MICROBIOLOGICAL HYDROXYLATION OF STEROIDS.

I. COMPARATIVE STUDY OF THE HYDROXYLATION OF CORTEXOLONE AND 16α-METHYLCORTEXOLONE BY A CULTURE OF *Tieghemella hyalospora*

L. K. Garcia-Rodriguez, K. N. Gabinskaya,
V. F. Shner, and O. V. Messinova

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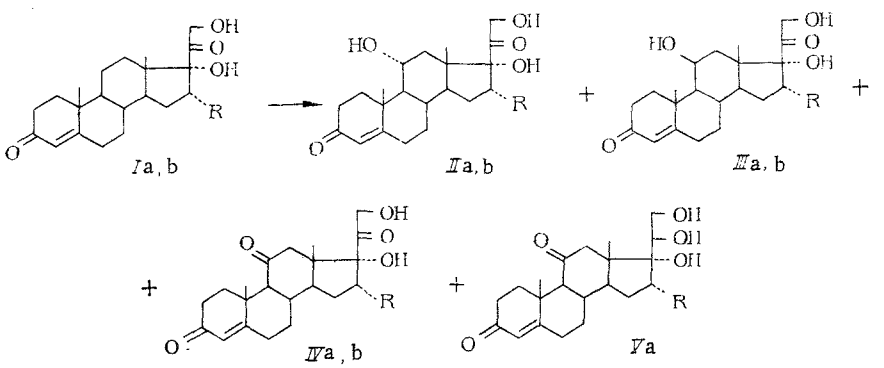
Microbiological hydroxylation of steroids [1] is a key step in the synthesis of high-activity glucocorticoids, which are typified by the presence of an oxygen function at position 11 of the steroid molecule. Among corticosteroid preparations great medicinal interest surrounds dexamethasone [2], which contains a 16α-methyl group. A Soviet culture of *Tieghemella hyalospora* is known [3], which induces the 11α-hydroxylation of cortexolone (Ia), forming epicortisol (IIa) as the major product, together with cortisol (IIIa) and cortisone (IVa) as minor products.

Our purpose here was to make a comparative study of the hydroxylation of Ia and of 16α-methylcortexolone (Ib), the intermediate in the synthesis of dexamethasone, by a culture of *T. hyalospora*. We decided to ascertain the influence of the 16α-methyl group on the course of this reaction. Introduction of a methyl group into the testosterone molecule has been reported [4] to modify the course of hydroxylation. The yield of 11α-hydroxy derivatives from the hydroxylation of methylated progesterones is less than that from progesterone itself, and the product distribution is different [5].

We identified the transformation products of II-V by thin-layer chromatography (TLC) using the relevant reference compounds; samples of compounds IIIb and IVb were prepared by independent synthesis.

We found that the transformation of steroid substrates Ia and Ib by the culture of *T. hyalospora* was practically complete after 20-25 h; less than 5% of the starting compound remained in the beer. Quantitative analysis by TLC showed that compound Ia formed up to ∿80% of IIa, ∿15% of IIIa, traces of IVa, and up to 10% of pregn-4-ene-17α,20β, 21-triol-3,11-dione (Va). In contrast compound Ib gave only 16α-methylepicortisol (IIb) in ∿75% yield, up to 20% of 16α-methylcortisol (IIIb), and ∿5% of 16α-methylcortisone (IVb); we did not detect the formation of a compound equivalent to triol Va.



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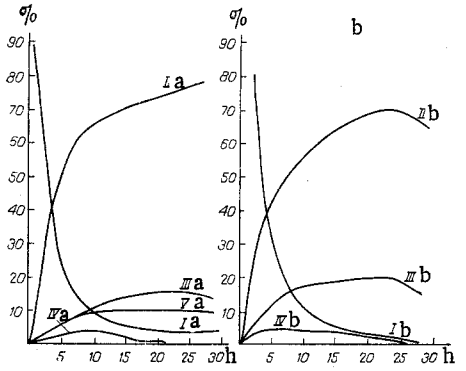

Fig. 1. Dynamics of the hydroxylation of Ia a) and Ib b).

This reveals a difference in the course of the transformation of compounds Ia and Ib. Hydroxylation of substrate Ia is rather more stereospecific than that of its 16α-methyl derivative and gives a higher yield of the 11α-hydroxy compound IIa.

