Journal of Steroid Biochemistry, 1977, Vol. 8, pp. 701–707. Pergamon Press. Printed in Great Britain

STEROID TRANSFORMING ENZYMES FROM MICROORGANISMS—III: PROPERTIES OF 4-ENE-3-OXOSTEROID-5α-REDUCTASE FROM *MYCOBACTERIUM SMEGMATIS*

C. HÖRHOLD, H. GROH, S. DÄNHARDT, N. N. LESTROVAJA AND K. SCHUBERT

Academy of Sciences of GDR, Research Centre of Molecular Biology and Medicine, Central Institute of Microbiology and Experimental Therapy, 69 Jena, GDR and
Academy of Sciences of USSR, Institute of Microbiology, Moscow, USSR

(*Received* 25 *July* 1976)

SUMMARY

Sonicated cell-free extract of *M. smegmatis* is capable of reducing 4-ene-3-oxosteroids to the corresponding 5α-dihydro-products. In the presence of sodium dithionite and with 4-androstenedione as substrate 5α-reduction takes place as the predominant transformation reaction. The enzyme remains in the 105,000 *g* supernatant and can be precipitated by ammonium sulfate at 30–45% saturation. The enzyme can be stored at −20° for at least 3 months without inactivation. At room temperature it loses about 50% activity in 48 h. In phosphate buffer the pH optimum is at 6.3 and the temperature optimum at 40°. Molecular weight is about 40,000. Thiol reagents, heavy metal ions and *o*-phenanthroline are strong inhibitors of the 5α-reductase. The inhibition by acriflavin and the increasing effect of sodium dithionite indicates the participation of flavin-nucleotides. Progesterone, testosterone and derivatives with additional double bonds and substituents in positions 4 and 17α, as well as both D- and L-19-nortestosterone are transformed to 5α-dihydrocompounds. The apparent K_M for androstenedione is 7.5×10^{-6} M. Cyproterone and Chlormadinonacetate are powerful steroid inhibitors of the enzyme.

INTRODUCTION

It is well known that, in comparison with the 4-ene-3-oxo compounds, the 5α-reduced derivatives of hormonal active steroids show interesting partial activities. In this connection we converted testosterone and 17α-methyltestosterone and their 4-chloro- and 1-ene-derivatives into the corresponding 5α-steroids by fermentation with *Rhodotorula glutinis* [1]. With *M. smegmatis* we obtained 5α-derivatives of cholesterol [2], 1,4-androstadiene-3,17-dione [3], progesterone [4] and 19-nortestosterone as well as ent-19-nortestosterone [5]. Recently, active cell-free extracts from microorganisms have been prepared [6, 7]. In the following we report in detail about the preparation of the extract from *M. smegmatis* and about some properties of the 5α-reductase.

EXPERIMENTAL PROCEDURES

Materials

M. smegmatis SG 98 was obtained from the Type Culture Collection of the Central Institute of Microbiology and Experimental Therapy, Jena, [4-^{14}C]-androst-4-ene-3,17-dione (56 mCi/mmol) from The Radiochemical Centre Amersham, Blue Dextran 2000 and Sephadex G-200 from Pharmacia, Uppsala, Kieselgel GF from Merck, Darmstadt, lysozyme from CAL-Biochem, Switzerland, pepsin and bovine serum albumin from Serva, Heidelberg, 2,5-diphenyloxazole (PPO) and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (Dimethyl-POPOP) from Packard Instrument GmbH, Frankfurt am Main, sodium dithionite, diisopropylether for chromatography and androsterone from Ferak, Berlin, 4-androstene-3,17-dione, testosterone, progesterone, 19-nortestosterone, ent-19-nortestosterone, oestradiol-17β, 6-chloro-17α-hydroxy-4,6-pregnadiene-3,20-dione acetate (Chlormadinonacetate), 17α-ethinyl-1,3,5(10)-estratriene-3,17β-diol (ethinyloestradiol), 3-methoxy-17α-ethinyl-1,3,5(10)-estratrien-17β-ol (Mestranol), 4-chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one (Oral-Turinabol®) and all other 17α-substituted steroids from VEB Jenapharm, Jena, 6-chloro-1α,2α-methylene-4,6-pregnadien-17α-ol-3,20-dione (Cyproteron) from Schering Berlin. 17β-Hydroxy-1,4,6-androstatrien-3-one was a gift of Prof. Dr. M. Kocor, Institute of Organic Chemistry of the Polish Academy of Sciences, Warszawa. All reagents were of analytical grade.

