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# INTERACTIONS OF STEROIDS AND FUNGI

## III*. 11α-HYDROXYLATION AND DEGRADATION OF PROGESTERONE-4-$^{14}$C BY A CELL-FREE PREPARATION FROM *ASPERGILLUS OCHRACEUS*

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SUMMARY

A 20-keto steroid 11α-hydroxylase and 17(20)-lyase active, cell-free preparation from *Aspergillus ochraceus* NRRL 405 has been obtained by rupture of the mycelia in a phosphate buffer at pH 7·2 in the presence of ethylenediaminetetraacetic acid and centrifugation of the homogenate at 43000 × g for 45 min. Hydroxylase and lyase activities were measured independently by two methods, reverse isotope dilution and gas chromatographic analysis, using progesterone-4-$^{14}$C as a substrate. The formation of radioactive 11α-hydroxyprogesterone and 11α-hydroxytestosterone have thus been demonstrated and their radiochemical purity verified by dilution with authentic carrier compounds and crystallization to constant specific activity. This paper constitutes the first report of the obtainment of an active, cell-free, crude enzyme system from *A. ochraceus*.

### INTRODUCTION

IN A PREVIOUS paper[1], it has been shown that the oral steroid contraceptive Norethisterone† was resistent against oxidative attack by *Aspergillus ochraceus* NRRL 405. Since this microorganism otherwise readily hydroxylates $\Delta^4$-3-keto steroids at various sites of the steroid nucleus[2–5], we have extended these observations by incubating with intact cells of *A. ochraceus* other contraceptive steroids, which also possessed the linear 17α-ethinyl side chain. The results which we obtained[6], only confirmed our previous negative findings. Before attempting to settle the question, whether the inhibitive effect exerted by the pseudoaxial 17α-ethinyl substituent upon the hydroxylation reaction may be attributed, for instance, to steric or electronic factors, it became imperative to investigate whether the inertness was not simply due to decreased solubility or membrane permeability effects. Thus, incubations conducted at the steroid substrate–enzyme level were called for.

The preparation of a hydroxylase and lyase active, cell-free extract from *A. ochraceus* has, to our knowledge, not been reported previously. The present study demonstrates that it is indeed possible to obtain such a cell-free enzyme system from *A. ochraceus*.

### EXPERIMENTAL

Progesterone, 11α-hydroxyprogesterone and 17α-methyltestosterone were purchased from Steraloids Inc., Pawling, N.J. Norethisterone and norethynodrel†

*References [1] and [5] are to be regarded as Parts I and II of these series.

†Trivial names: norethisterone = 17α-ethinyl-17β-hydroxy-4-estren-3-one; norethynodrel = 17α-ethinyl-17β-hydroxy-5(10)-estren-3-one.

were a gift from Syntex Corp., Palo Alto, Cal. Their purity was verified by gas chromatographic analysis, prior to use.

EDTA, NADH, NADPH, G-6-P, G-6-P-D and GSH were obtained from Sigma Chemical Co., St. Louis, Mo. All other chemicals used were analytical grade.

The *A. ochraceus* NRRL 405 strain was obtained through the courtesy of Dr. C. W. Hesseltine, Agricultural Research Service, Peoria, Ill.

Labelled progesterone-4-$^{14}$C was purchased from New England Nuclear Corp., Boston, Mass. Its radiochemical homogenity was verified with a Packard, Model 7201 chromatogram scanner.

Thin-layer chromatography (TLC) was conducted on $5 \times 20$ or $20 \times 20$ cm glass plates, coated with 0·1–0·2 mm Silica gel $HF_{254}$ (Merck, Darmstadt) and chloroform–methanol (9:1) as a solvent system. The spots were examined under ultraviolet light at 254 nm, prior to visualization by spraying with a 50% aqueous solution of sulfuric acid and heating at 110° for two minutes.

Infrared spectra were recorded on a Perkin Elmer double beam, Model 457 grating spectrometer, equipped with a beam condenser. Prior to measurements, the 1·5 mm potassium bromide micro wafers were dried under an infrared lamp. Steroid-KBr weight ratios were held at *ca.* 1:100.