Furthermore the transformation of compound Ia, unlike that of Ib, is accompanied by the side reaction of reduction of the C-20 carbonyl. Various microorganisms are known to possess the ability to reduce a keto group at position 20 of a steroid molecule [1, 2] but it has not been reported for the fungus *T. hyalospora*. Eroshin [4] found that hydroxylation of substrate Ia by this culture formed a mixture of IIa-IVa and one more compound, to which he assigned the structure of 6β-hydroxycortexolone. The 16α-methyl group is known [3] to block the C-20 carbonyl and inhibit its enzymic reduction in human and animal organisms. Our results demonstrate that this methyl group has the same effect on the course of the microbiological transformation.

We also studied the dynamics of the transformation. Figure 1a shows that in the course of hydroxylation of substrate Ia the content of the starting substance in the beer diminishes and the content of hydroxy compounds IIa, IIIa, and triol Va increases. The yield of compound IVa also increases in the initial stages of the transformation, but subsequently rapidly decreases; at the end of the reaction it is present only in traces. We therefore consider compound Va to be formed by the secondary reduction of the C-20 keto group of the primary product IVa.

The ability of the fungus *T. hyalospora* to degrade steroid substrates has been reported [7]. This we observed in the transformation of the methyl derivative Ib (Fig. 1b). Figure 1b shows that the yields of all the products IIb-IVb are reduced by the end of the reaction. However, ketone IVb is present in the beer until the end of the reaction, since unlike the equivalent compound IVa it is not involved in any further transformation.

Thus we can conclude that the 16α-methyl group in the cortexolone molecule rather inhibits the 11α-hydroxylase and blocks the action of the 20β-oxosteroid reductase of the culture of *T. hyalospora*.

EXPERIMENTAL

Culture Growth and Transformation Procedure. The strain of *T. hyalospora* was maintained on wort agar. The mycelium was grown on a medium of the composition, %: glucose 2, peptone 0.5, yeast autolysate 0.3, potassium dihydrophosphate 0.5; pH 5.6. An aqueous suspension of fungal spores (2 ml) was added to the medium (100 ml) in a 250-ml flask. The transformation was carried out with 48-h mycelium washed from the medium in 0.5% glucose solution; the mycelium was grown and the transformation carried out on a mechanical shaker at 28°C. Substrates Ia and Ib were introduced as solutions in dimethylformamide (0.5 ml) (100 mg per 100 ml medium). The transformation products were extracted from the beer four times with equal volumes of methylene chloride, separated by TLC on Silufol UF-254 plates in chloroform–acetone–cyclohexane (6:3:1), and determined spectrophotometrically with an SF-4 instrument at 242 nm or semiquantitatively assayed against the relevant reference compounds.

Identification of the Transformation Products. The extracted products (~100 μg of the mixture of steroids) were dissolved in chloroform–methanol (1:1) and applied to a Silufol UF-254 plate. They were separated (here and subsequently in the presence of reference compounds) in chloroform–acetone (3:2); the spots were detected with a UV lamp, transferred to another plate, and separated in chloroform–ethanol (9:1). For acetylation the mixture of

TABLE 1. Relative Chromatographic Mobilities of the Transformation Products of II-V on Silufol Plates

Compound	R_s	
	in chloroform–ethanol (9:1)	in chloroform–ethanol (3:2)
IIa	0,7	0,46
IIIa	1	1
IVa	1,41	1,17
Va	0,9	0,6
IIb	0,76	0,67
IIIb	0,87	1,16
IVb	1,4	1,71

steroids (∿400 μg) was separated in the first system, and the spots were removed and eluted with methanol. The solvent was evaporated from the eluate and anhydrous pyridine (0.5 ml) and acetic anhydride (0.5 ml) were added to the residue. Next day the reaction mixture was quenched with water, acidified with hydrochloric acid, and extracted with ethyl acetate. The extract was successively washed with water, twice with saturated sodium bicarbonate solution, and again with water; after drying, the solvent was evaporated. The residue was dissolved in chloroform–methanol (1:1) and the resulting solution was applied to a Silufol plate in a quantity equivalent to ∿100 μg of the mixture of steroids and separated in chloroform–alcohol (96:4). The substance was considered identified when its spot position coincided with that of a reference compound in two systems, and when the spot position of its acetate coincided with that of the reference acetate.

The relative chromatographic mobilities of the substances are summarized in Table 1.

