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Transformation of Progesterone by *Aspergillus niger* 100 and *Rhizopus nigricans* REF, 129

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The microbiological transformation of progesterone by the local moulds *Aspergillus niger* and *Rhizopus nigricans* was studied. To a 48 hr old culture, progesterone (0.4 g/*l* medium) dissolved in ethanol was added and the transformation was left to proceed for 72 hr. Thereafter, the different transformation products were resolved chromatographically. The identity of each product was established through the determination of mp, mixed mp, optical rotation and ultraviolet as well as infrared absorption spectra. A comparison of the R_f values of each product with that of the corresponding reference using different solvent systems as well as their colours with two spray reagents was used as a further proof for the identity of the isolated products. In case of *A. niger*, progesterone was converted to 17α-hydroxyprogesterone; 21-hydroxyprogesterone (cortexone); 11α-hydroxyprogesterone; 11α,17α-dihydroxyprogesterone; 6β,11α-dihydroxyprogesterone and 11α,17α, 21-trihydroxyprogesterone (epicortisol). These products together with 11α-hydroxy-5α-pregnane-3,20-dione were also identified in case of *R. nigricans*.

The microbiological oxidation of progesterone at one or more of the strategic carbon atoms 11, 17, and 21 was applied to the preparation of the cortical hormones. The introduction of a hydroxyl group at carbon 11 of progesterone was performed by many fungi, mostly Rhizopi and Aspergilli.[1,2] The 17α-hydroxyl group was introduced into steroids by *Trichothecium roseum*.[3,4] However, Meister *et al.*[5] announced that *Cephalothecium roseum* could accomplish this interesting tertiary hydroxylation along with simultaneous introduction of the biologically important oxygen at C-11. Meyster *et al.*[3] also found that *Ophiobolus herpotrichus* introduced a hydroxyl group at C-21. The microbial 21-hydroxylation of several susbtrates by *Aspergillus niger* was also reported.[6] Moreover, a strain of *A. niger* was able to introduce both 11α- and 21-hydroxyl groups simultaneously into progesterone.[7,8] Epicortisol (11α-,17α,21-trihydroxyprogesterone) was isolated by CIBA group[9] together with other transformation products when *R. nigricans* and *Cunninghamella blakesleeana* were acted on progesterone.

Many works have been carried out on hydroxylation for progesterone by *A. niger*, but in most work the strain ATCC 9142 was used, and hydroxylation occurred at C-6, 11 or 21. Screening of the ability of some microorganisms for the transformation of progesterone was carried out by the authors,[10] and some active moulds were found. Using the local strain *A. niger* 100, we observed that the hydroxylation of progesterone occurs at C-6, 11, 17 and 21. A similar hydroxylation was observed using the strain *R. nigricans* REF, 129. Qualitative as well as quantitative analyses were carried out on the different products which were obtained when these two active moulds were acted upon progesterone.

Experimental

Cultivation. The local strains; *Aspergillus niger* 100

1) D. H. Peterson, "Biochemistry of Industrial Microorganisms," ed. by Rainbow and Rose, Acad. Press, London and N. Y. (1963), Chap. 16, p. 537.

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4) L. E. Dulaney, W. J. McAleer, M. Kozlowski, E. O. Stapley and J. Jaglom, *Appl. Microbiol.*, **3**, 372 (1955).

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7) E. Weisz, G. Wix and M. Bodanszky, *Naturwissenschaften*, **43**, 39 (1956).

8) G. Wix, M. Natonek and M. Kovacs, *Acta Microbiol. Sci. Hungary*, **6**, 197 (1959).

9) Ciba Ltd., Brit. 816922, July 22 (1959); *Chem. Abstr.*, **54**, 1659 (1960).