Melting points were determined on a Kofler hot stage under microscopic magnification at barometric pressure and were not corrected.

Gas liquid chromatography (GLC) was performed on a F & M, Model 402 high efficiency instrument, equipped with a hydrogen flame ionization detector. A $180 \times 0{\cdot}3$ (i.d.) cm glass column was used, packed with 3% OV-1 or 3% QF-1 on 100–120 mesh silanized Gas-Chrom Q (Applied Science Laboratories, State College, Pa.). Columns were conditioned overnight at 250° prior to use. Operating parameters were: oven 250°, injection port 275°, detector 285°, attenuation $10 \times 16$. Helium carrier gas flow: 33 ml/min at an inlet pressure of 40 psi. Peak areas were quantitated by triangulation.

Radioactivity was measured with a Packard Model 3380 liquid scintillation counter, equipped with a Model 544 Absolute Activity Analyzer. The radioactive material was dissolved in a few drops of freshly distilled methanol, prior to mixing with 15 ml of the scintillation solution (4 g of PPO and 100 mg of POPOP in 1 L of distilled toluene). Efficiency for $^{14}$C was in the order of 90% (background 17·8 cpm).

*Preparation of cell-free extracts*

Sterilized media, containing 1% glucose and 1% yeast extract solutions[1, 5] were inoculated with *A. ochraceus* spores, grown on Czapek Dox agar. After initial 48 hr growth on a rotary shaker (New Brunswick Scientific Co., N.J.) at 250 rev/min and 29°, a few cells were transferred into a previously sterilized 5 L fermentation flask, equipped with a stirrer, air inlet and outlet, and which contained 4·5 L of the same glucose-yeast extract medium. Sterile air was bubbled into the flask via 2 drying towers, filled resp. with concentrated sulfuric acid and silica gel, and a sterilized cotton-packed $40 \times 2$ cm glass tube. The amount of air and the stirrer speed were adjusted to maintain a finely dispersed suspension. After periods of 24–48 hr of propagation, a solution of progesterone in dimethylformamide was injected to give a final concentration of 150 $\mu$g/ml. Induction of the steroid hydroxylase was then allowed to proceed for 48 hr. The abundantly

growing mycelia were then filtered on a sterile Buchner funnel, washed repeatedly with 0·5% sodium chloride solution, transferred twice to Erlenmeyer flasks, each containing 1·5 L of 0·5% sodium chloride solution, vigorously stirred, and filtered again. Finally, the thoroughly washed mycelia cake was blotted on filter paper (wet weight *ca.* 70 g). From this stage, all subsequent manipulations were conducted in a cold room at 0–4°.

Portions of the blotted mycelia (conc. ·14 g/50 ml) were homogenized in a mortar by grinding with analytical grade sand and a sterile medium, consisting of 5 mM of disodiumphosphate, 10 mM of EDTA and 250 ml of demineralized water (the pH of the media was adjusted previously to 7·2 with acetic acid). The homogenate was then transferred into eight 50 ml centrifuge tubes and centrifuged i.vac. at 43000 × g[7] and 0° for 45 min. The thus obtained clear supernatant was used immediately for the cell-free incubations. The absence of any intact cell was verified by microscopic examination and a streak-test on Czapek dox agar, both of which gave negative results.

*Incubations with cell-free extracts*

(A) *Progesterone-4-$^{14}C$.* To 15 ml of the crude cell-free extract was added 2 ml of a NADPH regenerating system, consisting of NADH (3 $\mu$M/ml), NADPH (3 $\mu$M/ml), G-6-P (10 $\mu$M/ml), G-6-PD (1·4 units/ml) and GSH (5 $\mu$M/ml)[8, 9]. After thorough mixing, 2 mg of carrier progesterone (dissolved in dimethylformamide at a concentration of 20 mg/ml) and 50 $\mu$L of a benzene solution of progesterone-4-$^{14}C$ (355·000 ± 710 dpm, spec. activity 57·3 mC/mM) were injected into each of in total eight 50 ml flasks. After incubation periods of 2–5 hr at 0–4° on a reciprocal shaker, the content of each flask was saturated with sodium chloride and immediately extracted with methylisobutylketone. The organic extract was washed with sodiumhydrogencarbonate and water, dried over sodium sulfate and evaporated i. vac. The residue was dissolved in 1–2 ml of a mixture of chloroform and methanol (2 : 1), filtered via a hypodermic syringe, equipped with a Swinny adapter and a membrane filter. Of the thus obtained clear filtrate, 0·6–1·6 $\mu$L was used for the GLC analysis while the remainder was set aside for purification by preparative TLC.

