# Conversion of cholesterol to testosterone by *Mycobacterium* sp. MB-3638.

**Sofía Borrego, María Esther Espinosa, Elena Martí and Magdalena Fonseca.**

Laboratorio de Esteroides, Centro Nacional de Investigaciones Científicas, Avenida 25 y 158, Apartado Postal 6990, Playa, Ciudad de La Habana, Cuba.

Recibido: 3 de julio de 1998. Aceptado: 28 de octubre de 1998.

Palabras clave: micobacteria, testosterona, biotransformación de esteroles, medios de cultivo.
Key words: mycobacteria, testosterone, sterol biotransformation, culture *medium*

**RESUMEN.** La etapa final en la biosíntesis de la testosterona es la reducción del 4-androsten-3,17-diona (AD) a testosterona. La reducción enzimática no sólo la realizan microorganismos y animales sino también suspenciones de células vegetales. Sin embargo, el AD puede producirse madiante un proceso de transformación microbiológica por la ruptura selectiva de la cadena lateral de los esteroles, mientras que la producción industrial de la testosterona a partir del AD ocurre mediante cuatro pasos de síntesis química. La producción de testosterona a partir de colesterol, AD ó 1,4-androstadien-3,17-diona (ADD) ha sido llevada a cabo por diferentes géneros de microorganismos. Se probaron diferentes medios de cultivo salinos en los cuales las micobacterias pueden biotrnaformar esteroles y variantes de éstos, con el objetivo de seleccionar la que mayores rendimientos de testosterona proporcionara. Se estudió el efecto del pH inicial en la producción de testosterona a partir del colesterol y el ADD. También se siguió la producción de androstanos y el crecimiento microbiano durante 168 h . La cepa mutante *Mycobacterium* sp. MB-3683 fue capaz de producir testosterona como producto mayoritario a partir del colesterol (Yp/s = 37,6 %) y del ADD (Yp/s = 45,1 %) empleando un medio salino enriquecido con glucosa al 3 % y urea a 0,4 g · $L^{-1}$ a pH inicial de 6 y 144 h de fermentación en zaranda. También esta cepa, que produce AD como producto mayoritario a partir de esteroles, fue capaz de producir ADD a partir de AD en presencia de glucosa, lo que demostró además, que este azúcar estimuló la 1,2-dehidrogenación. De los sustratos que se utilizaron en la biotransformación (colesterol, ADD y AD), el ADD resultó el mejor para obtener testosterona. La dehidrotestosterona, producto intermediario en la reacción doble del AD, no se detectó.

**ABSTRACT.** The final step in the testosterone biosynthesis is the reduction of 4-androstene-3,17-dione (AD) to testosterone. The enzymatic reduction has been not only in microorganisms, in animals but in vegetable cell suspension too. Although AD can be produced from the selective side chain cleavage of sterols via microbial transformation process, the industrial production of testosterone from AD is carried out via four steps of chemical synthesis. The production of testosterone from cholesterol, AD or 1,4-androstediene-3,17-dione (ADD) has been carried out by different genus of microorganisms. Different saline culture media in which the biotransformation of sterols by micobacteria is carried out and variants of some of them, were tested with the aim to select the media in whom the testosterone yield was the highest. The effect of the initial pH on the testosterone production from cholesterol and ADD was studied. Also the androstane production from cholesterol and microbial growth were monitored for 168 h . The mutant strain of *Mycobacterium* sp. MB-3683 was capable to produce testosterone from cholesterol (Yp/s = 37.6 %) and ADD (Yp/s = 45.1 %) as a major product using a saline medium supplemented with glucose at 3 % and urea (0.4 g . $L^{-1}$), at initial pH of 6 and 144 h of fermentation in shaker. Also, this strain which produce AD from sterols as a major product, was capable to produce ADD from AD in presence of glucose, hence this sugar stimulated the 1,2-dehydrogenation too. The cholesterol, the ADD and the AD were used as substrates in the biotransformation, and the ADD was the best to obtain testosterone. The dehidrotestosterone, intermediate product in the double reaction of AD, was not detected.

