Testosterone Metabolism in *Streptomyces hydrogenans*

Claus Markert, Brigitte Betz, and Lothar Träger

Zentrum für Biologische Chemie der Universität Frankfurt/M.,
Abteilung für Biochemie der Hormone

(Z. Naturforsch. **30 c**, 266–270 [1975]; received November 25/December 27, 1974)

Testosterone, Androst-4-en-3,17-dione, Enzyme Induction, *Streptomyces hydrogenans*

After cultivation of *Streptomyces hydrogenans* in the presence of ^{3}H-labelled testosterone, radioactive steroids were extracted separately from the cytosolic, ribosomal and cell wall-membrane fraction of the cells and from the culture medium, respectively.. The separation of the steroids was performed by one- and two-dimensional thin layer chromatography (TLC). The identification of the main metabolites was achieved by crystallization to constant specific radioactivity, specific staining procedures and acetylation. The oxidation of testosterone to androst-4-en-3,17-dione is by far the predominating reaction, which is almost finished after 3 h cultivation. Androst-4-en-3,17-dione is mainly transferred into the culture medium and partly accumulated within the cell wall-membrane fraction. High polar steroid metabolites and androstane derivatives are present in very small amounts only.

Introduction

After cultivation of *Streptomyces hydrogenans* in the presence of 17β-hydroxy-androst-4-en-3-one (testosterone) the steroid is taken up very rapidly by the cells [1]. Thereby the synthesis of 20β-hydroxysteroiddehydrogenase (EC 1.1.1.53) increases during the next 4 h [2]. Because there is a significant lag time in the course of enzyme induction by testosterone in comparison to the effect of other steroids [3], we supposed that testosterone must be converted to a metabolite, which functions as the real inducing steroid for 20β-hydroxysteroiddehydrogenase synthesis. Therefore the metabolism of testosterone and the distribution of its metabolites among the different cell fractions was investigated.

Materials and Methods

Steroids

Non-radioactive steroids were supplied by Fluka AG, Neu-Ulm, E. Merck AG, Darmstadt, Schering AG, Berlin, and Steraloids, Pawling, U.S.A. By addition of non-labelled testosterone the specific radioactivity of [1,2,6,7-^{3}H(N)]-testosterone (New England Nuclear, Dreieichenhain) was decreased to 1.2 μCi/μmol. Radioactive testosterone was purified by paper chromatography on Schleicher & Schüll, Nr. 2043 a [4].

Requests for reprints should be sent to Prof. Dr. L. Träger, Klinikum der J. W. Goethe-Universität Frankfurt a. M., *D-6000 Frankfurt/M.*, Theodor-Stern-Kai 7.

Thin-layer chromatography

Pre-coated silica gel F_{254} thin-layer plates, layer thickness 0.25 mm, supplied by E. Merck AG, Darmstadt, were utilized. They were stored at room temperature and dried over blue gel for at least 24 h. After migration the compounds were either located by U.V.-absorption, spraying with 0.2% 1,4-dinitrophenylhydrazine in 2 N HCl, staining in iodine atmosphere, or by scintillation counting.

Scintillation counting

After separation of the steroids by TLC 0.5 cm wide bands of silica gel were scraped off and 10 ml of toluene-based liquid scintillation cocktail added. Counting was performed in a Packard Tri-Carb, model 3375. Counting efficiency: $40 \pm 2\%$; background: $16 - 20$ cpm/0.5×4 cm silica gel band.

Cultivation of the microorganism

Streptomyces hydrogenans was cultivated as described earlier [2]. Cells were grown for 7 h. 1/4, 1/2 and 3 h before harvesting of the cells 100 μg [^{3}H]-testosterone/ml (0.34 μmol/ml; 0.4 μCi/ml) were added. Cells were harvested by centrifugation, washed 3 times with tris buffer (10 mM Tris, 1.5 mM Mg^{++}, 10 mM KCl, 1 mM NaN_3, pH 7.4) and resuspended in a small volume of the same buffer. Homogenization was performed by sonification (Branson sonifier, 4.5 A, 90 sec). The cell homogenate was separated into cell wall-membrane fraction (sediment after centrifugation at $35\,000 \times g$ for 20 min, sample II), ribosomes (III) and cytosol (IV) (centrifugation of the last supernatant at $105\,000 \times g$ for 3 h).