Methods

Growth conditions. M. smegmatis was grown in a gyratory shaker for 4 days at 37° in a medium containing per l. : 60 g glycerol, 4 g L-asparagine, 2 g citric acid, 0.5 g KH_2PO_4, 0.5 g $MgSO_4$, 0.05 g ferric ammonium citrate; pH 7.0.

Crude extract preparation. The cells were harvested by centrifugation at 6,000 *g* for 10 min at 4°, washed once with original vol. of 0.067 M phosphate buffer

(pH 7.0) and suspended in the same buffer (1/20 of original vol.). Cells were disrupted by 12 min ultrasonic treatment (20 khz; Sonifer®-B-12, Branson Sonic Power Company, Danbury, Connecticut). The broken cell suspension was centrifuged at 15,000 *g* for 20 min and sediment was discarded. The supernatant was centrifuged at 105,000 *g* for 60 min.

Purification procedures. The 105,000 *g* supernatant was treated with 1% streptomycin sulfate (final conc.) for nucleic acid precipitation at pH 7.0. The precipitate was separated at 15,000 *g* for 10 min. The supernatant was used for the protein fractionation with ammonium sulfate. Protein with 5α-activity was mainly precipitated between 30–45% saturation. The precipitate was resuspended in 0.067 M phosphate buffer (pH 7.0) to give a concentration of 30–50 mg protein/ml. All steps of the enzyme purification including gel filtration and ion exchange chromatography will be described in detail in the following paper [8].

Incubation and enzyme assay. The 105,000 *g* supernatant (1–3 mg protein) or the enzyme preparation (0.5–1 mg protein) were usually incubated with 10 μg (0.02 μCi) [4-^{14}C]-androstenedione in 20 μl methanol in a total vol. of 1.7 ml of 0.067 M phosphate buffer (pH 7.0) containing 5 mg $Na_2S_2O_4$. The incubations were carried out at 30° for 30 min. The samples were extracted three times with about 2 vol. of ether–chloroform (1 : 3, v/v). The extracts were combined and evaporated to dryness. The residue was redissolved in a small vol. of chloroform, applied to thin-layer plates of Kieselgel G 254 and chromatographed in the system diisopropyl ether–ethanol (95:5, v/v). If R_S for androstenedione is 1.0, we obtained $R_S = 1.3$ for 3α-hydroxy-5α-androstan-17-one and $R_S = 1.4$ for 5α-androstane-3,17-dione.

Radioactivity was determined on an integrating t.l.c.-scanner from Berthold Frieseke GmbH, Karlsruhe. The overall formation of 5α-products (5α-androstane-3,17-dione plus 3α-hydroxy-5α-androstan-17-one) was calculated from the substrate–product ratio. This method proved to be acceptable between 7 and 45% product formation (Fig. 1).

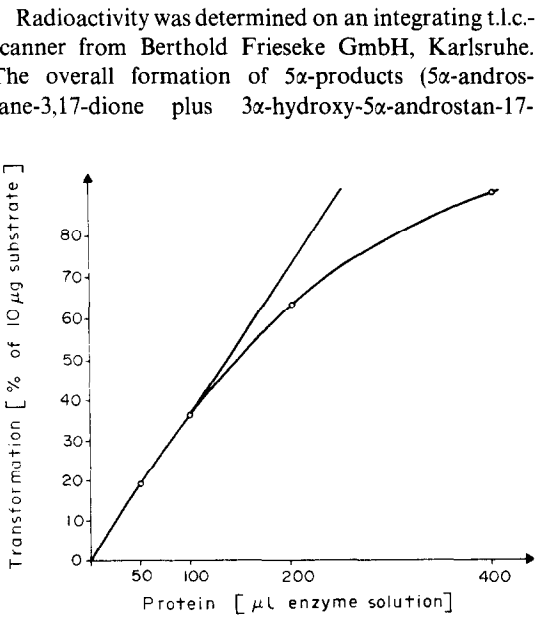

Fig. 1. Linearity of the assay of 5α-reductase activity with enzyme concentrations up to 40% transformation of 10 μg substrate per 1.7 ml phosphate buffer (pH 7.0).