(B) *17α-Methyltestosterone, norethisterone and norethynodrel.* Incubations with 17α-methyltestosterone, norethisterone and norethynodrel were carried out simultaneously in the same concentrations with the cell-free extracts as described above for progesterone. Subsequent extractions, purifications and GLC analysis were also performed in the same manner.

In all incubation experiments, a blank control sample, containing only the crude enzyme preparation was run and elaborated in an identical way. From these blank incubations, the "GLC-background" was determined and substracted from the gas chromatograms, obtained by incubations with (A) and (B).

*Preparation of 11α-hydroxytestosterone.* 11α-Hydroxytestosterone was synthesized by microbial hydroxylation of testosterone with *A. ochraceus* as previously described[1]. It was purified as its diacetate, which gave the correct melting point of 201–203°.

## RESULTS

(A) *Incubations with progesterone-4-$^{14}C$*

Figure 1 shows the gas chromatogram of progesterone after 5 hr exposure to

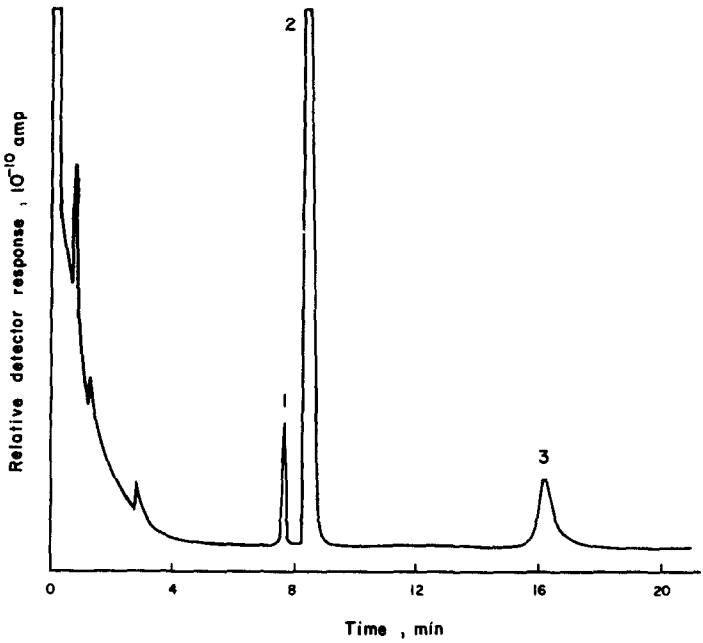


Fig. 1. Gas chromatogram of Progesterone-4-$^{14}$C incubation with a cell-free extract from *A. ochraceus* on 3% QF-1 at 250°.
1, Progesterone; 2, 11α-Hydroxytestosterone; 3, 11α-Hydroxyprogesterone.

the cell-free extract on a QF-1 column. There are 3 distinct peaks with the following retention times (relative to 5α-cholestane): progesterone (7·00), 11α-hydroxyprogesterone (15·06) and 11α-hydroxytestosterone (7·50). Their relative retention times on a OV-1 column are resp. 0·96, 1·61 and 1·22. The identity of each peak was established by subsequent co-injection of the same amount of sample with authentic reference compounds. In each case, no new peaks, but enlargement of a single, previously identified peak was observed. For further identification, the radioactive 11α-hydroxytestosterone was acetylated and subjected to GLC analysis. The relative retention times of the single peak thus obtained (1·36 on OV-1 and 10·63 on QF-1) were identical with those of authentic 11α, 17β-diacetoxy-4-androsten-3-one.

For proof of its radiochemical purity, the bulk of the progesterone incubation extracts were subjected to preparative TLC purification (20 × 20 cm glass plate, 1·5 mm coating) by means of a streaker. The plate was irrigated once with a mixture of chloroform and methanol (9 : 1). The plate was then examined under a 254 nm lamp and divided into 5 bands. Each band was scraped off, eluted with methanol, filtered and an aliquot of the eluate counted (see Table 1).