10) A. H. El-Refai, L. A. R. Sallam and I. A. El-Kady, *J. Gen. Appl. Microbiol* (Tokyo), **15**, 301 (1969)

and *Rhizopus nigricans* REF, 129 were collected from the Centre of culture of the Microbiological Chemistry Research Lab., National Research Centre, Cairo. The cultivation was performed in 250 m*l* Erlenmeyer flasks each containing 75 m*l* of the medium formulated by Capek *et al.*[11] The flasks were sterilized at 1 atm. for 20 min and inoculated with 2 m*l* inocula of a 48 hr culture of the pure organsim. The culture flasks were agitated on a reciprocal shaker (110 strokes/min, amplitude 7 cm) at 30±2°C for 48 hr. Thereafter, 30 mg of progesterone dissolved in 1 m*l* ethanol was added to each flask and fermentation was continued for another 72 hr.

Extraction. At the end of the fermentation periods, the contents of each flask (medium+mycelium) were homogenized in a blender (16000 rpm) with two volumes of chloroform (150 m*l*). The extraction was repeated 3 times in order to make sure that all the transformation products were extracted. The combined chloroform extracts were washed with half its volume 5% sodium bicarbonate, followed by an equal volume distilled water, dried over anhydrous sodium sulphate, filtered, then distilled to give a semi-solid residue.

Analysis of the Mixture of the Transformation Products. The semi-solid residue was dissolved in the minimal volume of benzene and then fractionated on standard activated alumina column (100 g). The following sequence of solvents was used: *n*-hexane : benzene (1 : 1); benzene containing different concentrations of chloroform and benzene containing different concentration of methanol. The fractions containing similar products were collected and crystallization from a suitable solvent was carried out whereby determination of mp; mixed mp; $[\alpha]_D$; IR and UV absorption spectra were made.

Further proof of the identity of each product was gained by the application of the thin-layer chromatographic technique.[12] The solvent systems used are: I: Cyclohexane : acetone : chloroform (75 : 25 : 20), II: Ethylene chloride : acetone (80 : 20), III: Benzene : ethyl acetate : acetone (60 : 20 : 20) and IV: Chloroform : cyclohexane : isopropanol (50 : 100 : 20).

The plates were then sprayed with two different colour reagents namely: I_2/KI,[13] and chlorosulphonic acid : acetic acid (3 : 1).[14]

Results and Discussion

Progesterone Bioconversion by *A. niger* 100. The chromatographic resolution of the mixture (1.3 g) containing the different transformation products obtained when *A. niger* was acted on progesterone (1 g) revealed the presence of unchanged progesterone (11%);*1 17α-hydroxyprogesterone (11.9%); 21-hydroxyprogesterone (cortexone) (13.3%); 11α-hydroxyprogesterone (22.4%); 11α,17α-dihydroxyprogesterone (13.1%); 6β,11α-dihydroxyprogesterone (10.8%) and 11α, 17α,21-trihydroxyprogesterone (epicortisol) (7.5%), respectively.

The fractions eluted with *n*-hexane : benzene (1 : 1) were evaporated and crystallized from methanol : chloroform to give crystals (110 mg), mp 128—129°C, $[\alpha]_D$+195°. This substance was found to be unchanged progesterone. The fraction eluted with 5% chloroform in benzene gave after repeated crystallization from chloroform: methanol crystals (119 mg), mp 218—220°C, $[\alpha]_D$ +120.3°, λ_{max}^{EtOH} 239 mμ (ε=12920). The material did not depress the mp of an authentic sample of 17α-hydroxyprogesterone. Reported[15] mp 210—214°C, identical IR spectra.

The solid residue eluted with 10% chloroform in benzene was repeatedly crystallized from methanol: chloroform to afford crystals (133 mg), mp 139—141°C, $[\alpha]_D$ +183°, λ_{max}^{EtOH} 241 mμ (ε=12700). This material was found to be 21-hydroxyprogesterone. Reported[6] mp 142—143°C, $[\alpha]_D$ +185°, identical IR spectra.

The combined fractions eluted with 20% chloroform in benzene on cyrstallization from chloroform: methanol gave crystals (224 mg), mp 165—168°C, $[\alpha]_D$ +177°, λ_{max}^{EtOH} 242 mμ (ε=12960). The material did not depress the mp of an authentic sample of 11α-hydroxyprogesterone. Reported[16] mp 166—168°C, $[\alpha]_D$ +176°, identical IR spectra.

