Hydroxylation of Progesterone by Extracts of *Rhizopus nigricans* NRRL 1477

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SUMMARY

Growing cultures of *Rhizopus nigricans* NRRL 1477 hydroxylate progesterone at the 11α -, 17α - and 6β -positions. Hydroxylation at the 11α -position seems to be predominant. At pH 7.5, extracts of this organism catalysed the hydroxylation of progesterone to 11α -hydroxyprogesterone and 11α -hydroxyallopregnane-3,20-dione. Ethylenediamine tetracetate inhibited and 2-mercaptoethanol stimulated this hydroxylation. While hydroxylation of progesterone at the 11α -position was optimal at pH 7.5, hydroxylation at the 17α -position was optimal at pH 6.5. The effect of some compounds and metal salts was studied.

INTRODUCTION

Steroid hydroxylation by extracts of micro-organisms has been described in only a few instances. Zuidweg, Van der Waard & DeFlines (1962) prepared from *Curvularia lunata* an extract capable of catalysing the hydroxylation of Reichstein's Compound S to hydrocortisone. A 9α -hydroxylase was demonstrated in extracts of *Nocardia restrictus* by Chang & Sih (1964). Wilson & Vestling (1965) obtained from *Bacillus megaterium* KM an extract that hydroxylates deoxycorticosterone mainly at the 15β -position. Smaller amounts of the 6β -, 11α - and 15α -monohydroxylated derivatives were also detected. Zuidweg (1968), using extracts of *C. lunata*, studied the nature of the hydroxylation of Reichstein's Compound S at the 11β -, and 14α -positions. It seemed that both hydroxylations are catalysed by one enzyme. Sallam, El-Refai & El-Kady (1971), using the supernatant solution of a cell-homogenate of *Rhizopus nigricans* REF 129, studied the hydroxylation of progesterone at the 17α - and 21 positions. They reported that the 11α -hydroxylating activity is bound to the cell-sediment fraction. However, the long incubation periods of the supernatant solution and progesterone (6 to 48 h at room temperature) that were adopted in their studies are rather unusual. Nguyen-Dang-Tam, Mayer & Janot (1971) showed that extracts of *Aspergillus niger* catalysed the hydroxylation of progesterone at the 11α -position. The present work describes an investigation of the hydroxylation of progesterone by extracts of *R. nigricans* NRRL 1477.

METHODS

Cultures. *Rhizopus nigricans* NRRL 1477 was cultivated in 250 ml Erlenmeyer flasks which contained 50 ml of the medium described by Capek, Tadra & Tuma (1964). The pH was adjusted to 5.6. After sterilization each flask was inoculated with 2 ml of a spore suspension made from a 3-day-old culture. The inoculated flasks were agitated on a reciprocating shaker at 30 °C. After 24 h of growth, the cultures were activated by the addition of 5 mg of progesterone/flask and reagitated for another 24 h.

Table 1. *Hydroxylation of progesterone by cultures of Rhizopus nigricans* NRRL 1477

Each culture flask contained 50 ml of the medium to which 50 mg of progesterone were added. Yields in mg.

Products	Time (h)			
	24	48	72	96
Residual progesterone	35.0	24.0	8.0	—
11 α -hydroxyprogesterone	6.5	10.0	16.7	11.5
11 α -hydroxyallopregnane-3,20-dione	1.3	2.0	4.7	6.3
17 α -hydroxyprogesterone	1.7	3.2	4.5	2.0
17 α -hydroxyallopregnane-3,20-dione	1.2	2.3	2.7	4.0
11 α -, 17 α -dihydroxyprogesterone	2.0	4.2	6.0	8.5
6 β -, 11 α -dihydroxyprogesterone	0.5	1.2	4.0	9.8

Preparation of extracts. The mycelia were harvested by filtration, washed thoroughly with 0.5 % NaCl solution, followed by distilled water, and then blotted dry with absorbent paper. These mycelia were then ground with cold sand and extracted with cold distilled water or 0.1 M-potassium phosphate buffer at pH 7.5. The slurry obtained was centrifuged at 4000 rev./min for 15 min and the supernatant used as the enzyme preparation.

Assay of progesterone-hydroxylating activity in extracts. To 5 ml portions of the extracts, in 25 ml Erlenmeyer flasks, were added 5 mg of progesterone in 0.1 ml of ethanol. The contents were mixed immediately and the flasks were left on a reciprocating shaker for 5 h at 30 °C. At the end of this period the contents of each flask were extracted with 10 ml of chloroform. Extraction was repeated three times and the combined chloroform extracts were then treated with half their volume of 5 % solution of sodium bicarbonate followed by an equal volume of distilled water. The chloroform extracts were then dried over anhydrous sodium sulphate, filtered and distilled. The residue was dissolved in a measured volume of a mixture of chloroform and methanol (1:1).

