ACTA CHEMICA SCANDINAVICA 21 (1967) 1297–1303

Mechanism of the Side Chain Degradation of Progesterone by Microorganisms

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The degradation of the side chain of progesterone by *Aspergillus flavus* and *Penicillium lilacinum* has been studied. *A. flavus* cleaves the side chain *via* an enzymatic Bayer-Williger-oxidation of progesterone to testosterone acetate. This intermediate is then hydrolyzed to acetic acid and testosterone. The testosterone is then oxidized to Δ^4-androstene-3,17-dione. *P. lilacinum* more directly oxidizes progesterone to Δ^4-androstene-3,17-dione and no testosterone acetate is formed. *P. lilacinum* easily oxidizes 17α-hydroxyprogesterone to Δ^4-androstene-3,17-dione, while *A. flavus* leaves this steroid unaffected.

Side chain degradation of progesterone, forming the C_{19}-steroids testosterone and Δ^4-androstene-3,17-dione, is carried out by human tissues as well as by several microorganisms. While the biologic function of these transformations in man is well known, their role in the microbial metabolism is still uncertain. The probable explanation is that they act as the initial step in the total oxidation of the steroid material to carbon dioxide and water.[1,2]

For the cleavage of the side chain two ways are known. The first pathway was demonstrated in human and animal tissues [3] and starts with an oxidation of progesterone to 17α-hydroxyprogesterone. This is followed by subsequent addition of oxygen to form a 17α-hydroxy-17β-peracetate. This unstable intermediate is then cleaved to acetic acid and Δ^4-androstene-3,17-dione, the latter being reduced to testosterone.

In the microbial side chain degradation the first intermediate to be demonstrated was testosterone acetate, which was found by Fonken *et al.*[4] after progesterone fermentations with *Cladosporium resinae*. The formation of this 17β-acetate evidently indicates a pathway similar to the non-enzymatic Bayer-Williger-oxidation of ketones to esters.[5] The testosterone acetate is then hydrolyzed to acetic acid and testosterone, which then can be oxidized to Δ^4-androstene-3,17-dione. Recently the same pathway has been demonstrated in this laboratory for *Streptomyces griseus*.[6] The two principal pathways for progesterone side chain degradation are given in Fig. 1.

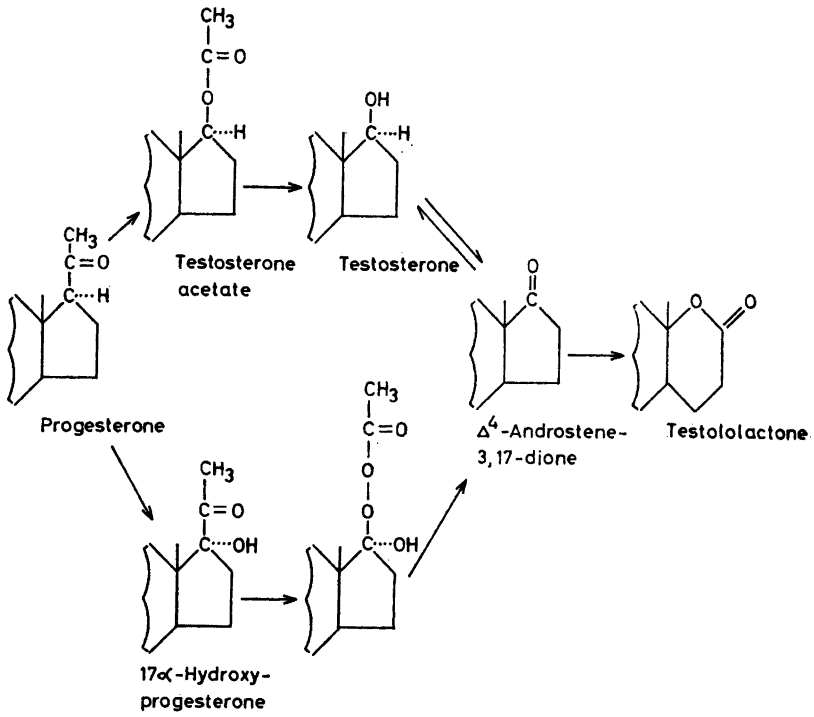

Fig. 1. The two principal pathways for the degradation of the side chain of progesterone.