Chemical identification of the products. The main product, [4-^{14}C]-5α-androstane-3,17-dione, was identified by dilution with inactive authentic 5α-androstane-3,17-dione and recrystallisation to a constant S.A. both in the crystal fraction and in the mother liquor. Radioactivity was measured on a LKB-Wallac 81,000 Liquid Scintillation Counter, using the toluene system containing 0.5% PPO and 0.03% dimethyl-POPOP. 5α-Androstane-3,17-dione was prepared by oxidation of androsterone with chromic acid [9], m.p. 132–34° (crystallized from cyclohexane–ether). Furthermore the gas chromatography and a combination of 3 t.l.c. systems were used for identification of the reaction products of the different substrates (Table 1). Gas chromatographic conditions: Varian 2700 gas chromatograph equipped with dual flame ionisation detector and 3 m × 2.5 mm glass column was used. The column was packed with 2% XE 60 on 80–100 mesh Gas Chrom Q. For examination of the various compounds the following conditions were used: air input flow 300 ml/min, argon input flow 50 ml/min and hydrogen input flow 40 ml/min. Samples were injected with a 1 μl Hamilton syringe and the following temperatures were used: column = 208°, injector and detector = 250°.

Molecular weight determination. For molecular weight determination of 5α-reductase gel filtration on Sephadex G-200 was used. Bed dimensions: 20 × 920 mm. Flow rate: 0.33 ml/min. Eluent: 0.067 M phosphate buffer (pH 7.0) containing 0.5 M NaCl. From the calibration curve, produced by chromatography of proteins of known molecular weight, the molecular weight of 5α-reductase was estimated.

pH-Measurement. In order to estimate pH during incubation 0.05 ml of assay mixture were used (Präzisions-Labor-pH-Meßgerät MV 85 VEB Präcitronic, Dresden).

Assay for protein. Protein concentrations were determined according to the method of Waddel [10] and Hesse *et al.*[11].

RESULTS

Steroid transforming activities of the 105,000 g extract. With androstenedione and testosterone as substrates the following transformations were detected: introduction of a 1,2-double bond and 5α-reduction of the 4,5-double bond followed by the reduction of the 3-oxogroup to a 3α-hydroxygroup. With testosterone oxydation of the 17β-hydroxygroup occurred. With 1,4-androstadiene-3,17-dione as substrate the reduction of the 1,2-double bond was additionally observed.

Influence of sodium dithionite ($Na_2S_2O_4$) on the reaction. Anaerobic conditions favoured the reduction reactions. After the addition of sodium dithionite the

Table 1. Thin-layer chromatography of several steroids of the androstane and 4-androstene type

Adsorbents, solvents and running distances for special separations:	Type of steroid	Migration distance (cm)	
Separation of 4-ene and 5α-steroids		4-ene	5α
Silica gel, diiso-propyl ether-ethanol (95 : 5, v/v), 2 × 16 cm	3,17-dione	8.5	12.5
	3α-hydroxy-17-one	8.5	11.0
	17β-hydroxy-3-one	8.0	12.0
	3α-17β-dihydroxy	8.0	9.5
Separation of 5α- and 5β-steroids		5α	5β
Aluminium oxide G, benzene–ethyl ether (4 : 1, v/v), 2 × 16 cm	3,17-dione	8.5	6.5
	17β-hydroxy-3-one	4.0	2.5
	3α-hydroxy-17-one	2.5	1.0
	3β-hydroxy-17-one	3.5	5.0
Separation of hydroxy and oxosteroids		17β-OH	17-oxo
Aluminium oxide G, benzene–ethyl acetate (1 : 1, v/v), 16 cm.	5α-3-one	7.5	12.0
	4-en-3-one	6.0	10.5
	1,4-dien-3-one	4.5	9.0
	3α-hydroxy-5α-	4.5	7.5
	3β-hydroxy-5α-	3.5	7.0
		3α-OH	3-oxo
	17β-hydroxy-5α-	4.5	7.5
	17-oxo-5α-	7.5	12.0
		3β-OH	3-oxo
	17β-hydroxy-5α-	3.5	7.5
	17-oxo-5α-	7.0	12.0

formation of 5α-products was 50 times higher. Controls without enzyme did not reduce 4-ene-3-oxysteroids. The amount of sodium dithionite influenced the pH of the incubation medium dependent on incubation time and buffer capacity. The addition of 0.3% $Na_2S_2O_4$ to 0.01 M phosphate buffer (pH 7.0) resulted in pH decreasing below pH 6.0. To guarantee a sufficient pH stability of 0.01 M and 0.067 M phosphate buffer $Na_2S_2O_4$ was used in concentrations of 0.06% and 0.3% respectively. The relation between the enzyme activity and $Na_2S_2O_4$ concentration at two pH values is demonstrated in Fig. 2. The highest activity was obtained in 0.067 M phosphate buffer (pH 6.2) with 0.3% $Na_2S_2O_4$.