Extraction of steroids

Culture medium (I) and the different fractions II – IV obtained from the cell homogenate were mixed with 10 vol. of aceton-methanol (1:1) and left for 18 h at room temperature. Precipitated material was resuspended in tris buffer and degraded by 1 mg trypsin/ml at 30 °C [5]. The clear supernatant after trypsin digestion was combined with the acteton-methanol containing extract and evaporated at 30 – 40 °C to 10 ml. The radioactivity of 0.1 ml was determined. Steroids were extracted three times with 1 vol. diethyl ether-chloroform (3:1). The lipophylic phase was washed successively by 2 N NaOH, 0.1 N acetic acid, and water (all solutions contained 10% Na_2SO_4) respectively and dried over Na_2SO_4.

Derivative formation and recrystallization

Steroids were extracted from silica gel by methylene dichloride and dried under a stream of nitrogen. 0.2 ml anhydrous pyridine and 0.2 ml acetic anhydride were added. After 18 h at room temperature the mixture was evaporated to dryness under nitrogen at 40 – 50 °C.

Recrystallization to constant specific radioactivity was performed after addition of the corresponding non-labelled steroid to the ^{3}H-labelled sample eluted from silica gel. After dissolution in methylene dichloride, radioactivity of 0.2 ml was estimated. Recrystallization was performed with methanol-H_2O, methanol-*n*-hexane, and acetone-*n*-hexane, respectively.

Results

Extraction of the labelled steroids

After cultivation of *Streptomyces hydrogenans* in the presence of 100 μg [^{3}H]testosterone/ml, radioactive steroids are distributed within the different cell fractions and in the culture medium (Table I). The main part of the radioactivity remains within the culture medium and only 4 – 10% of the total radioactivity can be found in the cells. These figures pretend that most of the steroids never enter the cell but stay unchanged in the culture medium.

To determine the composition of the different steroid samples extracted from the culture medium and the cell fractions, we looked for useful migra-

Table I. Distribution of total radioactive steroids in medium and cell fractions of *Streptomyces hydrogenans*. Steroids were extracted from culture medium (I), cell wall-membrane fraction (II), ribosomes (III), and cytosol (IV) of *Streptomyces hydrogenans* after cultivation in the presence of 100 μg [^{3}H]testosterone/ml (0.4 μCi/ml).

Time of cultivation [h]	Radioactivity in per cent of total testosterone administered. Fraction: I	II	III	IV
1/4	96.0	1.0	1.5	1.6
1/2	89.4	5.5	3.1	2.0
3	96.0	2.2	0.6	1.0

Table II. Separation of testosterone and 17 related steroids by thin-layer chromatography. Results are expressed as *Rf* values (precision ±0.02). Solvent systems used for migrations: A = benzene-aceton (4:1); B = benzene-aceton (3:1); C = cyclohexan-ethylacetate (1:2).

	A	B	C
Testosterone	0.25	0.30	0.28
Androst-4-en-3,17-dione	38	44	41
5α*H*-Androstan-3,17-dione	46	52	46
17β-Hydroxy-5α*H*-androstan-3-one	30	40	38
17β-Hydroxy-5β*H*-androstan-3-one	27	36	35
3α,17β-Dihydroxy-5α*H*-androstane	23	26	32
3α-Hydroxy-5α*H*-androstan-17-one	26	38	34
3β-Hydroxy-androst-5-en-17-one	27	36	42
3β,17β-Dihydroxy-androst-5-ene	19	26	35
17β-Hydroxy-androst-1,4-dien-3-one		17	24
6β-Hydroxy-androst-4-en-3,17-dione		13	15
3β-Hydroxy-androst-5,16-diene		44	52
Androst-5-en-3,17-dione		56	56
Testosterone acetate		34	
17β-Hydroxy-5α*H*-androstan-3-one acetate		48	
3α-Hydroxy-5α*H*-androstan-17-one acetate		44	
3β-Hydroxy-androst-5-en-17-one acetate		52	
3β,17β-Dihydroxy-androst-5-ene diacetate		54	