Δ^4-Pregnene-20β-ol-3-one has been demonstrated as a primary metabolite in the formation of C_{19}-steroids from progesterone by *Penicillium lilacinum*.[7,8] However, the role of this reductive transformation in the oxidative breakdown of the side chain is somewhat uncertain.

Contrary to human tissues, several microorganisms oxidize Δ^4-androstene-3,17-dione to testololactone *via* an enzymatic Bayer-Williger-oxidation.[9] However, this transformation is of less interest in a study of the initial steps of the side chain degradation.

Substrate specificity in the microbial side chain degradation has been studied by Sih and co-workers.[2] Using *Penicillium citrinum* and *Cylindrocarpon radicicola*, which both cleave the side chain of progesterone, they found that the side chain of 17α-hydroxy-progesterone was cleaved by *C. radicicola* but not by *P. citrinum*. From those results the conclusion was made that *P. citrinum* and *C. radicicola* cleave the side chain of progesterone in two different ways.

In order to study the initial steps in the oxidation of progesterone by microorganisms the fungi *A. flavus* and *P. lilacinum* were investigated. The results of this investigation show that the two fungi cleave the side chain in two quite different ways. *A. flavus* oxidizes progesterone *via* testosterone acetate to testosterone and Δ^4-androstene-3,17-dione as has been shown for

C. resinae[4] and *S. griseus*.[6] *P. lilacinum* more directly oxidizes progesterone to Δ^4-androstene-3,17-dione, probably *via* 17α-hydroxy-progesterone in the same manner as has been demonstrated for human and animal tissues.

During the investigation formation of testololactone and later metabolites was of no interest and therefore only the formation of testosterone, Δ^4-androstene-3,17-dione, and their precursors was studied.

MATERIALS AND METHODS

Strains of *A. flavus* (QM 380) and *P. lilacinum* (NRRL 895) were obtained from Centralbureau voor Schimmelcultures, Baarn. The fungi were grown up on beer wort-distilled water 1:1, harvested and washed as has been described previously.[6] After washing the mycelia were resuspended to original density in distilled water and distributed in 50 ml portions into 200 ml Erlenmeyer flasks. Steroid substrate, dissolved in methanol and in some cases DFP (di-isopropyl-fluorophosphate), dissolved in isopropanol, was pipetted into the flasks. The flasks were stoppered with cotton plugs and incubated on a shaking table at 23—25°C. At definite time intervals samples were withdrawn and extracted with two 50 ml portions of chloroform. Excepting the estimation of 17α-hydroxy-progesterone, the chloroform extracts were analyzed with thin-layer chromatography (TLC) and gas-liquid chromatography (GLC) as has been described previously.[6]

17α-Hydroxy-progesterone was impossible to estimate by GLC technique because of the decomposition of this steroid on the column. Therefore a colorimetric method was utilized. This method is developed from the method of Birke *et al.*[10] for estimation of 17-ketogenic steroids in urine. The steroid mixture, containing 17α-hydroxy-progesterone, Δ^4-androstene-3,17-dione, and traces of testosterone is first treated with KBH_4 in order to reduce the present 17-ketosteroid material to 17-hydroxy compounds. After that the 17α-hydroxyprogesterone is transformed to a 17-keto-steroid by oxidation with bismuthate. The 17-hydroxy-steroids are not oxidized by the bismuthate and therefore the product obtained from 17α-hydroxy-progesterone is the only 17-keto-steroid present. The amount of this 17-ketosteroid is finally measured, using the Zimmerman colour reaction with Δ^4-androstene-3,17-dione as standard. The estimation was carried out according to the following procedure: One fifth of the chloroform extract is evaporated to dryness. The residue is dissolved in 20 ml of methanol and 0.1 g of KBH_4 is added. The mixture is kept at 50°C for 30 min and after that cooled to room temperature. 8 ml of glacial acetic acid and 2 g of sodium bismuthate is added and the mixture is shaken in the dark for 2 h. After centrifugation 10 ml of the clear supernatant is transferred to a separatory funnel, containing 40 ml of water. The steroids are extracted with 75 ml of ether and the ether phase is washed with two 25 ml portions of 3 M NaOH and with two 25 ml portions of water. The washed ether phase is dried over sodium sulphate, filtered and evaporated to dryness. The residue is transferred with three 3 ml portions of chloroform to a Kober tube and evaporated to absolute dryness. Zimmerman colour reaction is made according to the paper of Birke *et al.*[10] and 50 μg of Δ^4-androstene-3,17-dione is used as standard.