## INTRODUCTION

Testosterone is a male sex hormone or androgen, which is produced throughout life by the testes. The final step in the biosynthesis of testosterone is reduction of 4-androstene-3,17-dione (AD) to testosterone.[1,2] This reaction is catalyzed by the microsomal enzyme 17-ketosteroid redcutase.[3]

The enzymatic reduction of AD to testosterone has been found not only in microorganisms,[4-7] in animals[3] but in vegetable cell suspension[8] too. Although AD can be produced from the selective side chain cleavage of sterols via microbial transformation process,[9-11] the industrial production of testosterone from AD is carried out via four steps of chemical synthesis.[12]

Wang *et al.*[13] reported that when a strain of genus *Mycobacterium* was grown in nutrient broth supplemented with glucose (4 %), the major transformation product of cholesterol was testosterone. Later Llanes *et al.*[5] and Hung *et al.*[14] reported other studies made with other *Mycobacterium* strain in which testosterone was the principal product of the biotransformation too.

The purpose of this study was to research the culture conditions the testosterone production from cholesterol by a *Mycobacterium* sp. mutant strain.

## MATERIALS AND METHODS

### Microorganism

*Mycobacterium* sp. MB-3683 degrades the sterol side chain to pro-

duce AD as the major product, as described in previous papers.[9-11]

### Media

A nutrient broth (NB) supplemented with glucose at 4 %,[13] the synthetic medium (MLL) reported by Liu *et al.*[4] and the saline medium (MSAL) consisting of 5 g NaCl, 5 g $NaNO_3$, 0.5 g $K_2HPO_4$, 0.5 g $KH_2PO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $CaCl_2$, per liter of distilled water was used as basal media for the production of testosterone. The cholesterol suspension was prepared in a similar form to Borrego *et al.*[11]

### Cultivation

The methodology employed was similar to Borrego *et al.*[11] When the biotransformation was carried out with AD or ADD as substrate, the experimental conditions were different. In this case, the reaction system total volume was 10 mL . The substrate (300 mg · $mL^{-1}$) was added by dissolving in methanol and solvent final concentration was 1 %.

### Steroid determination

The testosterone and AD levels were extracted and determined using the methodology reported by Borrego *et al.*[11]

### Growth determination

The growth was determined by measuring the protein content similar to Borrego *et al.*[11]

### Evaluation of experiments

The experiments were carried out by triplicate and the results were analyzed by the ANOVA-1 and Duncan tests.[15]

## RESULTS AND DISCUSSION

### Influence of the medium composition on the testosterone production

The effect of the medium composition on the testosterone production from cholesterol was examined. The main product of the transformation of this strain in both nutrient medium and synthetic medium was AD (Table 1). However the testosterone production was promoted by the addition of glucose only the saline medium (MSAL) and the concentration of 3 % of this sugar enhanced significantly the testosterone yield. On the other hand, the yield obtained was superior to the one reported by Wang *et al.*[13] and Liu *et al.*[4] The increase of glucose concentration did not enhance the testosterone production, it might be due to the regulatory effect of the glucose on the biotransformation process.

The effect of sugar on the reduction reactions can be explained by the regulatory mechanism mentioned by Llanes *et al.*[5,16] for steroid-1,2-dehydrogenation when an endogenous substrate is added in the culture media. The level of NADPH in the cell determines the reducing processes. Glucose as a carbon source provides reducing equivalents to *Mycobacterium* by glycolitic or monophosphate hexoses degradation pathway. For this reason, there is a competition between glucose and steroid substrate in order to get a final electron acceptor (respiratory chain). Glucose prevents oxidation reactions and enhances reduction reactions.

The testosterone yields in both NB with glucose and MLL media were very low. On the other hand, the peptone addition was not stimulated by the testosterone production; although when the urea was added to the medium, the testosterone was produced as the principal product.

### Effect of initial pH on the testosterone production from cholesterol

Another aspect to consider was the initial pH. It was studied in MSAL medium with urea and glucose (Fig. 1). The maximum testosterone yield was obtained at pH 6.0. This result is similar to the one obtained by Liu *et al.*[4] who worked with partial enzyme.

### Kinetics of production of testosterone

The androstanes production from cholesterol and growth were monitored for 168 h (Fig. 2). As shown in this figure, the growth (protein level) increased rapidly until 24 h, after 48 h it was constant.