Determination of hydroxylated products. The hydroxylated products of progesterone, in the prepared residue, were first identified by conventional thin-layer chromatography with authentic samples as controls and a solvent system consisting of cyclohexane-acetone-chloroform (75:25:20). The amounts of these products were determined by the preparative thin-layer chromatographic technique with the same solvent. The bands containing the compounds were outlined, scraped off, and eluted with ethanol. The concentration of each was then determined by u.v. spectrophotometric measurement. 11 α -Hydroxyallopregnane-3,20-dione was estimated by evaporating its eluate in a tared beaker and determining the weight.

RESULTS AND DISCUSSION

Hydroxylation of progesterone by growing cultures of Rhizopus nigricans NRRL 1477

In this experiment 50 mg of progesterone was added to each culture flask after 24 h of growth and the amounts of the hydroxylated products formed were determined in the culture media at specified time intervals. As shown in Table 1, after 72 h the amount of 11 α -hydroxyprogesterone was the main product and constituted about 32 %, on a molar basis, of the initially added progesterone. 11 α -Hydroxyprogesterone and its reduced product, 11 α -hydroxy-allopregnane-3,20-dione, constituted together about 42 % after the same time. Apparently hydroxylation at the 11 α -position predominates, especially if other products containing a hydroxyl group in the 11 α -position (11 α -, 17 α - and 6 β , 11 α -dihydroxyprogesterone) are considered.

Table 2. *Hydroxylation of progesterone by extracts of Rhizopus nigricans* NRRL 1447

Each reaction mixture contained: extracts in 0.1 M-potassium phosphate buffer at pH 7.5, 5 ml; progesterone in 0.1 ml of ethanol, 5 mg; temp 30 °C; reaction time as indicated, with shaking conditions. Yields in mg.

Incubation period (h)	Residual progesterone	11 α -hydroxy-progesterone	11 α -hydroxy-allopregnane-3,20-dione
1	4.50	0.30	—
2	3.55	1.10	0.15
3	2.82	1.42	0.35
4	2.20	1.67	0.65
5	1.80	1.81	0.72

Table 3. *Hydroxylation of progesterone at different pH values*

Reaction mixture contained: extract in distilled water, 4 ml; progesterone, 5 mg; buffer, as indicated, 500 μ moles, total vol, 6 ml; temp. 30 °C; reaction time, 5 h with shaking conditions. Yields in mg.

pH	11 α -hydroxy-progesterone	11 α -hydroxy-allopregnane-3,20-dione	17 α -hydroxy-progesterone	17 α -hydroxy-allopregnane-3,20-dione	11 α -, 17 α -dihydroxy-progesterone
Phosphate buffer					
5.5	0.23	0.20	1.08	0.27	0.22
6.0	0.27	0.25	1.33	0.36	0.25
6.5	0.40	0.30	1.62	0.45	0.25
7.0	1.17	0.37	0.52	0.22	—
7.5	1.75	0.65	—	—	—
8.0	1.56	0.45	—	—	—
Tris buffer					
8.0	1.37	0.35	—	—	—
8.6	1.20	0.30	—	—	—
9.0	1.10	0.27	—	—	—

Hydroxylation of progesterone by extracts of Rhizopus nigricans NRRL 1477

The results, presented in Table 2, show that hydroxylation at only the 11 α -position took place when progesterone was incubated with the extracts under the experimental conditions used. 11 α -Hydroxyprogesterone was the only hydroxylated derivative detected in the reaction mixture in the first hour of incubation. After that, 11 α -hydroxyallopregnane-3,20-dione started to be formed. After 5 h both hydroxylated products constituted about 50 % of the added progesterone.

In this experiment the reaction mixture did not contain any additives such as ethylenediaminetetra-acetate (EDTA), glutathione or Tween 80 which have been used either separately or together in most of the studies made on the hydroxylation of steroids by extracts of micro-organisms (Zuidweg *et al.* 1962; Chang & Sih, 1964; Zuidweg, 1968; Sallam *et al.* 1971). In consequence, the effect of some of these reagents on the hydroxylating activity of the extracts was studied. The presence of EDTA, at concentrations as low as 1 mM, inhibited (15 %) of the 11 α -hydroxylase activity. Higher concentrations of EDTA inhibited more. This suggests that a metal cation is involved in this hydroxylation. The addition of 2-mercaptoethanol at a concentration of 5 mM stimulated (14 %), and reduced glutathione, at the same concentration, produced some inhibition (17 %) as also did sodium thioglycollate

Table 4. *Effect of some compounds and metal salts on the hydroxylation of progesterone*

Reaction mixture contained: extract in 0.1 M-phosphate buffer, pH 7.5, 5 ml; progesterone 5 mg; compound or metal salt, as indicated, total vol. 6 ml; temp. 30 °C; reaction time, 5 h with shaking conditions. Yields in mg.

Compound or metal salt	11 α -hydroxy-progesterone	11 α -hydroxy-allopregnane-3,20-dione
—	1.90	0.70
NADPH (10^{-4} M)	1.80	0.75
NADPH (2×10^{-4} M)	1.45	1.35
NADPH (4×10^{-4} M)	1.25	1.80
Menadione (10^{-3} M)	—	2.35
H ₂ O ₂ (10^{-3} M)	—	—
ZnSO ₄ (10^{-3} M)	2.20	1.00
NaCN (10^{-3} M)	—	—
CoSO ₄ (10^{-3} M)	—	—

(29 %). These results suggest that the hydroxylating system of the present organism differs from those used by other workers.