Identification of the steroids formed during the fermentations was carried out by comparing the R_F-values in two different TLC systems,[6] the GLC retention times on two different liquid phases,[6] and the infrared spectra with the corresponding properties of the authentic substances. In some cases formation of derivatives and comparison of their properties with the corresponding derivatives of the reference substances was carried out for further identification.

Analytical samples for the IR-spectroscopy were obtained by column chromatography on neutral alumina, using benzene, containing increasing amounts of ethyl acetate as solvent, as has been described previously.[6] The testosterone acetate appeared in the benzene-ethyl acetate 10:1 fraction, the Δ^4-androstene-3,17-dione in the 10:1.5 fraction and the testosterone in the 10:2 fraction. The IR-spectra were recorded on a Perkin-Elmer 237 IR-spectrometer.

RESULTS

1. *Incubation of progesterone with* A. flavus. Into each flask containing 50 ml of mycel suspension 29.0 mg of progesterone, dissolved in 1.0 ml of methanol, was pipetted. The composition of the steroid mixture during the fermentation is given in Fig. 2. Following steroids were formed: Testosterone acetate (identified by TLC, GLC, and hydrolysis to testosterone), testosterone (IR, TLC, GLC, and acetylation to testosterone acetate), Δ^4-androstene-3,17-dione (TLC, GLC) and a fourth product not identified. This unidentified product appeared in the later stages of the fermentation and it had the same TLC and GLC properties as a compound formed in the later steps of the progesterone fermentation by *P. lilacinum*.

2. *Incubation of progesterone with* A. flavus *during simultaneous inhibition of esterase activity with DFP*. The conditions were the same as under 1 except that 0.2 ml of 10 % DFP in isopropanol (giving a DFP concentration of 2.15×10^{-3} M) was added. During this fermentation only testosterone acetate was formed (IR, TLC, GLC, and hydrolysis to testosterone). The composition of the steroid mixture during the fermentation is given in Fig. 3. No other steroid, except the substrate progesterone, could be detected.

3. *Incubation of progesterone with* P. lilacinum. The fermentation was carried out as described for *A. flavus* under 1. Following steroids were detected: Testosterone (TLC, GLC, and acetylation to testosterone acetate), Δ^4-andros-

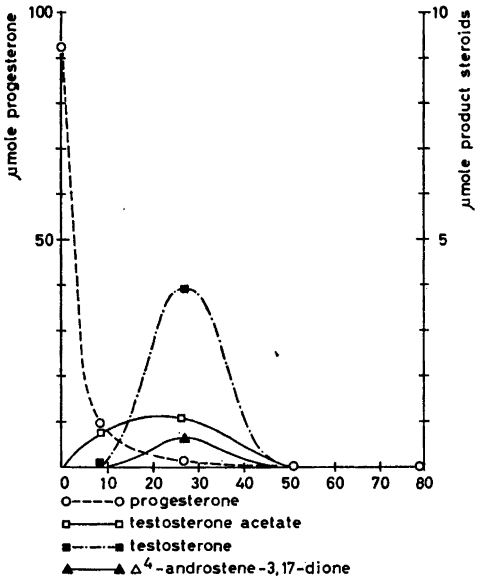

Fig. 2. Composition of the steroid mixture during the fermentation of progesterone with *A. flavus*.