The AD was formed at 24 h and the maximum accumulation was at 72 h (24.7 %), then the consumption rate of this product is slowly because most of the AD was converted to tes-

**Table 1.** Effect of medium composition on the production of androstanes.

| Medium | Yp/s (%)a | | |
|---|---|---|---|
| | Testosterone ± SD | AD ± SD | Total |
| Nutrient broth (NB) | 0 | 16.3 ± 1.5 | 16.3 |
| NB + Glucose (4 %) b | 3.1 ± 1.5 (c) | 19.9 ± 2.0 | 23.0 |
| MLL c | 1.5 ± 0.5 (e) | 8.3 ± 1.0 | 9.8 |
| MSAL + Glycerol d | 1.8 ± 0.5 (e) | 33.0 ± 3.1 | 34.8 |
| MSAL + Glucose (1 %) | 10.2 ± 2.3 (d) | 21.8 ± 3.0 | 32.0 |
| MSAL + Glucose (2 %) | 14.7 ± 1.8 (c) | 19.9 ± 2.1 | 34.6 |
| MSAL + Glucose (3 %) | 18.8 ± 2.5 (b) | 16.6 ± 3.1 | 35.4 |
| MSAL + Glucose (4 %) | 12.5 ± 2.0 (cd) | 21.1 ± 2.8 | 33.6 |
| MSAL + Peptone + Glucose e | 13.6 ± 1.5 (cd) | 14.2 ± 2.0 | 27.8 |
| MSAL + Urea + Glucose f | 37.6 ± 2.8 (a) | 1.8 ± 0.2 | 39.4 |

a: Yp/s, Yield product/substrate added; SD, Standard deviation.
b: Culture medium reported by Wang *et al.* (1982).
c: Culture medium reported by Liu *et al.* (1994).
d: Saline medium (MSAL) reported by Borrego *et al.* (1997) was utilized as Control.
e: MSAL supplemented with peptone (1 %) and glucose (3 %).
f: MSAL without $NaNO_3$ but Urea (SIGMA, 0.4 g · $L^{-1}$) and glucose (3 %) were added.
The value (b), (c), (d), (cd) and (e) are significantly different than test with (a) (Test of Duncan $p \leq 0.05$).

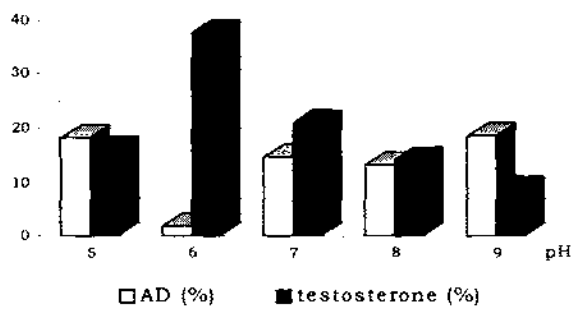


*Fig. 1. Influence of initial pH on the testosterone production from cholesterol.*

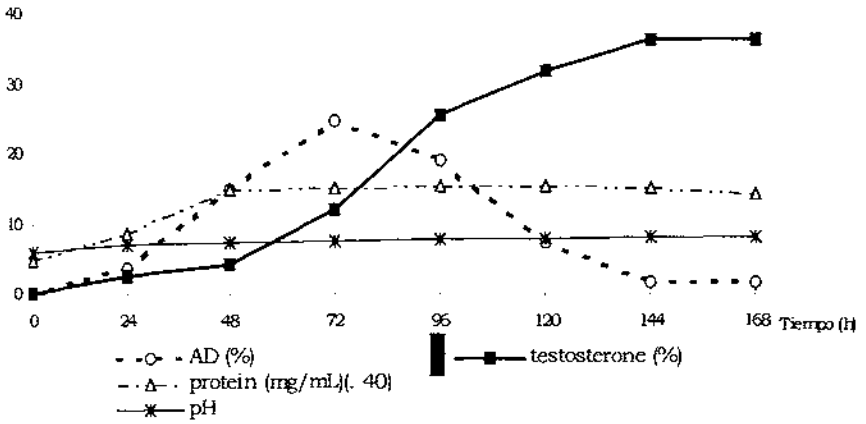


*Fig. 2. Kinetic of testosterone production from cholesterol in shaker.*

Table 2. Bioconversion of AD and ADD in presence of glucose and urea at initial pH 6.0.

