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Transformation of Cortisone-21 Acetate with *Streptomyces atroolivaceus*

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ABSTRACT. The enzymatic hydrolysis and $\triangle^1$ – dehydrogenation of cortisone-21-acetate with *S. atroolivaceus* were evaluated. Highest yields of prednisone were obtained when the transformation process was conducted using cells in their logarithmic growth phase (60 hr old culture). Optimal conversion estimates of cortisone-21-acetate were obtained with 5 mg substrate/2 g cell fresh weight/50 ml reaction mixture. Supplementation of the reaction mixture with adenine and adenosine retarded the formation of prednisone while menadione and riboflavine led to the formation of high yields of prednisone. Similarly, Fe^{++} and Mg^{++} encouraged the formation of high yields of prednisone while Mn^{++} gave comparatively lower product yield.

Microbial $\triangle^1$ – dehydrogenations are essential to the manufacture of corticosteroid analogues as chemical dehydrogenation methods do not compete effectively. The microbial $\triangle^1$ – dehydrogenation reaction is influenced by several factors. Assessment studies were made by several workers on elucidating the physiological aspects of the $\triangle^1$ – dehydrogenation reaction. Of these factors, the age of the culture (Marsheck 1971, Richter group 1972, El-Rffai *et al.* 1975, and Smith 1984); the composition and the supplementation of the fermentation medium with iron and mangnesium ions (Sutter *et al.* 1957 and El-Refai *et al.* 1974); the addition of a variety of compounds including organic acids, growth promoting substances, purines and pyrimidines (Sallam *et al.* 1975) and the effect of some redox agents, enzyme inhibitors and stimulators (Sih and Bennett 1960, 1962, Lystrovaya *et al.* 1967, El-Refai *et al.* 1976, Naim *et al.* 1978, and Ohlson *et al.* 1978).

We have previously reported (Sallam *et al.* 1987) our findings on cortisone-21-acetate transformation with *S. atroolivaceus*. In this report, the effect of some factors that influence the enzymatic hydrolysis and $\triangle^1$–dehydrogenation of

cortisone acetate will be given. Although a number of transformation products had been encountered throughout the present study (20 β-hydroxy cortisone and 20 β-hydroxy prednisone), attention was only paid to the products of specific reactions, *i.e.* hydrolysis and $\triangle^1$−dehydrogenation.

Materials and Methods

Microorganism

Streptomyces atroolivaceus was isolated and identified by El-Shanshoury (1985). The organism was locally isolated from Egyptian soil following the method described by Tsao *et al.* (1960) and stored either on slants at 4°C or in 10% (W/V) glycerol at −20°C (Wellington and Williams 1978). Characterization of the organism was carried out by the procedure of Becker *et al.* (1965), and that from Williams *et al.* (1983). Following the key of Nonomura, survey of literatures on the description of *Streptomyces* species, ISP (1974) and Bergey's Manual (1974), the experimental organism concluded to be identified as *Streptomyces atroolivaceus*. The organism was used by El-Sayed *et al.* 1987 for biosynthesis and metabolism of indole-3-acetic acid.

Cultivation

Streptomyces atroolivaceus was grown on 50 ml portions of a medium containing (g/l): glucose, 40; yeast extract, 5 and peptone 10, pH 6.8. The flasks were agitated on an orbital shaker 180 rpm for 16 hr whereafter 0.1 mg cortisone-21-acetate dissolved in 0.1 ml ethanol was added to each flask. After 24 hr the content of each flask was filtered under aseptic conditions, washed with distilled water, physiological saline solution and resuspended in 50 ml portions of phosphate - citrate buffer pH 6.6. 5 mg cortisone-21-acetate in 1 ml 96% ethanol were then added to each flask and the reaction was allowed to proceed for another 24 hr. The details will be given at its appropriate position in the text.