The fifth product eluted with 5% methanol in benzene afforded after crystallization from methanol crystals (131 mg) mp 217—220°C, $[\alpha]_D$ +72°, λ_{max}^{EtOH} 242 mμ (ε=16100). This material was found to be 11α,17α-dihydroxyprogesterone. Reported[17] mp 220—222°C, $[\alpha]_D$ +76°, identical IR spectra.

The substance eluted with 10% methanol in benzene gave upon crystallization from methanol: water, crystals (180 mg), mp 244—246°C, $[\alpha]_D$ +140°, λ_{max}^{EtOH} 239 mμ (ε=13480). The material did not depress the mp of the authentic sample of 6β,11α-dihydroxyprogesterone. Reproted[16] mp 245—248°C, $[\alpha]_D$ +144°, identical IR spectra.

Final strip of the column with 25% methanol in benzene gave a solid material which yielded on repeated crystallization from methanol: water,

11) A. Capek, M. Tadra and J. Tuma, *Folia Microbiol. Prague*, **9**, 380 (1964).

12) L. A. R. Sallam, A. H. El-Refai and I. A. El-Kady, *J. Gen. Appl. Microbiol.* (Tokyo), **15**, 309 (1969).

13) E. Tumova, Z. O. Siblikova and O. Hanc, *Biochemika Oxydance Pregnaovych Steroidu*, **4**, 65 (1955).

14) D. Waldy, "Thin-Layer Chromatography, A Laboratory Handbook, Egon Stahl," Acad. Press. Inc., New York, London (1965). p. 249.

*1 Yields are represented by weight % based on added progesterone.

15) W. J. McAleer and E. L. Dulaney, *Arch. Biochem. Biophys.*, **62**, 111 (1956).

16) D. H. Peterson, H. C. Murray, S. H. Eppstein, L. M. Reineke, A. Weintraub, P. D. Meister and H. M. Leigh, *J. Amer. Chem. Soc.*, **74**, 5933 (1952).

17) P. D. Meister, D. H. Peterson, H. C. Murray, G. B. Spero, S. H. Eppstein, A. Weintraub, L. M. Reinke and H. M. Leigh, *ibid.*, **75**, 416 (1953).

Table 1. The R_f values and colours of the different transformation products obtained from progesterone bioconversion by *R. nigricans* and *A. niger*

	$R_f \times 100$ with solvent system				I_2/KI	Chlorosulphonic/acetic acid	
	I	II	III	IV		Day light	UV
17α-Hydroxyprogesterone	88	83	97	85	weak pink	brown	bluish violet
21-Hydroxyprogesterone (cortexone)	90	89	85	78	rust brown	deep blue	brilliant brick red
11α-Hydroxy-5α-pregnane-3,20-dione*	75	65	74	75	grey	brown	brilliant blue
11α-Hydrxoyprogesterone	75	61	74	72	deep blue	brown	greenish yellow
11α,17α-Dihydroxyprogesterone	48	46	67	57	deep blue	brown	yellow
6β,11α-Dihydroxyprogesterone	39	45	48	40	pale brown	brown	sky blue
11α,17α,21-Trihydroxyprogesterone (epicortisol)	17	12	33	28	deep blue	pale brown	yellow

* 11α-Hydroxy-5α-pregnane-3,20-dione was detected in case of *R. nigricans* only.

crystals (75 mg) mp 208—210°C, $[\alpha]_D$ 109°, λ_{max}^{EtOH} 241 mμ (ε=14700). This substance did not depress the mp of an authentic sample of 11α, 17α,21-trihydroxyprogesterone (epicortisol). Reported,[18] mp 210—212°C, $[\alpha]_D$ +113°, identical IR spectra.

Futher proof of the identity of the different compounds produced from the bioconversion of progesterone by *A. niger* was made by application of thin-layer chromatographic analysis. The results of this study are presented in Table 1.