| Substrate | AD(D) | | Testosterone (%)[a] | | Total | |
|---|---|---|---|---|---|---|
| | Control[b] | Glucose 3 % | Control[b] | Glucose 3 % | Control[b] | Glucose 3 % |
| AD | 7.2 | 20.4 | 7.0 | 29.6 | 14.2 | 50.0 |
| ADD | 36.2 | 45.0 | 19.8 | 45.1 | 56.0 | 90.1 |

[a] Yield product/substrate added.
[b] Saline medium (MSAL) only with steroidal substrate.

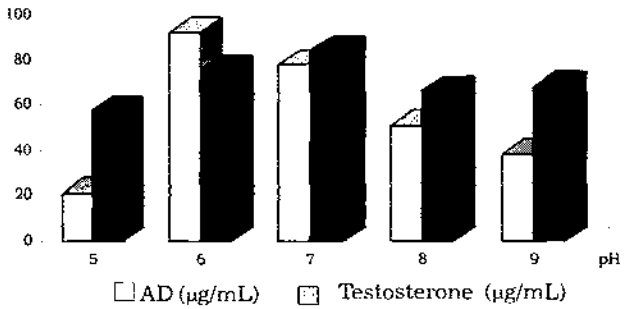


*Fig. 3. Influence of initial pH on the testosterone production from ADD.*

tosterone after 144 h . This bioconversion was about 98 % in 72 h of cultivation (of 72 until 144 h). The pattern of cell growth, AD and testosterone production were similar to the one reported by Liu *et al.*[4] in a bioreactor.

The pH of culture has increased from 6 to 8.3, but this is a consequence of the glucose degradation. In cultures without cholesterol the pH had a similar tendency.

### Conversion of AD and ADD in presence of glucose and urea

Table 2 shows the results of AD and ADD conversion with and without glucose and urea in MSAL medium at initial pH 6.0. The MB-3683 strain produces AD as a principal product from sterols and ADD is obtained as trace,[9-11] besides the steroid-1,2-dehydrogenase activity was high because the ADD production was stimulated when the AD was added as substrate.

When the glucose and urea were added, the ADD was formed in higher quantities than the control and the testosterone yield obtained from AD was lower than from ADD, however in this case, it was possible to obtain this compound from AD, contrary to the one reported by Hung *et al.*[13] and Llanes *et al.*[7] for the *Mycobacterium* sp. NRRL B-3683 strain. These results suggest that these nutrients enhanced the reduction reactions in general, hence not only more testosterone was obtained but the $D^1$ reduction of AD and ADD is higher also, similar to the report by Llanes *et al.*[8]

On the other hand, the use of ADD as substrate increases the carbon 17-reduction too, and the testosterone production was significantly higher. Hung *et al.*[14] and Llanes *et al.*[6,7] explained that this compound is not formed by a single reduction step from AD but by the double reduction of ADD, with the formation of $\Delta^1$-dehydrotestosterone as intermediate product. Although in these experimental conditions the intermediate mentioned above was not observed.

### Effect of initial pH on the testosterone production from ADD

This study was made in MSAL medium and testosterone was determined at 144 h . The maximum testosterone yield was obtained at pH of 7 (Fig. 3). This result is similar to the one obtained by Llanes *et al.*[6,7]

## CONCLUSIONS

Significant effect on the testosterone production from cholesterol was observed when different culture media were used. The saline medium MSAL with glucose stimulated the testosterone formation but the urea addition increased significantly this production. The ADD formation from AD was stimulated by the glucose addition too, although the *Mycobacterium* sp. MB-3683 produces AD as a major product from cholesterol, hence, also this sugar enhanced the 1,2-dehydrogenation reaction.

The testosterone production from cholesterol was stimulated by the initial pH 6, which is increased with time as a consequence of the glucose degradation, not the sterol biotransfromation.