Linearity of the assay of 5α-reductase activity as a function of time under one of these conditions is illustrated in Fig. 3. By repeated additions of $Na_2S_2O_4$ the reaction could be prolonged up to 20 h. $Na_2S_2O_4$ could not be replaced by Na_2S, $Na_2S_2O_3$, Na_2SO_3, hydroquinone or ascorbic acid.

Substrates of 5α-reductase. The following steroids have been transformed to 5α-compounds with resuspended ammonium sulphate precipitate: Androstenedione, 1,4-androstadiene-3,17-dione, testosterone, 17β-hydroxy-1,4-androstadien-3-one, 17β-hydroxy-1,4,6-androstatrien-3-one, 17α-methyl-17β-hydroxy-4-androsten-3-one, 17α-methyl-17β-hydroxy-1,4-androstadien-3-one, 4-chloro-17β-hydroxy-4-androsten-3-one, 4-chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one, 19-nortestosterone, ent-19-nortestosterone and progesterone. 19-Nortestosterone and ent-19-nortestosterone have been reduced to diastereomers.

The reduction product of [4-^{14}C]-androstenedione was identified by crystallisation with 5α-andros-

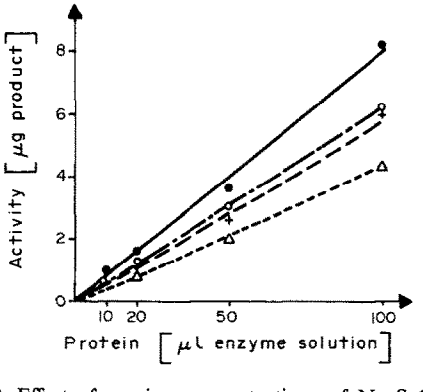

Fig. 2. Effect of varying concentrations of $Na_2S_2O_4$ and pH of the 0.067 phosphate buffer on 5α-reductase activity: (●——●) 0.3% $Na_2S_2O_4$, pH 6.2, (○—·—○) 0.3% $Na_2S_2O_4$, pH 7.0, (+– – –+) 0.06% $Na_2S_2O_4$, pH 6.2, (△– – –△) 0.06% $Na_2S_2O_4$, pH 7.0.

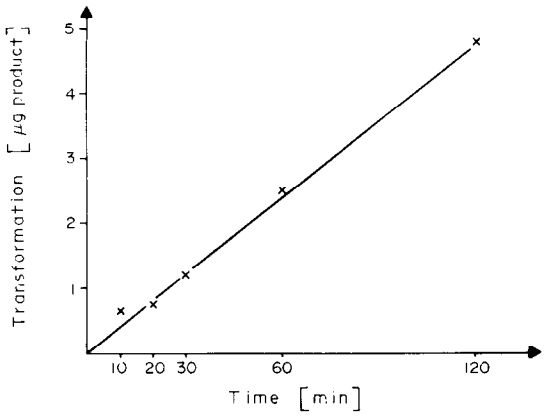

Fig. 3. Linearity of the assay of 5α-reductase activity with time (pH 7.0, 0.06% $Na_2S_2O_4$, 0.067 M phosphate buffer).

tane-3,17-dione to constant specific radioactivity. After the third crystallisation the radioactivity in the crystalline substance was 112 d.p.m./mg (m.p. 134°) and in the mother liquor 114 d.p.m./mg. The other 5α-products were identified by comparison of t.l.c. and g.l.c. behaviour with authentic substances. The reduction products of the following steroids have been detected by means of comparative t.l.c. (Table 1): 17β-hydroxy-1,4,6-androstatrien-3-one, 17α-methyl-17β-hydroxy-4-androsten-3-one, 17α-methyl-17β-hydroxy-1,4-androstadien-3-one, 4-chloro-17β-hydroxy-4-androsten-3-one, 4-chloro-17α-methyl-17β-hydroxy-1,4-androstadien-3-one, 19-nortestosterone, ent-19-nortestosterone and progesterone.

Michaelis constant for androstenedione. The K_M for androstenedione as substrate was determined. The results are presented as Lineweaver–Burk plot in Fig. 4. It gave a K_M for androstenedione of $7.5 \pm 0.8 \times 10^{-6}$ M.