Progesterone Bioconversion by *R. nigricans* REF, 129. Inspection of the mixture (1.4 g) resulting from the bioconversion of progesterone (1 g) by *R. nigricans* revealed the presence of unchanged progesterone (6%), 17α-hydroxyprogesterone (8.2%), 21-hydroxyprogesterone (7.9%), 11α-hydroxy-5α-pregnane-3,20-dione (7.4%), 11α-hydroxyprogesterone (24.4%), 11α,17α-dihydroxyprogesterone (16.7%), 6β,11α-dihydroxyprogesterone (9.5%) and finally 11α,17α,21-trihydroxyprogesterone (epicortisol) (10.5%).

The first fraction eluted with *n*-hexane : benzene (1 : 1) gave upon crystallization from chloroform: methanol, crystals (60 mg) mp 126—128°C, $[\alpha]_D$ +194°. This was found to be unchanged progesterone. Evaporation of the fractions eluted with 5% chloroform in benzene followed by crystallization from chloroform : methanol gave crystals (82 mg), mp 209—212°C, $[\alpha]_D$ +120—123°, λ_{max}^{EtOH} 239 mμ (ε=12960). This material did not depress the mp of an authentic sample of 17α-hydroxyprogesterone. Reported[15] mp 210—214°C, identical IR spectra.

The solid substance removed with 10% chloroform in benzene gave crystals (79 mg) mp 140—142°C, $[\alpha]_D$ +180°, λ_{max}^{EtOH} 241 mμ (ε=12700). This substance was found to be 21-hydroxyprogesterone as no depression in the mp was observed upon admixture with an authentic sample of cortexone. Reported[14] mp 142—143°C, $[\alpha]_D$ +185°, identical IR spectra. The compound eluted with 15% chloroform in benzene gave on crystallization from chloroform : methanol crystals (74 mg) mp 194—196°C, $[\alpha]_D$ +81°. No depression in the mp of an authentic sample of 11α-hydroxy-5α-pregnane-3,20-dione with the isolated crystals. Reported[16] mp 198—200°C, $[\alpha]_D$ +84°, identical IR spectra. The fractions collected with 20% chloroform in benzene yield upon crystallization from methanol crystals (244 mg), mp 165—167°C, $[\alpha]_D$ +175°. This substance did not depress the mp of an authentic sample of 11αhydroxyprogesterone. Reported[16] mp 166—168°C. $[\alpha]_D$ +176°, identical IR spectra.

Elution with 5% methanol in benzene gave a compound which upon crystallization from methanol gave crystals (167 mg), mp 216—218°C, $[\alpha]_D$ +75°, λ_{max}^{EtOH} 243 mμ (ε=16200). This compound is 11α,17α-dihydroxyprogesterone. Reported[17] mp 220—222°C, $[\alpha]_D$ +76°, identical IR spectra.

The fractions eluted with 10% methanol in benzene gave on crystallization from methanol : water crystal (95 mg), mp 242—245°C, $[\alpha]_D$+139°, λ_{max}^{EtOH} 239 mμ (ε=13480). This material was found to be 6β,11α-dihydroxyprogesterone. Reported[16] mp 245—248°C, $[\alpha]_D$ +144°, identical IR spectra.

The fractions collected with 25—50% methanol in benzene was crystallized from methanol: water to give crystals (105 mg), mp 208—210°C, $[\alpha]_D$ +105°, λ_{max}^{EtOH} 241 mμ (ε=14700). This product did not depress the mp of an authentic sample of epicortisol. Reported[18] mp 210—212°C, $[\alpha]_D$ +113°, identical IR spectra.

Further establishment of these different products was made through thin-layer chromatographic analysis as presented in Table 1.

18) D. H. Peterson, S. H. Eppstein, P. D. Meister, B. J. Magerlin, H. C. Murray, H. M. Leigh, A. Weintraub and L. M. Reineke, *ibid.*, **75**, 412 (1953).

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