*Reverse isotope dilutions.* The eluate from band No. 2 was diluted with 31·1 mg of carrier 11α-hydroxyprogesterone ($\nu_{max}^{KBr}$: 3430, 1708, 1660, 1606, 1052 cm$^{-1}$) and recrystallized; while the eluate from band No. 4 was evaporated to dryness ($\nu_{max}^{KBr}$: 3425, 1650, 1600, 1062 cm$^{-1}$), acetylated overnight at room temperature under nitrogen, diluted with 41 mg of carrier 11α, 17β-diacetoxy-4-androsten-3-one ($\nu_{max}^{KBr}$: 1724, 1660, 1602, 1250 cm$^{-1}$) and recrystallized (Table 2).

Table 1. Preparative TLC purification of progesterone-4-$^{14}$C incubation with a cell-free extract from *A. ochraceus*

| Parameters | Band No. | | | | |
|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 |
| $R_f$ (chloroform–methanol 9 : 1) | 0·84 | 0·62 | 0·48 | 0·29 | 0·12 |
| Total activity (dpm) | 125500 | 4450 | 1780 | 1575 | 10200 |
| RRT* (on OV-1) | | 1·61 | | 1·22 | |
| RRT* (on QF-1) | | 15·06 | | 7·50 | |

*Retention time, relative to 5$\alpha$-cholestane.

Table 2. Recrystallizations of 11$\alpha$-hydroxyprogesterone-4-$^{14}$C (*A*) and 11$\alpha$,17$\beta$-diacetoxy-4-androsten-3-one-4-$^{14}$C (*B*)

| No. | Solvent | Specific activity | |
|---|---|---|---|
| | | dpm/mg | dpm/$\mu$M |
| 1*A* | Chloroform–methanol (9 : 1) | 34·0 ± 1·2 | 11·2 ± 0·4 |
| 2*A* | Acetone–petrolether | 33·8 ± 1·1 | 11·2 ± 0·4 |
| 3*A* | Ethanol–hexane | 34·2 ± 1·2 | 11·3 ± 0·4 |
| 1*B* | Acetone–hexane | 1705 ± 40 | 661 ± 15 |
| 2*B* | Ether | 1709 ± 40 | 663 ± 15 |

(B) *Incubations with 17$\alpha$-methyltestosterone, norethisterone and norethynodrel*

GLC-analysis of the 17$\alpha$-methyltestosterone incubation with the cell-free extract showed a metabolite in 7·8% yield with a relative retention time of 1·16 (OV-1) and 7·66 (QF-1). Co-injection with authentic 11$\alpha$-hydroxy-17$\alpha$-methyltestosterone gave peak enhancement and thus established its identity.

From the incubation extracts using norethisterone and norethynodrel as substrates, the sole GLC peaks obtained were those of the starting material. No other peaks were observed.

## DISCUSSION

The smooth conversion of progesterone into its 11$\alpha$-hydroxy derivative and into its degradation product, 11$\alpha$-hydroxytestosterone, as well as the identification of 11$\alpha$-hydroxy-17$\alpha$-methyltestosterone in yields of 8·3%, 74·2% and 7·8% resp. at 0–4° clearly was a proof that we had obtained a hydroxylase active, cell-free preparation of *A. ochraceus*. The major progesterone metabolites obtained upon prolonged incubations with *intact* cells of *A. ochraceus* were shown to be the mono and dihydroxylated $C_{21}$-steroids[1, 10, 11], whereas the $C_{19}$-metabolite could be isolated in only very small yields[1]. In contrast, when exposed to a cell-free extract, the yields were the reverse and 11$\alpha$-hydroxytestosterone was gaschromatographically shown to be the major product formed (>70%). Since we have already demonstrated earlier by kinetic studies[1] that the conversion of progesterone to 11$\alpha$-hydroxytestosterone proceeds via 11$\alpha$-hydroxyprogesterone as an intermediate, the above result confirms that our cell-free preparation indeed contained an effective hydroxylating system. The discrepancy in the yields