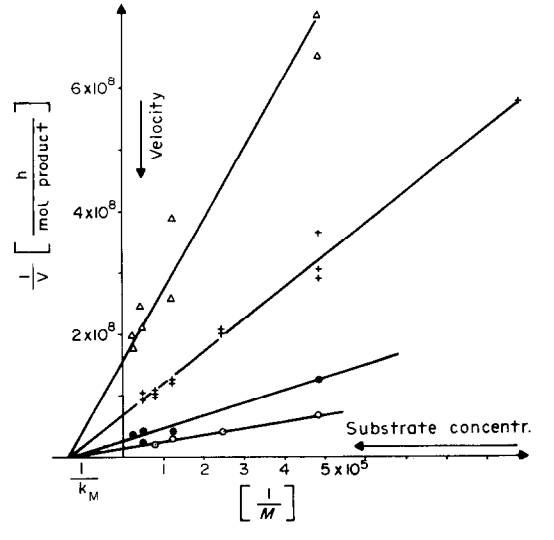

Fig. 4. Lineweaver–Burk plot of the effect of increasing concentrations of 4-androstene-3,17-dione on the rate of 5α-androstane-3,17-dione formation by the following concentrations of protein of the 30–45% ammonium sulfate precipitation: (Δ——Δ) 0.38, (+ —— +) 0.76, (●——●) 1.90, (○——○) 3.80 mg protein/1.7 ml phosphate buffer (pH 7.0).

Inducibility of enzyme. All attempts to enhance 5α-reductase activity with the addition of androstenedione, testosterone, progesterone and Oral-Turinabol® (2–150 mg steroid/50 ml medium or buffer) were without success. The changing of medium components, temperature and pH did not increase activity.

Stability, temperature and pH optimum. The 105,000 *g* supernatant as well as the resolved ammonium sulfate precipitate containing about 10 or 50 mg protein/ml, respectively, in 0.067 M phosphate buffer (pH 7.0) could be stored at −18° at least for 3 months without inactivation. At 25° the enzyme solutions have been found to lose about 50% activity within 48 h.

The temperature optimum of the 5α-reductase has been found to be about 40° (Fig. 5). When the temperature was raised further the enzyme activity started to decrease sharply.

The effect of pH on 5α-reductase activity was tested with 0.067 M Sörensen phosphate buffer and universal buffer according to Britton and Robinson[12] of pH values ranging from 4.8–7.6 and from 5.3–9.0, respectively (Fig. 6). The pH activity profile indicates a pH optimum of 6.3 in phosphate buffer and the rate of 5α-androstanedione formation is reduced by 20% at pH 7.0. In universal buffer the 5α-reductase activity was lower and the enzyme showed a broad pH optimum between 6.5 and 9.0.

Inhibitors. The effect of various inhibitors on 5α-reductase is summarized in Table 2. The enzyme was strongly inhibited by Hg^{2+} and Cu^{2+} and to a lesser extent by Mn^{2+} and Cr^{3+}. Zn^{2+} had no influence on the enzyme activity. The sulphydryl reagent iodoacetamide in concentration 5×10^{-3} M caused almost complete inhibition. Potassium cyanide, sodium azide and some other metal-binding reagents were without significant effect, with the exception of *o*-phenanthroline, which strongly inhibited the enzyme at higher concentrations. The inhibi-

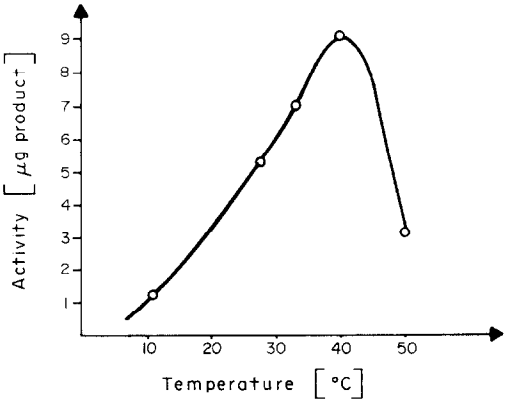

Fig. 5. The temperature dependence of the rate of the 5α-reductase activity.

