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Short contribution

Transformation of microcrystalline hydrocortisone by free and immobilized cells of *Arthrobacter simplex*

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Summary. The transformation of microcrystalline hydrocortisone by free and immobilized cells of *Arthrobacter simplex* resulted in formation of prednisolone. The effect of medium composition, non-ionogenic surfactants and exogeneous electron acceptors on the Δ^1-dexydrogenase activity of free and immobilized cells is described.

Introduction

Microbial Δ^1-dehydrogenation of hydrocortisone is one of the most important reactions in the microbial transformation of steroid compounds (Atrat 1982; Koshcheenko et al. 1981; Kondo and Masuo 1961; Spassov et al. 1983; Vlahov et al. 1985). At present, along with free cells, microorganisms immobilized in various carriers are very often used to achieve Δ^1-dehydrogenation (Sonomoto et al. 1980; Koshcheenko et al. 1981). The aim of the present study was to determine the conditions of microbial transformation, ensuring a 95% conversion of hydrocortisone to prednisolone.

Materials and methods

Reagents. Acrylamide, methylene-bis-acrylamide, ammonium persulphate, *N,N,N',N'*-tetramethyl ethylenediamine (TEMED), menadione, sucrose palmitate stearate-15 (SPS-15), Tween 40, 60, 80, and polyethylene glycol (PEG) 1500, 2000 were obtained from Serva (Heidelberg, FRG). Hydrocortisone (11β,17α,21-trioxy-4-pregnene-(3), 20-dione) was supplied by the Industrial Microbiology Works, Razgrad, Bulgaria.

Cultivation conditions. Arthrobacter simplex ATCC 6946 was grown in 1-l flasks containing 200 ml nutrient medium. Two media were used: (medium 1) corn steep liquor (CSL), 1%; glucose, 1%; tap water; pH after sterilization 6.8–7.2: (medium 2) CSL, 1%; glucose, 1%; amino acid bioproduct containing 25% L-lysine, 1%; $MgSO_4$, 0.2%; tap water; pH after sterilization 7.2.

The culture was grown at 180–200 rpm and 28° C for 24 h. As an inductor of steroid dehydrogenase, we used hydrocortisone (0.02%), which was added together with the inoculum. After 20–24 h growth, the cells were centrifuged at 5000 *g* for 15–20 min, washed with 0.06 *M* Na_2HPO_4—KH_2PO_4 buffer, pH 7.2, and used for hydrocortisone transformation.

Hydrocortisone transformation and determination of Δ^1-dehydrogenase activity. Transformation of hydrocortisone into prednisolone by free and immobilized cells was carried out in 1-l flasks containing 100 ml of 0.06 *M* phosphate buffer solution, pH 7.2, at 180 rpm and 28° C. The hydrocortisone content in the medium varied from 1 to 30 g/l. The non-ionogenic surfactants Tween 40, 60, 80, PEG 1500, and 2000 were introduced into the buffer solution at concentrations of 0.01%–1.0%.

Δ^1-Dehydrogenase activity of free and immobilized cells was determined by the amount of the product formed and expressed in percentage of the initial amount of substrate. In certain cases the enzymatic activity was expressed in units of specific activity.

Analysis of the transformation product. Samples (1 ml) of medium were taken for analysis at certain intervals; 40 mg steroids were extracted by 15 ml ethylacetate. Separation of the steroids was performed by thin layer chromatography on DC-Alufolien Kiesel gel 60 F_{254} plates in a system of methylene chloride:acetone = 2:1. Identification and quantitative determination of steroids were carried out densitometrically using UV light at 245 nm. Qualitative identification of steroid compounds was effected by developing the plates with cerium sulphate solution.

The steroids were isolated from the reaction medium by extraction from the water phase with a double volume of ethyl acetate. The extraction was repeated three times. Upon evaporation, the dry steroid residue was recrystallized. The isolated product prednisolone was analysed according to the requirements of the British Pharmacopoeia (1980; BP-80).

Immobilization. Immobilization into polyacrylamide gel (PAG) was carried out under conditions described in (Koshcheenko et al. 1981). The entrapment of cells into Ca-alginate gel was performed according to the method of Ohlson et al. (1979); the conditions of immobilization into carrageenan gel were described by Takata et al. (1980). In all cases the total volume of the cell suspension and the solution of the carrier was 20 ml.