Extraction

At the end of the transformation period, the content of each flask was homogenized in a blender (16000 rpm) with two volumes of chloroform (100 ml). The extraction was repeated three times in order to assure that all the transformation products were extracted. The combined chloroform extracts were washed with half its volume of distilled water, dried over anhydrous sodium sulphate, then distilled to give a semi-solid residue "test material".

Analysis

The test material was dissolved separately in a measured volume of chloroform: methanol mixture (1:1 v/v). Analysis was carried out by thin-layer chromatography on silica gel G plates. For resolution of the different products

encountered during this work the following two solvent systems proved to be suitable: (a) toluene: acetone: ethyl acetate (40:20:20, /v/v/v) and (b) cyclohexane: acetone: ethyl acetate (50:30:20, v/v/v). The transformation products as well as cortisone-21-acetate were identified from thin-layer chromatograms of each product compared with the authentic steroids using different colour reagents (Sallam *et al.* 1974). Quantitative estimation of the products was carried out by the preparative thin-layer chromatography using standard equipment. The concentration of each compound was determined colorimetrically as described by Mizsei and Szabo (1961).

Results and Discussion

The Age of the Culture

This was approached by evaluating the different growth phases following by performing the transformation process with the experimental cultures representing these phases; 12 hr (lag phase), 60 hr (logarithmic phase), 48 hr (stationery phase) and 96 hr (decline phase). The data (Table 1) showed that the age of the culture influenced not only the transformation rate but also the resulting metabolites. The bioconversion of cortisone acetate to its different transformation products and also prednisone formation attained their maximal rate with the cultures aged 60 hr (logarithmic phase). On the other hand, cortisone yield reached the minimum level. The obtained results may be attributed to the fact that, in that phase of

Table 1. Transformation of cortisone acetate (5 mg/50 ml medium) with different aged *Streptomyces atroolivaceus* cultures

Culture age (hr)	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
4	61.11 ± 1.2	35.25 ± 0.3	2.25 ± 0.2
12	55.50 ± 1.1	29.75 ± 0.6	7.75 ± 0.2
24	53.30 ± 1.5	21.25 ± 0.6	16.25 ± 0.3
36	48.80 ± 1.4	17.50 ± 0.5	20.00 ± 0.4
48	46.10 ± 2.5	15.50 ± 0.4	22.00 ± 0.5
60	43.80 ± 0.8	9.75 ± 0.3	27.75 ± 0.7
72	47.22 ± 0.5	12.75 ± 0.3	23.75 ± 0.5
84	51.66 ± 0.8	13.75 ± 0.4	23.75 ± 0.3
96	61.10 ± 0.8	23.00 ± 0.6	14.50 ± 0.3

Medium: 50 ml phosphate - citrate buffer pH 6.6.

growth, the enzymes responsible for cell division, cell mass production, deacetylation and dehydrogenation are in their higher activity and therefore higher product yields were obtained.

Transformation of Cortisone-21-acetate

The bioconversion of cortisone-21-acetate with different fresh weights of the experimental organism (Table 2) showed that the consumption of the substrate increased by increasing the fresh weight of the experimental organism. However, highest prednisone yield (28%) was obtained with 2 g fresh weight.

Table 2. Transformation of cortisone acetate by different fresh weights of *Streptomyces atroolivaceus* cells

Fresh weight (g)	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
0.25	58.33 ± 0.6	21.75 ± 0.6	15.75 ± 0.2
0.5	53.77 ± 0.7	15.25 ± 0.0	22.25 ± 0.6
1.0	52.22 ± 0.7	12.00 ± 0.2	25.50 ± 0.3
1.5	50.50 ± 0.5	12.00 ± 0.2	25.50 ± 0.4
2.0	47.70 ± 0.7	9.50 ± 0.2	28.00 ± 0.2
3.0	47.70 ± 0.6	15.25 ± 0.2	22.25 ± 0.3
4.0	45.00 ± 0.7	16.25 ± 0.4	21.25 ± 0.3
5.0	45.50 ± 0.3	16.90 ± 0.5	20.60 ± 0.5
6.0	42.00 ± 0.4	34.25 ± 1.2	3.25 ± 0.1

The different weights of the organism were suspended in 50 ml phosphate - citrate buffer pH 6.6.