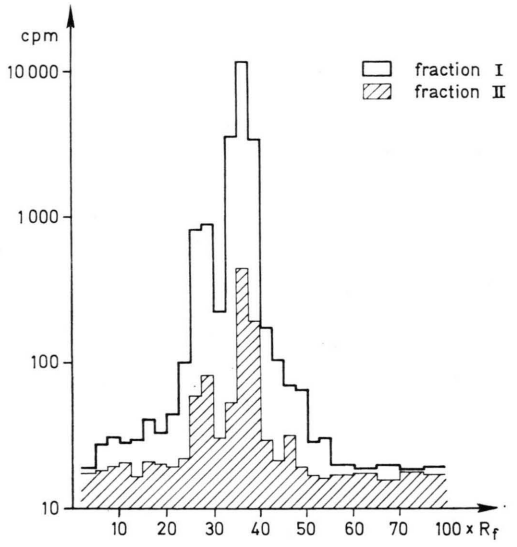

Fig. 1. Thin-layer chromatography of steroids extracted from culture medium and cell wall-membrane fraction of the cells. Separation by TLC in solvent system A. Radioactivity is plotted as log cpm. Cells were cultivated in the presence of 100 μg [^{3}H]testosterone/ml for 3 h. Fraction I: culture medium; fraction II: cell wall-membrane fraction.

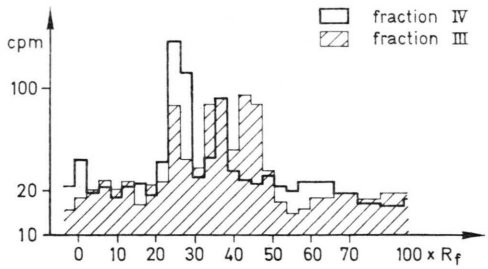

Fig. 2. Thin-layer chromatography of steroids extracted from ribosomes and cytosol of the cells. Further details as described in Fig. 1. Fraction III: ribosomes; fraction IV: cytosol.

tion systems to separate testosterone and its metabolites by TLC. Sufficient separations were obtained by TLC in the solvent systems A, B and C (Table II). A clear cut separation of the main radioactive compounds could be achieved (Figs 1 and 2). Radioactivity in the band with R_f 0.25 could be identical with testosterone, the next high radioactive zone with R_f 0.38 may be androst-4-en-3,17-dione. However, metabolites from the ribosomal fraction (III) show a third radioactive spot with R_f 0.46, which could be identical with 5α-androstan-3,17-dione.

Very similar distributions of the radioactive metabolites were established at shorter incubation times (Table III). The radioactive pattern confirms the rapid turnover of testosterone into androst-4-en-3,17-dione in the culture medium (fraction I) during the first hour. Correspondingly the amount of androst-4-en-3,17-dione increases in the cell wall-membrane fraction (II). The accumulation of this metabolite in fraction II is obvious in comparison to the content of the same steroid in the cytosolic fraction (IV), but is still less than in the culture medium (fraction I). Furthermore, the relative high percentage of unpolar steroid(s) in the area of R_f 0.42 – 0.49 within the ribosomal fraction (III) is surprising.

Table III. Distribution of radioactive metabolites of testosterone after thin-layer chromatography. Separation by TLC in solvent system A. Steroids were extracted from the different fractions I–IV.