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Results and discussion

Composition of the nutrient medium in relation to Δ^1-dehydrogenase activity

We carried out a comparative study of *A. simplex* cells grown on two nutrient media. The data, characterizing the process of Δ^1-dehydrogenation by washed-out cells, are shown in Fig. 1 (curves A, B). Analysis of the data obtained and subsequent calculations indicate that the specific Δ^1-dehydrogenase activity of the cells grown in medium 1 (curve A) was 0.011–0.013 μmol mg^{-1} cells per minute, and in medium 2 (curve B), containing the bioproduct, 0.018 μmol mg^{-1} cells per minute. Thus, the specific Δ^1-dehydrogenase activity of the cells grown in the CSL-glucose medium containing L-lysine bioproduct was higher by 30% on average, which apparently indicates a more active synthesis of the enzyme due to the presence of amino acids in the medium (Cejka 1976).

The transformation of hydrocortisone of the two media at a rate of 5 g/l took about 8–10 h (Fig. 1A). During this period of time 90% of the hydrocortisone was converted into prednisolone. Longer duration of the process did not lead to a more complete conversion of the substrate. Similar data have been reported by other authors (Borman et al. 1975; Skryabin et al. 1979; Constantinides 1980).

Effect of non-ionogenic surfactants on Δ^1-dehydrogenase activity and completeness of hydrocortisone-to-prednisolone conversion

The possibility of increasing the Δ^1-dehydrogenase activity and reducing the residual hydrocortisone in the medium was checked by transformations carried out in the presence of surfactants. The latter can improve permeability resulting in increased quantity of the permeated substrate.

The results obtained indicate that all surfactants, Tween 40, 60, 80, PEG 1500, 2000, SPS-15, applied in concentrations of 0.01% to 1.0% had no positive effect on the rate of Δ^1-dehydrogenation and completeness of the substrate conversion. The specific Δ^1-dehydrogenase activity of *A. simplex* cells was 0.017–0.018 μmol mg^{-1} per minute. The content of residual hydrocortisone was 10%–15% at concentrations of 5–30 g/l.

The effect of menadione on the rate of Δ^1-dehydrogenation and on the completeness of hydrocortisone conversion

Figure 1 (curve D) shows that 60% prednisolone is accumulated in menadione solution during 60 min against 15% in the control (curve C). Δ^1-Dehydrogenation in the presence of menadione was completed 5–6 h earlier. However, in both cases the concentration of microcrystalline hydrocortisone was 10 g/l and the product contained 90% prednisolone and 10% hydrocortisone.

Increasing the menadione concentration from 0.035% to 0.15% did not lead to a more complete conversion of the substrate. Thus, the presence of menadione in the medium had no effect on the completeness of hydrocortisone conversion. These results are in agreement with those of Constantinides (1980).

Transformation of hydrocortisone in the presence of menadione, SPS-15 and L-lysine bioproduct

The data chracterizing hydrocortisone Δ^1-dehydrogenation at 5–30 g/l of buffer medium are shown in Fig. 2. Free cells (curve 1) produced 60% prednisolone after

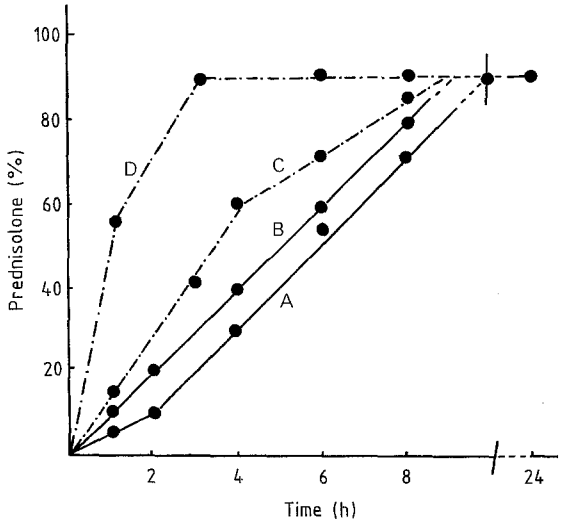

Fig. 1. Δ^1-Dehydrogenation of hydrocortisone by free cells of *Arthrobacter simplex*. *A* and *B:* cells grown on medium 1 *(A)* and medium 2 *(B)*; hydrocortisone, 5 g/l; biomass dry weight 2 g/l. *C* and *D:* effect of menadione (*C*, 0%; *D*, 0.025%); hydrocortisone, 10 g/l; biomass dry weight 6 g/l