In view of the stimulating effect of 2-mercaptoethanol, the effect of different concentrations of this sulphhydryl compound on the 11 α -hydroxylating activity was tested. 2-Mercaptoethanol, up to a concentration of 40 mM, produced 26 % increase in hydroxylation at the 11 α -position of progesterone. At higher concentrations (60 to 100 mM), gradual inhibition of this hydroxylation took place with the formation of small amounts of 17 α -hydroxyprogesterone, 17 α -hydroxylapregnane-3,20-dione, and 11 α -17 α -dihydroxyprogesterone. Hayaishi (1969) reported that sulphhydryl compounds, such as glutathione or 2-mercaptoethanol, are implicated as ultimate electron donors in certain monooxygenase-catalysed reactions. 2-Mercaptoethanol may play the same role in the hydroxylation of progesterone by extracts of *Rhizopus nigricans* NRRL 1477.

The stability of the 11 α -hydroxylating activity was tested in extracts stored at 5 and -10 °C. Extracts kept at 5 °C lost most of their activity (about 85 %) in 24 h and extracts kept at -10 °C lost 54 % of their activity in the same period. After 48 h all the activity was lost at either temperature. In most of the studies reported in the literature, microbial steroid-hydroxylating systems were shown to be unstable.

Hydroxylation of progesterone at different pH values by extracts of Rhizopus nigricans NRRL 1477

Table 3 shows that while hydroxylation of progesterone at the 11 α -position was optimal at pH 7.5, the hydroxylation of the same substrate at the 17 α -position was optimal at pH 6.5. At pH 7.5, 17 α -hydroxyprogesterone could not be detected in the reaction mixture. These results suggest that these two hydroxylations are catalysed by two different enzymes. The data indicate that at the respective optimal pH values, 11 α -hydroxyprogesterone constituted about 35 % of the added progesterone and 17 α -hydroxyprogesterone about 33 %. If the amounts of the reduced products of these derivatives are considered, the percentage conversions are 48 and 41 respectively. The Table also shows that at pH values ranging from 5.7 to 6.5 only small amounts of the dihydroxylated derivative 11 α -, 17 α -dihydroxyprogesterone could be detected in the reaction mixture.

Effect of some compounds and metal salts on the hydroxylation of progesterone

The effect of NADPH on the hydroxylation of progesterone was tested in view of its known role as an external electron carrier in certain reactions catalysed by mixed-function oxygenases. As shown in Table 4, it seems that NADPH participated mainly in the reductive reaction converting 11α -hydroxyprogesterone into 11α -hydroxyallopregnane-3,20-dione, as increasing the amount of NADPH increased the latter compound. A similar role could be ascribed to menadione, as 11α -hydroxyallopregnane-3,20-dione was the only detectable hydroxylated product when it was added to the reaction mixture. With Zn^{2+} , 11α -hydroxyprogesterone was the main product. Co^{2+} , CN^- and H_2O_2 completely inhibited the hydroxylation of progesterone.

REFERENCES

- CAPEK, A., TADRA, M. & TUMA, J. (1964). Separation of androst-17-hydroxy-epimers. *Folia microbiologica* **9**, 380–382.
- CHANG, F. N. & SIH, C. J. (1964). Mechanisms of steroid oxidation by microorganisms. VII. Properties of the 9α -hydroxylase. *Biochemistry* **3**, 1551–1557.
- HAYAISHI, O. (1969). Enzymic hydroxylation. *Annual Review of Biochemistry* **38**, 21–44.
- NGUYEN-DANG-TAM, MAYER, M. & JANOT, M. M. (1971). Hydroxylation of progesterone by a cell-free extract of *Aspergillus niger*. *Compte rendu des séances de l'Académie des sciences D* **272**, 2032–2033.
- SALLAM, L. A. R., EL-REFAI, A. H. & EL-KADY, I. A. (1971). The *in vitro* transformation of progesterone by *Rhizopus nigricans* REF 129. *Zeitschrift für allgemeine Mikrobiologie* **11**, 325–330.
- WILSON, J. A. & VESTLING, C. S. (1965). A cell-free steroid hydroxylating system from *Bacillus megaterium*, strain KM. *Archives of Biochemistry and Biophysics* **110**, 401–403.
- ZUIDWEG, M. H. J., VAN DER WAARD, W. F. & DEFILINES, J. (1962). Formation of hydrocortisone by hydroxylation of Reichstein's compound S. with an enzyme preparation from *Curvularia lunata*. *Biochimica et biophysica acta* **58**, 131–133.
- ZUIDWEG, M. H. J. (1968). Hydroxylation of Reichstein's compound S with cell-free preparation from *Curvularia lunata*. *Biochimica et biophysica acta* **152**, 144.