Testosterone was obtained from AD but ADD was a better substrate than AD in the formation of this product, although the $\Delta^1$-dehydrotestosterone, intermediate product in the double reduction of AD, was not detected.

## BIBLIOGRAPHY

1. Zucker T.P., Higashiura K., Mathur R.S. and Halushka P.V., Androstenedione increases thromboxane A(2) receptors in human erythroleukemia cells. **Life Science, 58**, 683, 1996.
2. Berthaut I., Portois M.C., Cussenot O. and Mowszowicz I., Human prostatic cells in culture: Different testosterone metabolic profile in epithelial cells and fibroblasts from normal and hyperplastic prostates. **J. Steroid Biochem. Mol. Biol., 58**, 235, 1996.
3. Bogovich K. and Payne A.H., Purification of rat testicular microsomal 17-ketosteroids reductase. **J. Biol. Chem., 255**, 5552, 1980.

4. Singer Y., Shity H. and Bar R. Microbial transformation in ciclodextrin medium. 2. Reduction of androstenedione to testosterone by *Saccharomyces cerevisiae*. **Appl. Microbiol. Biotechnol.**, 35, 731, 1991.
5. Liu W.H., Kuo C.W., Wu K.L., Lee C.Y. and Hsu W.Y. Transformation of cholesterol to testosterone by *Mycobacterium* sp. **J. Ind. Microbiol.**, 13, 167, 1994.
6. Llanes N. Hung B., Falero A., Pérez C. and Aguila B., Glucose and lactose effects on AD and ADD bioconversion by *Mycobacterium* sp. **Biotechnol. Lett.**, 17, 1237, 1995.
7. Llanes N., Hung B.R., Falero A., Pérez C. y Aguila B. Obtención de testosterona a partir de precursores esteroidales. **Rev. CENIC Ciencias Biológicas, 28**, 63, 1997.
8. Hamada H. and Kawabe S., Biotransformation of AD by green cell suspension of *Marchantia polymorfa*: stereoselective reduction at carbon 17. **Life Science, 48**, 613, 1991.
9. Borrego S., Espinosa M.E., Pérez I., Guillén F., Fonseca M. and Niubó E., Influencia de la fuente de carbono sobre el crecimiento microbiano, la agregación celular y la capacidad biotransformadora de la cepa *Mycobacterium* sp. MB-3683 para producir AD a partir de los fitosteroles de la caña de azúcar. XII Seminario Científico del Centro Nacional de Investigaciones Científicas, 1995.
10. Szykula J., Hebda C. and Orpiszewski J., Isolation and identification of a new metabolite of microbial conversion of upgraded neutral fraction of Polish tall oil by *Mycobacterium* sp. MB-3683. **Biotechnol. Lett., 13**, 917, 1991.
11. Borrego S., Pérez I., Espinosa M.E., Martí E. and Fonseca M., Influence of inorganic ions and vitamins on the biotransformation of cholesterol to androstenedione by *Mycobacterium* sp. MB-3683. **Revista CENIC Ciencias Biológicas, 28**, 59, 1997.
12. Herzog H. and Oliveto E.P., A history of significant steroid discoveries and developments originating at the Schering Corporation (USA) since 1948. **Steroid, 57**, 617, 1992.
13. Wang K.C., Gan C. and Chen R.R., Microbial oxidation of sterols. I. Conversion of cholesterol and sitosterol to 17-hydroxy steroids. **J. Taiwan Pharm. Assoc., 34**, 129, 1982.
14. Hung B., Falero A., Llanes N., Pérez C. and Ramírez M.A., Testosterone as biotransformation product in steroid conversion by *Mycobacterium* sp. **Biotechnol. Lett., 16**, 497, 1994.
15. López R., Finalidad del diseño de experimentos. Generalidades. En: Diseño Estadístico de Experimentos., Editorial Científico-Técnica, La Habana, 106-111, 1984.
16. Llanes N., Falero A., Hung B.R., Pérez C., Aguila B. y Fong O., Efecto de los azúcares sobre la actividad 17b-hidroxiesteroide dehidrogenasa de la cepa de levadura YSD 196-2 inmovilizada en pectato de calcio. **Revista CENIC Ciencias Biológicas, 31**, 2000.