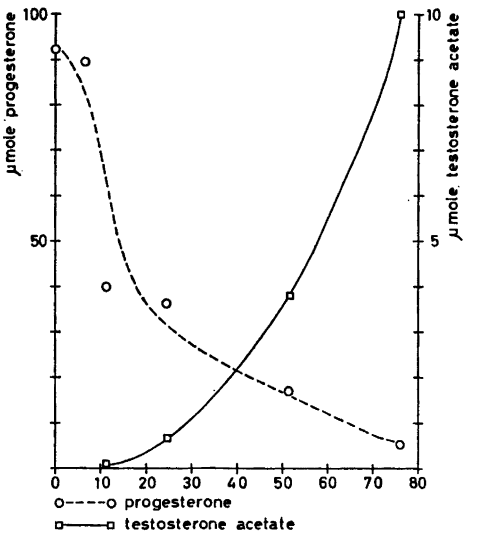

Fig. 3. Composition of the steroid mixture during the fermentation of progesterone with *A. flavus*. DFP is added as an esterase inhibitor.

Table 1. R_F-values and retention times for progesterone, testosterone acetate, Δ^4-androstene-3,17-dione, testosterone, and the unidentified compound.
TLC I: Silica gel G; Ethyl acetate:benzene:hexane 1:1:1.
TLC II: Al_2O_3 G; 0.5 % ethanol in benzene.
GLC I: 1 % XE-60 on AW-HMDS-Chromosorb W, 80—100 mesh. Column 2 m × 1/8″ stainless steel, column temperature 230°C, nitrogen flow 50 ml/min.
GLC II: 3 % SE-30 + 0.1 % Versamid 900 on AW-HMDS-Chromosorb W, 80—100 mesh. Column as in GLC I.
Column temperature 240°C, nitrogen flow 50 ml/min.

Steroid	R_F-value, TLC I	R_F-value, TLC II	Retention time, GLC I, sec	Retention time, GLC II, sec
Progesterone:	0.38	0.48	174	432
Testosterone acetate:	0.47	0.50	162	408
Δ^4-Androstene-3,17-dione:	0.29	0.26	138	258
Testosterone:	0.20	0.10	144	264
Unknown compound:	0.06	0.08	448	—

tene-3,17-dione (IR, TLC, and GLC) and a third unidentified compound. This compound appeared in the last samples of the fermentation and it had the chromatographic properties described in Table 1. Formation of testololactone by *P. lilacinum* is well known and it is probable that the unknown compound may be testololactone. The composition of the steroid mixture during the fermentation is given in Fig. 4.

4. *Incubation of progesterone with* P. lilacinum *during simultaneous inhibition of esterase activity with DFP.* The conditions were the same as for *A. flavus* under 2. The same steroids were formed as under 3. No greater difference from 3 in the composition of the steroid mixture was observed.

5. *Incubation of 17α-hydroxy-progesterone with* P. lilacinum. 5.6 mg of 17α-hydroxy-progesterone, dissolved in 1.0 ml of methanol was added to 50 ml mycel suspension and the fermentation was carried out as under 1. During the fermentation Δ^4-androstene-3,17-dione (TLC and GLC), testosterone (TLC and acetylation to testosterone acetate) and in the later stages of the fermentation traces of a compound with the same TLC-properties as the unknown substance in 3 and 4 were formed. Composition of the fermentation mixture is given in Fig. 5.

6. *Incubation of 17α-hydroxy-progesterone with* A. flavus. The conditions were the same as for *P. lilacinum* under 5. During the fermentation no transformation of the steroid substrate to any known product could be demonstrated.

DISCUSSION

From the results of this work it is clear that *A. flavus* and *P. lilacinum* cleave the side chain of progesterone in quite different ways. This confirms the results of Sih *et al.*[2] that several ways of the side chain scission exist in microorganisms.