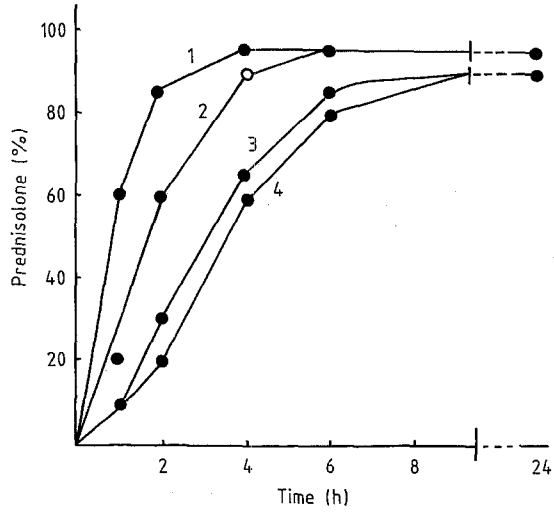

Fig. 2. Transformation of hydrocortisone by free cells of *A. simplex*. Concentration of hydrocortisone: *1*, 5 g/l; *2*, 10 g/l; *3*, 20 g/l; *4*, 30 g/l. Content of glucose, 0.05%; sucrose palmitate stearate-15 (SPS), 0.01%; menadione, 0.025%; cells, 6–8 g/l dry weight

1 h hydrocortisone transformation (5 g/l) in the presence of the L-lysine bioproduct. Complete transformation took 4 h and the end-product formed contained 94% prednisolone and 6% hydrocortisone. Similar data were obtained upon transformation of hydrocortisone at a concentration of 10 g/l (Fig. 2, curve 2). Recrystallization of the product obtained by Δ^1-dehydrogenation of hydrocortisone at 5–10 g/l concentration of the medium, yielded prednisolone with 3.5% hydrocortisone content. The high degree of hydrocortisone (5–10 g/l) conversion was achieved at a ratio of hydrocortisone:SPS-15:menadione = 50–100:1:2.5.

Increasing the content of hydrocortisone in the medium up to 30 g/l while using the same quantities of SPS-15 and menadione, resulted in a 90% conversion of the substrate (Fig. 2, curves 3, 4). The proportional increase of the amount of hydrocortisone up to a ratio of 100:1:2.5 only the presence of L-lysine bioproduct in the medium reduces the amount of residual hydrocortisone in the product to 5%–6%. Prednisolone obtained after a single recrystallization from organic solvent is in conformity with the requirements of BP-80. In this respect our results exceed the ones reported by the USA patent (1985). This effect was not observed when menadione was used in a complex with other non-ionogenic surfactants such as Tween 40, 60, 80 and PEG 1500, 2000.

Study of Δ^1-dehydrogenation by immobilized cells of A. simplex

Entrapped in PAG and carrageenan, the cells of *A. simplex* lost 30%–40% and 40%–50% of their initial activity respectively (curve 1B). The Δ^1-dehydrogenase activity of cells in Ca-alginate gel was only 30% (curve 1C) of the free cell activity (curve 1A). The reason for the drastically lower enzymatic activity of the Ca-alginate entrapped cells is not clear. The results obtained are not in agreement with the existing literature, where it is assumed that soft immobilizing conditions and optimal diffusion of the gel provide high Δ^1-dehydrogenase activity of microbial cells (Ohlson et al. 1979).

Data characterizing hydrocortisone Δ^1-dehydrogenation by PAG-entrapped cells are shown in Fig. 3. The hydrocortisone transformation was carried out at a concentration of 5–10 g in the presence of a complex of menadione, SPS and glucose (w/w 100:1:2.5). The immobilized cells converted 94% of the substrate as illustrated on Fig. 3 (curves 1B, 1C).

The menadione concentration varied from 1.4 m*M* to 0.17 m*M* in order to balance the process of cell death and propagation in the carrier. A threefold increase in stability upon 95% conversion of hydrocortisone (5 g/l) into prednisolone was observed at a menadione concentration of 0.35 m*M*.