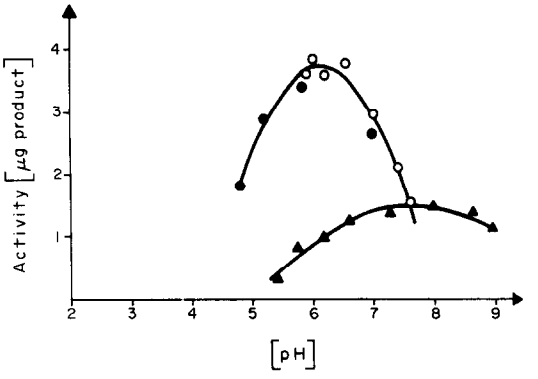

Fig. 6. Optimal pH for 5α-reductase activity. (● and ○) phosphate buffer, (●——●) $Na_2S_2O_4$ dissolved in H_2O, (○——○) $Na_2S_2O_4$ dissolved in pH 7 buffer, (▲——▲) universal buffer (0.04 mol phosphoric acid, 0.04 mol acetic acid and 0.04 mol boric acid/l. with 19–48% 0.2 M NaOH).

Table 2. Percentage of inhibition of the 5α-reductase by various inhibitors

Inhibitor	3×10^{-4} M	10^{-3} M	5×10^{-3} M
Iodoacetamide	41	67	92
NaN_3	20	20	—
KCN	24	31	37
$HgCl_2$	12*	19*	81*
$CuSO_4$	27	52*	92*
$CrCl_3$	0	11	17*
$ZnSO_4$	0	0*	0*
$MnSO_4$	—	—	12
α.α'-Dipyridyl	17	17	27
o-Phenanthroline	26	—	66
EDTA	0	0	0
Acriflavin	0	0	40

$$\% \text{ Inhibition} = 100 \times \left(1 - \frac{\mu\text{g prod. with inhib.}}{\mu\text{g prod. without inhib.}}\right)$$

* Precipitation.

tion by acriflavin indicates that flavin nucleotides may participate at the enzyme reaction.

Inhibition by steroids. Different steroids which inhibit the 5α-reductase from rat liver microsomes [13], were examined for their effectiveness towards the microbial 5α-reductase. Threefold excess of inhibitor concentration relative to substrate concentration was used. Effect of these steroid compounds on microbial and rat liver 5α-reductase is shown in Table 3. Progesterone exhibited a strong inhibitory action on both enzymes. The microsomal enzyme was hardly inhibited by oestradiol-17β and 17α-ethinyl-oestradiol-17β. The microbial 5α-reductase is relatively insensitive to these steroids. The 3-methoxy derivative, Mestranol, had only small inhibitory effect on the microsomal enzyme and had no influence on 5α-reductase activity of *M. smegmatis*. In contrast Chlormadinonacetate and Cyproteron had no significant effect on the rat liver enzyme, but were the most potent inhibitors of the microbial enzyme.

Table 3. Inhibition of 5α-reductase from *M. smegmatis* by steroids in comparison to that of the 5α-reductase from rat liver microsomes

Inhibitor	Inhibition of 5α-reductase (%) from M. smegmatis	from rat liver microsomes
Progesterone	80	94
Ethinyloestradiol	40	90
Oestradiol-17β	50	80
19-Nortestosterone	50	35
Mestranol	0	30
Chlormadinonacetate	95	22
Cyproterone	90	10

$$\% \text{ Inhibition} = 100 \times \left(1 - \frac{\mu\text{g prod. with inhib.}}{\mu\text{g prod. without inhib.}}\right)$$

Estimation of the molecular weight. The apparent molecular weight of 5α-reductase was determined by descending gel filtration through a Sephadex G-200 column (see Methods). The column was calibrated by use of bovine serum albumin, pepsin and lysozyme. The molecular weight of the 5α-reductase was estimated by a plot of the logarithmic values of the mol-

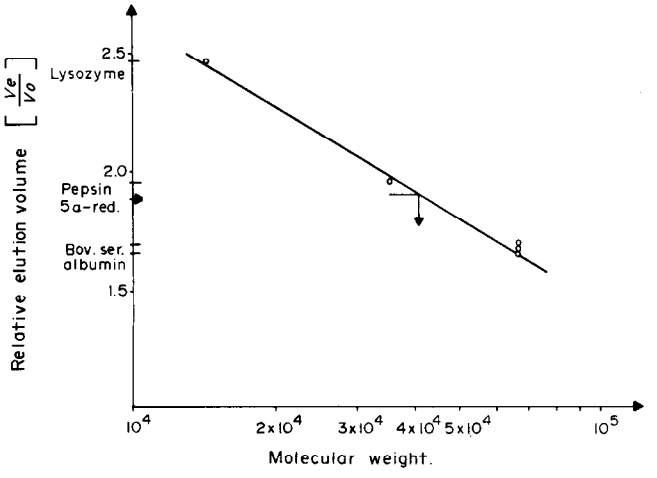

Fig. 7. Relationship between relative elution volumes v_e/v_0 of proteins and the molecular weight (on a logarithmic scale) for calculation of the molecular weight of the 5α-reductase.

ecular weights and the elution volumes. Activity could be found in the region of pepsin. The molecular weight of the 5α-reductase was calculated to be about 40,000 when the logarithmic values of the molecular weights were plotted against the relative elution vol. (Fig. 7).