16α-Methylpregn-4-ene-17α,21-diol-3,11,20-trione 21-Acetate (VI). A mixture of IIb (2 g) and acetic acid (22 ml) was warmed to effect dissolution (∿ 60°C) and then cooled to 25°C; after addition of barium acetate (0.716 g), acetic anhydride (5.6 ml) was added dropwise. The reaction mixture was stirred for 4 h and then warmed to 50°C; after addition of water (7.36 ml), the mixture was cooled to ∿20°C, poured onto ice, and extracted with ethyl acetate. The extract was washed with sodium bicarbonate solution until neutral, and then with water. After drying, the solvent was evaporated under vacuum. The residue was triturated with ether and the precipitate (2.03 g) was filtered off. After addition of Kiliani's solution [8] (4.06 ml) to a solution of the precipitate in acetone (35 ml), the mixture was warmed to 50°C, stirred for 0.5 h (the reaction is endothermic), cooled to 10°C, and poured into ice water (350 ml). The precipitate was filtered off, washed with water, and recrystallized from methanol to give VI (1.17 g), mp 207-210°C. IR spectrum, ν: 1675, 1705, 1735 and 1755 (C=O), 3380 (OH) cm^{-1}. Found, %: C 69.02; H 7.86. $C_{24}H_{32}O_6$. Calculated, %: C 69.23; H 7.68.

Literature mp is 207-210°C [9].

16α-Methylpregn-4-ene-17α,21-diol-3,11,20-trione (IVb). After addition in an atmosphere of argon of 40% potash solution (5 ml) to a solution of VI (1 g) in freshly distilled methanol (20 ml), the reaction mixture was stirred for 2 h, neutralized with acetic acid, and precipitated with water. The precipitate was filtered off, washed with water, and recrystallized from methanol to give IVb (0.60 g), mp 218-221°C. IR spectrum, ν: 1665, 1695 and 1720 (C=O) and 3440 (OH) cm^{-1}. Found, %: C 69.89; H 8.07. $C_{22}H_{30}O_5$. Calculated, %: C 70.59; H 8.02.

16α-Methylcortisol (IIIb). The culture of *T. hyalospora* was prepared for the transformation by the method of [10]. After the transformation of Ib (1.165 g) the beer was extracted with methylene chloride; after concentration of the extract, the residue contained IIb and IIIb in ∿1:1 ratio (TLC). It was washed with warm hexane and dissolved in ethyl acetate; the solution was treated with activated carbon and filtered, and the solvent was evaporated under vacuum. The residue was washed with ether and then triturated with ether, whereupon the mixture of steroids (0.47 g) was filtered off. It was dissolved in chloroform–methanol (1:1) (7.5 ml) and separated by preparative TLC on 18 × 24 cm plates with a

layer of LL-254 silica gel (gypsum binder; with a fluorescent indicator), by applying the mixture of steroids (~35 mg) to each plate. The corresponding spots were collected and eluted with methanol; evaporation of the solvent gave the residue (90 mg). It was dissolved in a mixture of chloroform (0.9 ml) and methanol (0.22 ml); after addition of water (6.75 ml), the mixture was left overnight at 0°C, being periodically agitated. Filtration gave chromatographically pure IIIb, mp 240-244°C. Found: MW 376 (mass spectrometry), $C_{22}H_{32}O_5$. Calculated: MW 376.

Literature mp is 220-222°C [11].

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MICROBIOLOGICAL HYDROXYLATION OF STEROIDS. II. STUDY OF THE SIDE REACTIONS ACCOMPANYING THE HYDROXYLATION OF CORTEXOLONE BY CULTURES OF *Tieghemella orchidis* AND *Tieghemella hyalospora*

L. K. Garcia-Rodriguez, K. N. Gabinskaya,
L. A. Krasnova, Yu. N. Korobova,
V. F. Shner, and O. V. Messinova

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The microbiological hydroxylation of cortexolone (Ia) or its acetate (Ib) by Soviet cultures of *Tieghemella orchidis* [1] and *Tieghemella hyalospora* [2] forms cortisol (IIa) and epicortisol (IIIa) as the respective major products. These enzymatic processes are accompanied by various side reactions, the chief of which is the formation of cortisone (IVa). Some other side reactions in the hydroxylation of substrate Ia by a culture of *T. orchidis* have also been described [3].

Here we are concerned with the side reactions accompanying the hydroxylation of Ia and Ib by cultures of *T. orchidis* and *T. hyalospora* and the conversion in the course of the reaction of the primary transformation products, corticosteroids IIa-IVa with a C_{11} oxygen

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