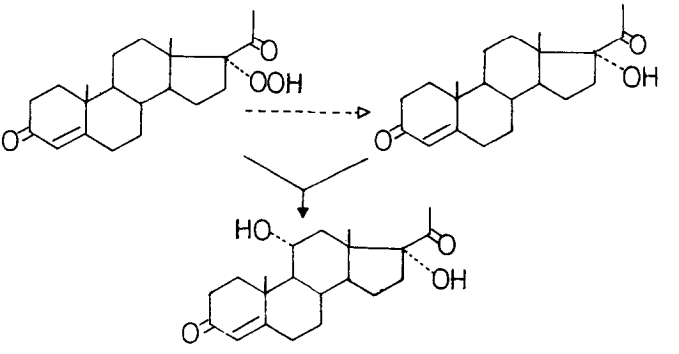
Fig. 1. Mass spectrum of crystalline metabolite, obtained from incubation of 17α-hydroperoxyprogesterone with A.ochraceus.

The molecular ion at m/e 346 clearly is too high for a C_{19} compound. The M + 1/M ratio of 23.6% also indicates that the C_2 side-chain is still intact. The base peak occurs at m/e 43, consistent with the presence of a CH_3CO side-

chain in the parent molecule. Other significant fragment ions at m/e 328 ($M-H_2O$), 318 (M-CO), 310 ($M-2H_2O$), 300 ($M-CO-H_2O$) and 286 ($M-CH_3CO-OH$) are entirely in agreement with the structure of 11α,17α-dihydroxyprogesterone ($C_{21}H_{30}O_4$, mol wt 346) for the unknown metabolite. As a further proof, the compound was oxidized with Jones reagent (13) to yield the well-known 4-androsten-3,11,17-trione (adrenosterone). GLC analysis with authentic adrenosterone as a reference confirmed the identity of this chemical degradation product.

As earlier described (3), the C-17(20)-lyase, inducible in growing cultures of A.ochraceus, produced side-chain scission in only very small yields. In this case, GLC analysis carried out with a detection sensitivity at the submicrogram level, failed to indicate the presence of hydroxylated testosterone metabolites. We assume that the induction period for the incubations as applied to this particular batch, proved to be insufficient.

On the other hand, we consider the conversion of both 17α-hydroperoxyprogesterone and 17α-hydroxyprogesterone to a single metabolite of identical structure as significant. Reduction of the hydroperoxy group by artifact formation can be excluded, since in the control incubation with 17α-hydroperoxyprogesterone in the absence of cells, but otherwise carried out under identical conditions, no such conversion could be observed and the starting material was recovered unchanged. The concomitant introduction of a hydroxyl group at an inactive site of the nucleus in a stereospecific manner, namely at 11α, also lends support to our contention that the tertiary hydroperoxy group at C-17 has indeed been enzymically reduced by our A.ochraceus culture according to the following scheme.



Despite numerous reports in the literature (14), the exact sequence of events by which steroid hydroxylations in biological systems are effected, or the acetyl side-chain of progesterone is split off enzymatically, is not really fully understood. At least 3 steps seem to be involved: I. binding of molecular oxygen, II. activation and lysis of a σ-bond, and III. substitution by OH. Studies with stable O^{18} isotopes have shown that molecular oxygen is incorporated at C-17 in the formation of testosterone from progesterone by rat testicular and adrenal enzyme preparations (15). This finding is not in disagreement with our hypothesis of the existence of a pathway in certain biological hydroxylations, involving prior formation of hydroperoxide intermediates according to the sequence:

$$\geq C-H \xrightarrow[O_2]{(1)} \geq C-OOH \xrightarrow[NADPH]{(2)} \geq C-OH + H_2O$$

In the case of tertiairy hydrogen atoms activated by a vicinal ketone group or by other electron capturing chromophores (16), reaction step 1 may very well be of a chemical, non-enzymic nature.

This study seems to be the first report on the biological conversion of 17α-hydroperoxyprogesterone.

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