Cortisone-21-acetate Level

The data (Table 3) show that lower substrate level (1 mg) was completely consumed. Higher levels of cortisone-21-acetate were accompanied by achieving elevated amounts of unchanged substrate. However, best bioconversion estimates were obtained upon using 5 mg cortisone-21-acetate. Under this condition the highest prednisone output (28%) was obtained. The decrease in prednisone yield with the parallel increase of the cortisone-21-acetate levels may indicate that the action of the enzymes catalyzing the bioconversion reactions appeared to proceed in the reverse direction when the obtained products reached definite levels. The reversibility of the oxidation-reduction reaction of the C-1,2 and C-20 functional groups of Δ 1,4–3,20 diketones was demonstrated by Goodman *et al.* (1960).

Table 3. Transformation of different concentrations of cortisone acetate by the *Streptomyces atroolivaceus* resting cells

Added cortisone acetate (mg)	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
1	0.00 ± 0.0	0.00 ± 0.0	23.90 ± 0.2
2	2.80 ± 0.0	1.25 ± 0.0	25.00 ± 0.3
3	14.80 ± 0.1	12.50 ± 0.2	20.83 ± 0.3
4	34.72 ± 0.3	9.75 ± 0.2	21.50 ± 0.4
5	45.00 ± 0.7	9.50 ± 0.2	28.00 ± 0.6
6	51.10 ± 1.3	16.08 ± 0.4	15.16 ± 0.3

The transformation process was conducted in 50 ml phosphate - citrate buffer pH 6.6.

Role of Some Additives

Biological bases

The evaluation of the role of some biologically active compounds on the deacetylation and Δ^1−dehydrogenation of cortisone-21-acetate is presented in Table 4. The data suggested that supplementation of the reaction mixture with adenine and its nucleoside (adenosine) led to supression of cortisone-21-acetate conversion as well as prednisone production. In accordance to our results Naim *et al.* (1978) reported that C 1-2 dehydrogenation of cortisol with cell free extract of *B. cereus* was suppressed by adenine. However, adenosine was reported by the same author to activate the C 1-2 dehydrogenation reaction.

Table 4. Transformation of cortisone acetate with the resting cultures of *Streptomyces atroolivaceus* in the presence of adenine and adenosine

Additives	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
No additives (control)	45.27 ± 0.6	9.50 ± 0.2	28.00 ± 0.4
Adenine	16.10 ± 0.2	20.75 ± 0.5	16.75 ± 0.5
Adenosine	51.70 ± 1.1	11.90 ± 0.3	25.60 ± 1.1

Both adenine and adenosine were individually supplemented to the reaction mixture (50 ml phosphate-citrate buffer pH 6.6) at 1.0 mg % concentration at the same time of substrate addition.

Some vitamins and redox agents

The data given in Table 5 showed that addition of 8-hydroxyquinoline to the reaction mixture led to suppression in the yield of prednisone. Contrary to our findings Naim *et al.* (1978) reported that 8-hydroxyquinoline enhanced the C 1-2 dehydrogenation reaction of cortisol with cell free extract of *B. cereus*.

Table 5. Transformation of cortisone acetate with the resting cultures of *Streptomyces atroolivaceus* in the presence of some vitamins and redox agents

Additives	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
No additives (control)	45.27 ± 0.6	9.50 ± 0.2	28.00 ± 0.4
Riboflavine	43.33 ± 1.1	6.14 ± 0.3	31.36 ± 0.3
Menadione	41.66 ± 1.3	4.80 ± 0.2	32.70 ± 0.4
8-Hydroxyquinoline	53.30 ± 1.2	15.25 ± 0.3	22.25 ± 0.6

Each compound was separately supplemented in 1 mg % to the reaction mixture (50 ml phosphate-citrate buffer pH 6.6) at the same time of substrate addition.