Comparative analysis of the rate and stability of Δ^1-dehydrogenation by free and immobilized cells indicates that the use of menadione in combination with sucrose palmitate stearate providing 95% conversion of the substrate was only expedient for free cells. Rapid

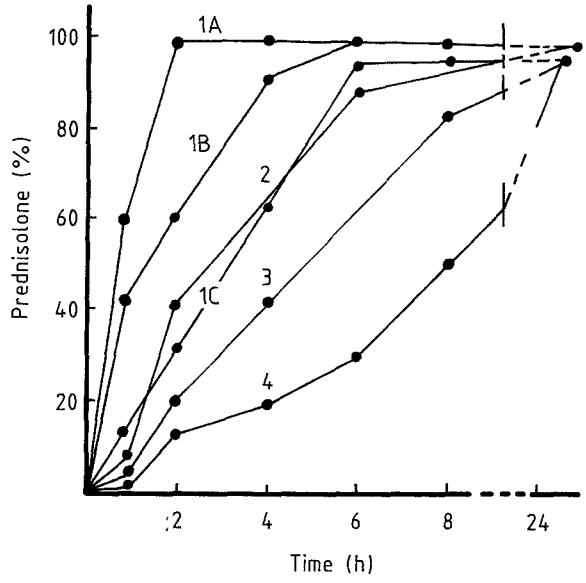

Fig. 3. Transformation of hydrocortisone by polyacrylamide-gel-entrapped cells of *A. simplex*. Concentration of hydrocortisone: *1A**, *1B*, *1C***, 5 g/l; *2*, 10 g/l; *3*, 20 g/l; *4*, 30 g/l. Content of glucose, 0.05%; SPS, 0.01%; menadione, 0.02%; cells, 6 g/l dry weight. * free cells (cf. Fig. 2), ** immobilized cells in the buffer without glucose, SPS and menadione

decrease in enzymatic activity in the presence of menadione renders the use of immobilized cells quite meaningless. Further research is required to find out the conditions for stability of immobilized systems in the presence of menadione for the purpose of reaching 95% conversion of hydrocortisone into prednisolone. We believe the use of antioxidants would probably preserve immobilized cells and improve their enzymatic activity.

References

Artrat P (1982) Steroidumwandlung mit immobilisierten Microorganismen. Z Allg Microbiol 10:723–761

Borman EA, Koshcheenko KA, Sokolova AV, Kovylkina NF (1975) Microbial dehydrogenation of microcrystalline Δ^4-3-ketosteroids. Izv Akad Nauk SSSR Ser Biol 1:143–145

British Pharmacopoeia (1980) Her Majesty's Stationery Office, London, 1980, University Press Cambridge 1:362–363

Ceijka A (1976) The effect of corn steep liquor on the transformation of steroids. Eur J Appl Microbiol 3:145–156

Constantinides A (1980) Steroid transformation at high substrate concentration using immobilized *Corynebacterium simplex* cells. Biotechnol Bioeng 22:119–136

Kondo E, Masuo E (1961) "Pseudo-crystallofermentation" of steroids: a new process for preparing prednisolone by a microorganism. J Gen Microbiol 7:113–117

Koshcheenko KA, Sukhodolskaya GV, Tyurin VS, Skryabin GK (1981) Physiological, biochemical and morphological changes in immobilized cells during repeated periodical hydrocortisone transformation. Eur J Appl Microbiol Biotechnol 12:161–169

Ohlson S, Larsson PO, Mosbach K (1979) Steroid transformation by living cells immobilized in calcium alginate. Eur J Appl Microbiol Biotechnol 7:103–110

Skryabin GK, Sukhodolskaya GV, Koshcheenko KA (1979) The peculiarities of hydrocortisone transformation by PAG entrapped cells. Izv Akad Nauk SSSR, Ser Biol 2:165–172

Sonomoto K, Sin I-N, Tanaka A, Fukui S (1980) Application of urethane prepolymers to immobilization by *Arthrobacter simplex* cells entrapped with urethane prepolymers. Agric Biol Chem 44:1119–1126

Spassov G, Krützfeldt R, Sheldrick WS, Wania W, Vlahov R, Snatzke G (1983) Crystallographic monitoring of microbiological steroid transformations. Eur J Appl Microbiol Biotechnol 17:80–84

Takata J, Yamamoto K, Tosa T, Chibata I (1980) Immobilization of *Brevibacterium flavum* with carrageenan and its application for continuous production of 4-malic acid. Enzyme Microbiol Technol 2:30–36

USA patent (1985) No. 4524134

Vlahov R, Spassov G, Krainova JL, Kovachev IT, Tarpanov VI, Krikorian DA, Pramatarova VK, Terezov VP, Milanova RK, Vassilev ChD, Ivanov ZCh (1985) Bulgarian patent no. 40420