Time of cultivation [h]	Fraction	Radioactivity in % of total steroids in the whole culture R_f-values x 100 00–17	17–27	27–42	42–49
1/4	I	0.7	78.7	11.2	7.2
1/2		0.8	71.4	14.6	2.2
3		0.2	8.6	86.3	0.7
1/4	II	<0.1	0.7	0.1	0.1
1/2		0.1	4.4	0.7	0.3
3		<0.1	0.5	1.4	0.1
1/4	III	0.1	0.9	0.4	0.2
1/2		0.4	1.4	0.4	0.5
3		<0.1	0.1	0.3	0.2
1/4	IV	0.1	0.6	0.8	<0.1
1/2		0.1	1.0	0.9	<0.1
3		0.2	0.5	0.3	<0.1

To get a more detailed separation of the steroids hitherto summed up within the R_f areas as described in Table III, the steroids were separated by two-dimensional TLC in the solvent systems B and C (Fig. 3). There are at least 27 different metabolites,

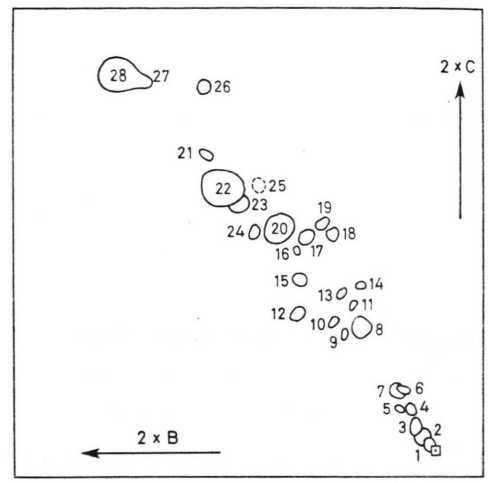

Fig. 3. Two-dimensional thin-layer chromatography of testosterone metabolites extracted from culture medium of *Streptomyces hydrogenans*. *Streptomyces hydrogenans* was cultivated in the presence of 100 μg [^{3}H]testosterone/ml for 3 h. Steroids were extracted from the culture medium and separated by two-dimensional TLC in solvent system B and C. Migration was performed twice in each direction. The metabolites were located by their UV-absorption, staining in an iodine atmosphere, and spraying with 1,4-dinitrophenylhydrazine, successively.

which are mainly arranged on the 45° line. A few deviations from this distribution may be due to the different behaviour of androst-5-ene-derivatives in comparison to androst-4-ene- and androstane-metabolites, which show higher R_f values in system C in comparison to system B. Table IV shows the relative radioactivity of every single spot shown in Fig. 3 in comparison to the total metabolites from the culture medium. Additionally a few properties referring to staining and UV-absorption are mentioned. Table IV confirms that out of 28 compounds, so far separated, two comprise more than 93% of the total steroids. Presumably spot number

Table IV. Distribution of testosterone metabolites in the culture medium after two-dimensional thin-layer chromatography. The numerical order corresponds to Fig. 3, where further details are described.

Spot number	R_f value * in solvent system B	C	UV-absorption	Hydrazone formation	Per cent of total radioactivity
1–4	0.02–0.04	0.01–0.04	+	–	1.4
5–7	0.06–0.07	0.05–0.08	–	–	0.5
8	0.13	0.17	+	–	0.3
9–11	0.15–0.19	0.16–0.19	+	+	each <0.1
12	0.26	0.17	+	+	0.1
13	0.17	0.21	+	–	0.3
14	0.14	0.22	+	–	<0.1
15	0.26	0.23	+	+	0.4
16	0.26	0.28	+	–	0.5
17	0.24	0.31	+	+	0.5
18–19	0.19–0.21	0.31–0.33	–	–	each <0.1
20	0.30	0.32	+	+	10.4
21	0.49	0.44	–	–	0.1
22	0.43	0.41	+	+	83.0
23	0.40	0.37	–	+	2.1
24	0.36	0.31	–	+	0.1
25	0.34	0.42	–	–	2.4
26	0.47	0.66	–	–	0.1
27–28	0.68–0.88	0.68–0.72	+	+	each <0.1

* R_f values are calculated for double migration in each solvent system and direction by the formula

$$R_f = \frac{f_1+f_2}{2\cdot f_1}\left(1-\sqrt{1-\frac{4\cdot f_1\cdot s_2}{(f_1+f_2)^2}}\right)$$

f_I distance of front, s_I distance of spot } to start spot.