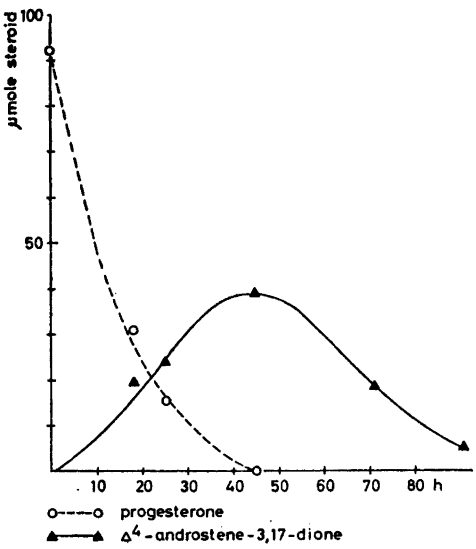

Fig. 4. Composition of the steroid mixture during the fermentation of progesterone with *P. lilacinum*. Testosterone was only present in trace amounts.

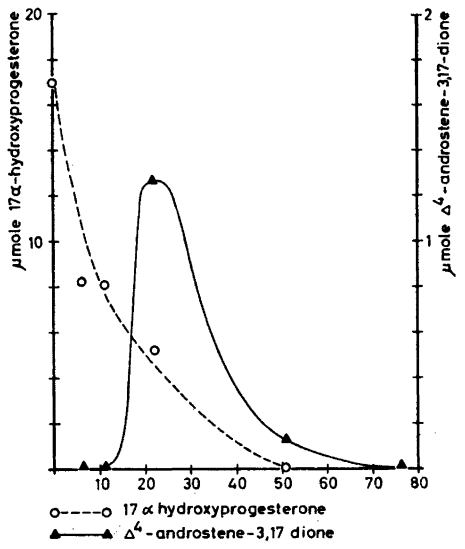

Fig. 5. Composition of the steroid mixture during the fermentation of 17α-hydroxy-progesterone with *P. lilacinum*. Testosterone was only present in trace amounts.

A. flavus oxidizes progesterone in the same manner as has been demonstrated for *C. resinae* [4] and *S. griseus*,[6] *i.e. via* an enzymatic Bayer-Williger-oxidation to testosterone acetate. During incubation of progesterone with *A. flavus* in presence of DFP, which is a powerful inhibitor of the steroid esterase,[11] testosterone acetate is accumulated and the subsequent transformation to testosterone and Δ^4-androstene-3,17-dione is completely inhibited. When 17α-hydroxy-progesterone is incubated with *A. flavus* no transformation takes place.

The progesterone metabolism of *P. lilacinum* was not influenced by DFP. This indicates that the side chain degradation by *P. lilacinum* can not proceed in the same way as by *A. flavus*. *P. lilacinum* readily oxidizes 17α-hydroxy-progesterone, giving the same products as from progesterone. From this it may be assumed that the cleavage of the side chain of progesterone by *P. lilacinum* proceeds in the same way as has been demonstrated in human and animal tissues, *i.e. via* 17α-hydroxy-progesterone.

During the fermentation of progesterone with *P. lilacinum* no 17α-hydroxy-progesterone could be detected. This may be explained by the rapid conversion of this intermediate into Δ^4-androstene-3,17-dione.

From the results of Sih *et al.*[2] and of this work the conclusion can be made that the two *Penicillium* fungi *P. lilacinum* and *P. citrinum* cleave the side chain in quite different ways. *P. citrinum* was found to be incapable of cleaving the side chain of 17α-hydroxy-progesterone.[2] However, some other workers [12,13] have found that this steroid is readily transformed by *P. citrinum*

into testosterone and 1-dehydro-testololactone. The mechanism of the side chain degradation is in no way specific for the genus of the microorganism. Even different strains of the same species seem to oxidize progesterone in quite different ways.

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Received February 20, 1967.