of the metabolites observed, when comparing the two types of incubations with intact cells and with cell-free extracts may be interpreted in terms of selective binding of the more polar 11$\alpha$-hydroxytestosterone by cellular membranes, or, more likely, because our concentrated cell-free preparation contained per unit volume much more of the C-17(20)-lyase. For our purposes, however, this question is irrelevant. What we intended to show was that, if our cell-free preparation showed sufficient hydroxylase activity towards two different steroid substrates (progesterone and 17$\alpha$-methyltestosterone) and yet it would still leave those steroids with an ethinyl group intact, then the inertness of these contraceptive steroids can no longer be regarded as caused by solubility or membrane permeability effects. Furthermore, since the non-substrate specificity of the hydroxylases induced in *A. ochraceus* has been amply demonstrated[1–5, 12–14], the observed resistance of norethisterone and norethynodrel towards enzymic hydroxylation must be sought in an, as yet unrecognized, characteristic of the ethinyl moiety itself. It should be noted that biologically derived 11-hydroxylated derivatives of the above two contraceptive steroids have not been described thus far. The only known metabolites, demonstrated in mammalian tissues were the 10$\beta$-hydroxy derivative of norethisterone[15–18] and the reduced epimeric C-3 alcohols[19]. Formation of 10$\beta$-hydroxynorethisterone from norethynodrel can, however, be explained by a non-enzymic mechanism via nuclecphilic attack of an $OH^-$ at C-10 in a protic medium, or by oxidation via a 10$\xi$-hydroperoxide along a pathway recently postulated for cholesterol autoxidation[20]. Indeed, norethynodrel is not very stable. Treatment of this compound at room temperature in methanolic solution with active charcoal was sufficient to transform it quantitatively into the more stable conjugated norethisterone, as we have established by GLC analysis. On the other hand, reduction of the keto group at C-3 in norethisterone and norethynodrel clearly does not implicate a hydroxylating enzyme.

Stereospecific biological hydroxylation must involve formation of a substrate-enzyme complex by prior rigid conformational fixation necessitating at least 2 sites of the steroid substrate. It has been postulated[21, 22] that the polar functions at C-3 and C-17 may act as the centers of binding in question. In the case of 17$\alpha$-ethinylated steroids, however, the binding surface of the enzyme is in the order of 2·60 A°[23] further away from the C-17 carbon atom than in steroids devoid of such a protruding linear substituent (see Fig. 2). Consequently, any nonbonded type interaction between the C-17 OH-group and the binding site

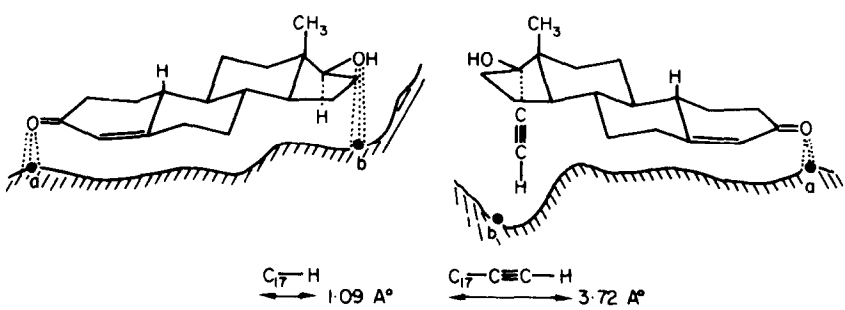


Fig. 2. Comparison between 19-nortestosterone and 17$\alpha$-ethinyl-19-nortestosterone of possible interaction with receptor sites *a* and *b*.

is weakened by a factor of at least 5. Thus, while fixation at C-3 may not be affected, rigid positioning of a norethisterone or norethynodrel molecule, involving the 17-OH group may well become impossible. Further support of the above hypothesis based upon steric considerations may perhaps be furnished by exposing steroids with other 17$\alpha$-substituents, such as ethyl and nitrile, or 15$\alpha$- and 16$\alpha$-ethinylated substrates to incubations with hydroxylase active, cell-free enzyme preparations from *A. ochraceus*. We intend to continue explorations in this direction.

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