DISCUSSION

The capability of microorganisms to reduce 4-ene-3-oxosteroids yielding dihydrocompounds has been well known for a long time [14]. However, in contrast to the extensive investigations of 4-ene-3-oxosteroid-5α-reductases of mammalian tissues [15–23] corresponding microbial enzymes were not explored till recently.

Levy and Talalay[24] described a 4-ene-3-oxosteroid-5α-dehydrogenase from *P. testosteroni*. Attempts to demonstrate reversal of the reaction under different conditions were unsuccessful. A cell-free preparation of a 5α-reducing enzyme of microbial origin (*Nocardia corallina*) was first reported in 1972 [6].

The present investigation has shown the existence of a 5α-reductase activity in extracts from *M. smegmatis*. The reaction was found to be a predominant process in the presence of sodium dithionite. Favourable conditions have been found for the estimation of 5α-reductase activity.

Investigation of substrate specificity shows that additional double bonds in positions 1,2 and 6,7 as well as 4-chloro- and 17α-methyl substituents do not prevent the 5α-reduction. The experiments were carried out with enzyme preparation able to reduce the 1,2-double bond. Therefore reduction of this double bond as first reaction step is possible. 19-Nortestosterone and ent-19-nortestosterone were transformed to two different reduction products. This means that the enzyme attacks the 5α-side of substrates regardless of their configuration: 19-nortestosterone is transformed to a dihydroproduct with *trans* configuration of the rings A and B and the enantiomer is reduced to a dihydroproduct with *cis* configuration of the rings A and B as Schubert and Hobe[5] reported for the reduction of 19-nortestosterone enantiomers by whole cells of *M. smegmatis*.

Some characteristics of the 5α-reductases of *M. smegmatis*, such as temperature and pH dependence, resemble the characteristics of 4-ene-3-oxosteroid-5α-reductases of different mammalian tissues. The temperature optimum of the microbial enzyme has been found to be at 40° and that of mammalian 5α-reductases in the most cases was reported to be at 35–40° [15–17]. Optimum pH for the enzyme of *M. smegmatis* was determined to be 6.3 in phosphate buffer. The 5α-reductase of mammalian tissues has an apparent pH-optimum of 6.5 ± 0.5 [16–20]. Furthermore the enzymes from both sources appear to have a broad substrate specificity for several 4-ene-3-oxosteroids. The apparent K_M values for different steroid substrates in most cases are about 10^{-6}–10^{-7} M [15–19, 23].

However some properties of microbial 5α-reductase are strikingly different from the mammalian enzymes: 1. Sulphhydryl agents hardly inhibited 5α-reductase of *M. smegmatis* (see Table 2) and *N. corallina* [25], but not the enzyme activity of nuclear and cytoplasmic membranes of rat ventral prostate [26]. 2. There was no inhibition of the microbial 5α-reductase with Zn^{2+} in contrast to the very powerful effect on mammalian enzymes preparations [26]. 3. Cyproterone and Chlormadinonacetate were not effective in inhibiting of rat prostate and liver 5α-reductase activity [13, 16], but they practically completely inhibited the enzyme of *M. smegmatis*. In both cases the strong inhibitory activity of progesterone is caused by substrate competition. 4. 5α-Reductase of *M. smegmatis* is a relative low molecular weight protein (M.W. approximately 40,000) whereas the molecular weight of nuclear and microsomal rat prostate enzymes were reported to be 250,000–350,000 [19].

The 5α-reductase from *N. corallina* [6, 25] and the reductases from mammalian tissues are NADPH dependent. But the increasing of the 5α-reductase activity from *M. smegmatis* in the presence of sodium dithionite and the acriflavin inhibition points to reduced flavin nucleotides participating in the reaction [27]. Some results on cofactor requirement will be reported in connection with purification of the enzyme in the following paper [28].