Indices are referring to the sequence of migrations in one direction.

20 corresponds to testosterone, number 22 to androst-4-en-3,17-dione, and number 21 to 5αH-androstan-3,17-dione.

Identification of the steroid metabolites

For further identification recrystallization to constant specific radioactivity was accomplished (Table V). The course of specific radioactivities determined after each recrystallization shows that the assumptions for the three main metabolites mentioned before are correct. Besides, both hydrazone formation for spot 20 and 22 and detection of an acetate in the case of spot 20 only let conclude that spot 20 and 22 are identical with testosterone, and androst-4-en-3,17-dione respectively. 5αH-Androstan-3,17-dione (spot 21) may be one of the metabolites produced in very small amounts. The failure to detect the expected hydrazone for this metabolite is due to the pale yellow colour of this steroid hydrazone.

Table V. Recrystallization of three steroids to constant specific radioactivity. Details of the procedure as described in methods.

Number of crystallization	Specific radioactivity [cpm/mg] Spot Nr. 20	21	22
0	13 330	2 600	100 060
1	11 620	1 220	94 570
2	12 410	550	99 530
3	13 020	410	97 790
4		440	
5		420	

Discussion

Either by one- or by two-dimensional TLC two main steroids can be detected after cultivation of Streptomyces hydrogenans in the presence of testosterone. Both steroids contain a 3-keto-Δ^4-structure, which was proved by four different procedures. Both compounds show UV-absorption and form hydrazones. Their mobility on TLC corresponds in different solvent systems to testosterone and androst-4-en-3,17-dione, respectively. Three recrystallizations to constant specific radioactivity prove their radiochemical purity and identity. Last not least, testosterone only can be converted to the corresponding acetate. The third metabolite which seems to be 5αH-androstan-3,17-dione with high probability, can be detected in all cell fractions and in the culture medium as well, but seems to be especially enriched in fraction III (ribosomes).

If testosterone concentration or 1 minus androst-4-en-3,17-dione concentration in the total cell culture

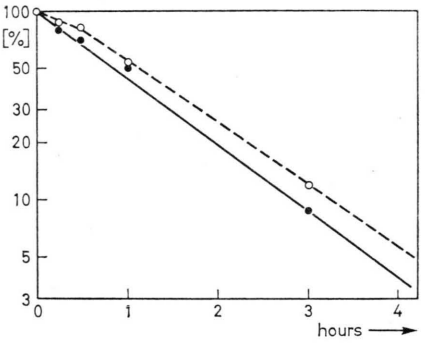

Fig. 4. Testosterone oxidation or androst-4-en-3,17-dione formation as a function of time of cell cultivation. Logarithms of total concentration of testosterone (–●–●–) or 1-androstendione (- - ○ - - ○ - -) concentration in the culture (medium + cells) are plotted against time of cultivation.

is plotted semilogarithmically against time of cultivation the sequence of the 4 available (Fig. 4) plots for each system approach a straight line. Fig. 4 is in agreement with the assumption that testosterone conversion follows first order kinetics. Furthermore the rapid metabolism of the starting steroid points to a very extensive transfer of testosterone into the cells and export of androst-4-en-3,17-dione into the culture medium. Therefore the relative low radioactivity within the cells after cultivation in the presence of labelled steroids does not reflect the real magnitude of steroid exchange.

Microbial oxidations of different steroids at C-17 were already described for *Streptomyces albus*, *Actinomyces albus* and *Proactinomyces species* [6–8]. The formation of 5α-androstan-3,17-dione was proved in the related microorganisms *Streptomyces species* and *Nocardia corallina* [9]. As a similar reduction could be possible in *Streptomyces hydrogenans*, 17β-hydroxy-5αH-androstan-3-one (5α-dihydrotestosterone) could be produced. This steroid may be identical with spot 23 of Fig. 3. Because 5α-dihydrotestosterone is accepted as a substrate of 20β-hydroxysteroid-dehydrogenase (reduction of carbonyl at C-3 to 3α-hydroxyl [10]) the formation of 3α.17β-dihydroxy-5αH-androstane and 3α-hydroxy-5αH-androstan-17-one must be considered. Both metabolites could be identical with spots 17 and 24, respectively. Moreover, *Streptomyces hydrogenans* contains a specific receptor for 5α-dihydrotestosterone [1].