The deacetylation and C 1-2 dehydrogenation of cortisone-21-acetate were activated in the presence of riboflavine and menadione where 31.1 and 32.7% yields of prednisone were obtained, respectively. The stimulatory action of riboflavine and menadione may also be explained through the findings of Talalay (1965) who reported that flavines work as intermediates in the oxidation reduction chain of C 1-2 of steroids.

Mineral salts

The data of Table 6 showed that the bioconversion efficiences of cortisone-21-acetate were markedly initiated in the presence of magnesium and ferrous salts. In both cases highest prednisone yields (34 and 32%, respectively) were obtained (in comparison to the control treatment).

A little increase in prednisone yield than that of the control was however observed with $MnSO_4$. These results are in accordance to those previously reported by Naim *et al.* (1978).

Table 6. Transformation of cortisone acetate with *Streptomyces atroolivaceus* the resting cultures in the presence of some mineral salts

Additives	Transformation products		
	Residual cortisone acetate %	Cortisone %	Prednisone %
No additives (control)	45.27 ± 0.6	9.50 ± 0.2	28.00 ± 0.4
$MgSO_4$	40.00 ± 0.4	3.50 ± 0.1	34.00 ± 0.4
$FeSO_4$	42.70 ± 0.6	5.16 ± 0.1	32.34 ± 0.6
$MnSO_4$	44.40 ± 0.7	7.36 ± 0.3	30.14 ± 0.6

$MgSO_4$, $FeSO_4$ and $MnSO_4$ were individually added to the reaction mixture (50 ml phosphate-citrate buffer pH 6.6) in 1.8 mmol equivalent of their constituents of Mg^{++}, Fe^{++} and Mn^{++}.

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خواص التحول الانزيمي لخلات الكورتيزون بواسطة استربتوميسس أتروأوليفاسيس

عبدالرحيم رمضان الشنشورى و لطفي عبدالرؤوف سلام
و مصطفى السيد و عصام عزب

معمل كيمياء المنتجات الطبيعية والميكروبية بالمركز القومي للبحوث ـ الدقي ـ القاهرة
قسم النبات بكلية العلوم ـ جامعة طنطا ـ مصر

تمت دراسة تأثير بعض العوامل على عملية تحويل خلات الكورتيزون بواسطة استربتوميسس اتروأوليفاشيس، دلت الدراسة على أن عملية تحويل خلات الكورتيزون إلى البريدنيزون تتأثر بعمر المزرعة وكان أعلى معدل للتحويل باستخدام مزرعة عمرها ٦٠ ساعة كما دلت التجارب على أن الوزن الطازج للخلايا يؤثر أيضا في عملية التحول وكان أعلى معدل للبريدنيزون عند استعمال ٢ جم من الخلايا الطازجة لكل ٥٠ مللي من محلول التفاعل.

وتأثرت كمية البريدنيزون المتكون بدرجة تركيز خلات الكورتيزون وكانت أفضل النتائج عند استخدام ٥ مجم من خلات الكورتيزون لكل ٥٠ مللي من وسط التفاعل. كما أدى إضافة كل من الادنين والادينوزين ٨ هيدروكسي كينولين إلى وسط التفاعل إلى تثبيط عملية التحول الانزيمي لخلات الكورتيزون إلى البريدنيزون بينما أدت إضافة الريبوفلافين والمينادیون إلى زيادة ملحوظة في كمية البريدونيزون المتكونة، كذلك اختلف تأثير إضافة بعض أيونات العناصر إلى وسط التفاعل على عملية التحول بينما لم تؤد إضافة أيونات المنجنيز إلى زيادة ملحوظة في كمية البريدنيزون وأدت إضافة كلا من أيونات الحديدوز والماغنسيوم إلى زيادة في كمية المركب الناتج.