The 5α-reductase of *M. smegmatis* as the enzyme of *N. corallina* [6, 25] is not induced by steroids. 1-Dehydrogenation and side chain degradation in spores but not in vegetative cells of *Fusarium solani* appear to be constitutive [29]. The 20α-hydroxysteroid-oxidoreductase of *Bacillus megaterium* seems to be a constitutive enzyme too [30]. The physiological significance of microbial steroid transforming enzymes is not clear, but the constitutive nature may reflect the importance of these enzymes for microbial cells.

Acknowledgements—We would like to thank Mrs. Uta Tinney for skilful technical assistance, Ing.Chem. Franz Ritter for gas chromatographic measurements and Mr. Kurt Kummer for scanning of more than a thousand radioactive thin-layer chromatograms.

REFERENCES

1. Schubert K., Schlegel J., Groh H., Rose G. and Hörhold C.: *Endocrinology* **59** (1972) 99–114.
2. Schubert K. and Kaufmann G.: *Biochim. biophys. Acta* **106** (1965) 592–597.
3. Hörhold C.: Untersuchungen über Wechselwirkungen zwischen Steroiden und Mikroorganismen. Dissertation Jena (1966).
4. Schubert K., Böhme K.-H. and Hörhold C.: *Z. Naturf.* **16B** (1961) 595–597.
5. Schubert K. and Hobe G.: *Steroids* **14** 3 (1969) 297–300.
6. Pierre G., Lefebvre G., Gay R. and Bena B.: *C.r. hebd. Séanc. Acad. Sci., Paris* **D276** (1972) 600–602.
7. Hörhold C., Groh H., Dänhardt S., Lestrovaja N. N. and Schubert K.: *Z. Allg. Mikrobiol.* **15** (1975) 563–564.

8. Lestrovaja N. N., Groh H., Hörhold C. and Dänhardt S.: *J. steroid Biochem.* **8** (1977), in press.
9. Djerassi C., Engel R. R. and Bowers A.: *J. org. Chem.* **21** (1956) 1547–1549.
10. Waddel W. J.: *J. Lab. clin. Med.* **48** (1956) 311–314.
11. Hesse G., Lindner R. and Müller R.: *Z. Allg. Mikrobiol.* **11** (1971) 585–594.
12. Britton H. T. S. and Robinson R. A.: *J. chem. Soc.* (1931) 1456–1462.
13. Schubert K., Strecke J., Schumann G., Rose G. and Ritter F.: Unpublished data.
14. Charney W. and Herzog H. L.: *Microbial Transformations of Steroids* (A Handbook). Academic Press, New York and London (1967).
15. Gustafsson J.-A. and Ponsette Å.: *Biochemistry* **13** (1974) 875–88.
16. Nozu K. and Tamoaki B.-I.: *Acta Endocr., Copenh* **76** (1974) 608–624.
17. Fouchet C. and Ozon R.: *Biochimie* **55** (1973) 899–905.
18. Fredericsen D. W. and Wilson J. D.: *J. biol. Chem.* **246** (1971) 2584–2593.
19. Moore R. J. and Wilson J. D.: *Biochemistry* **13** (1974) 450–456.
20. Shimazaki J., Haraguchi T., Ohki Y. and Shida K.: *Endocr. jap.* **18** (1971) 179–187.
21. Verhoven G. and De Moor P.: *Endocrinology* **91** (1972) 54–64.
22. Cheng Y.-J. and Karavolas H. J.: *J. biol. Chem.* **250** (1975) 7997–8003.
23. Roy A. B.: *Biochimie* **53** (1971) 1031–1040.
24. Levy H. R. and Talalay P.: *J. biol. Chem.* **234** (1959) 2015–2021.
25. Germain P., Lefebvre G., Bena and Gay R.: *C.r. Séanc. Soc. Biol. Fil.* **166** (1973) 1123–1128.
26. Moore R. J. and Wilson J. D.: In *Methods in Enzymology* (Edited by B. W. O'Malley and J. G. Hardman). Academic Press, New York, San Francisco, London, Vol. 36 (1975) pp. 466–474.
27. Burch H. B.: In *Methods in Enzymology* (Edited by S. P. Colowick and N. O. Kaplan). Academic Press, New York, Vol. 3 (1957) pp. 961–62.
28. Lestrovaja N. N., Groh H., Hörhold C., Dänhardt S. and Schubert K.: *J. steroid Biochem.* **8** (1977) 313–317.
29. Hafez-Zedan H. and Plourde R.: *Biochim. biophys. Acta* **326** (1973) 103–115.
30. Krasnova L. A.: *Reduction of 20-oxosteroids by Bacillus megatherium.* Dissertation, Moscow, (1974).