Several strains of *Streptomyces or Nocardia* form Δ^1-metabolites [9, 11, 12]. Δ^1-Testosterone and spot 15 have identical R_f values in the solvent systems used in these studies. Moreover, 6β-hydroxytestosterone [13] and 6β-hydroxyandrost-4-en-3,17-dione have comparable mobilities with spots 8 – 12. If there is any ring opening reaction to seco-derivatives this metabolic way must be very insignificant in comparison to the main conversion of testosterone to androst-4-en-3,17-dione. Other microorganisms show a high rate of steroid degradation to seco-derivatives, *e. g. Mycobacterium smegmatis* [14]. Besides, hydroxylation to high polar androgen metabolites (spots 1 – 7) takes place to a very minute extent only.

The results show that the oxidation of testosterone to androst-4-en-3,17-dione is the main metabolic reaction catalyzed by the cells. Further experiments have shown that the enzyme which is responsible for this conversion is a NAD^+-dependent 17β-hydroxysteroid dehydrogenase (EC 1.1.1.63). Its activity (or synthesis) increases in the presence of testosterone [15]. Additionally, a steroid reductase must be present yielding 5αH-androstan-3,17-dione.

Testosterone induces the synthesis of 20β-hydroxysteroid-dehydrogenase with a significant lag time. After 2 h its enzyme activity is rather low, although 80% of testosterone are already converted to androst-4-en-3,17-dione at this time. Therefore we may suggest, that testosterone must be converted to the real active inducing metabolite, the identity of which must be clarified by further investigations. Androst-4-en-3,17-dione is the most probable candidate, because enzyme induction in the presence of this steroid shows no lag time. Besides, the formation of androst-4-en-3,17-dione is by far the predominant reaction concerning testosterone metabolism in *Streptomyces* hydrogenans.

This work was made possible by financial support from the Deutsche Forschungsgemeinschaft, for which we would like to thank gratefully.

[1] L. Träger, Hoppo-Seyler's Z. Physiol. Chem. **354**, 1077 [1973].
[2] A. Wacker, B. Bauer, and L. Träger, Hoppe-Seyler's Z. Physiol. Chem. **351**, 320 [1970].
[3] J. Betz and L. Träger, Hoppe-Seyler's Z. Physiol. Chem., in press.
[4] I. E. Bush, The Chromatography of Steroids, Pergamon Press, London 1961.
[5] B. Palmowski and L. Träger, Hoppe-Seyler's Z. Physiol. Chem. **355**, 1070 [1974].
[6] M. Welsh and C. Heusghem, C. R. Soc. Biol. **142**, 1074 [1948].
[7] P. Talalay and N. Dobson, J. Biol. Chem. **205**, 823 [1953].
[8] G. E. Turfitt, Biochem. J. **40**, 79 [1946].
[9] G. Lefebvre, P. Germain, and R. Gay, C. R. Acad. Sci. **274**, 449 [1972].
[10] W. Gibb and J. Jeffery, Biochem. J. **135**, 881 [1973].
[11] M. Hayano, H. J. Ringold, V. Stefanovic, M. Gut, and R. J. Dorfman, Biochem. Biophys. Res. Commun. **4**, 454 [1961].
[12] I. Belic, V. Kramer, and H. Socic, J. Steroid Biochem. **4**, 363 [1973].
[13] N. Sato, M. Ota, and K. Obara, Experientia **26**, 100 [1970].
[14] C. Hörhold, K.-H. Böhme, and K. Schubert, Z. allg. Mikrobiol. **9**, 235 [1969].
[15] C. Markert and L. Träger, in preparation.