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Sequential 11α-Hydroxylation and 1-Dehydrogenation of 16α-Hydroxycortexolone

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Summary

Two-step microbial transformation of 16α-hydroxycortexolone to its 1-dehydro-11α-hydroxy derivative, without isolating an intermediate, was achieved with an overall yield of 72% of product at a steroid substrate concentration of 3 mg/ml. The process included formation of the cycloborate complex of the substrate, hydroxylation of the borate complex with a suspension of *Aspergillus ochraceus* mycelium in phosphate buffer, and dehydrogenation of the 11α-hydroxylated intermediate with acetone-dried *Arthrobacter simplex* cells. The desired product was then obtained by breaking the resultant borate complex through acidification.

INTRODUCTION

The feasibility of microbiological hydroxylation and 1-dehydrogenation of corticosteroids has been established; for example, 11α-hydroxylation of various steroids by *Aspergillus* species and *Rhizopus nigricans* has been reported by many workers: Dulaney *et al.*,[4] Dulaney and MacAleer,[3] Dulaney,[2] Weaver *et al.*,[23] Weaver,[22] and Fried *et al.*[6] Similarly, 1-dehydrogenation of the steroid nucleus has been accomplished with intact cells or cell-free preparations from different microbial species, including *A. simplex*, Herzog et al.,[10] Kondo,[12] and Penasse and Peyre;[16] *Nocardia restrictus*, Sih and Bennett;[18] *Pseudomonas testosteroni*, Levy and Talalay;[15] *Bacillus sphaericus*, Hayano *et al.*,[9] and *Bacillus cyclooxydans*, Goodman *et al.*[8] and Iida *et al.*[11]

The present study was undertaken to develop a practical method of microbial transformation of 16α-hydroxycortexolone (I) by 11-hydroxylation and 1-dehydrogenation to its 11α-hydroxy-1-dehydro derivative (III); these and other steroids referred to are identified in

TABLE I
Identification of Steroids

Steroid	Derivative
16α-hydroxycortexolone	I
11α-16α-dihydroxycortexolone	II
1-dehydro-16α-hydroxycortexolone	IId
1-dehydro-11α-, 16 α-dihydroxycortexolone	III
cycloborate ester of I	Ib
cycloborate ester of II	IIb
cycloborate ester of III	IIIb

Table I. Since two transformation reactions are involved, a number of alternate processes exist. These include: a) varying the sequence of the two reactions, with or without isolating the intermediate, b) the use of a mixed culture system to carry out both reactions, and c) the use of either growing or resting cells. The extremely low solubility (<10 μg/ml) of I in fermentation broth proved to be an immediate problem, for it prevented transformation from proceeding in good yield at relatively high concentrations.

We solubilized I as the borate complex (Ib) and developed a practical two-step process for converting Ib to the borate complex of the desired product (IIIb), via the borate complex of the 11α-hydroxy intermediate (IIb), in a high yield at a relatively high concentration. Acidification of the broth converted IIIb to III, (Fig. 1).

METHODS AND MATERIALS

Organisms

The organisms used were *A. ochraceus* (Squibb Culture No. 4057), as the 11α-hydroxylator, and *A. simplex* (Squibb Culture No. 6062), as the 1-dehydrogenator. The former was maintained on glucose–agar slants, and the latter on yeast–beef–agar slants. Stock cultures were stored as suspensions in skimmed milk, frozen and held over liquid nitrogen at about -150°C.

Steroid Substrate

16α-Hydroxycortexolone (I) was used throughout the studies (Syntex Laboratories, Mexico).

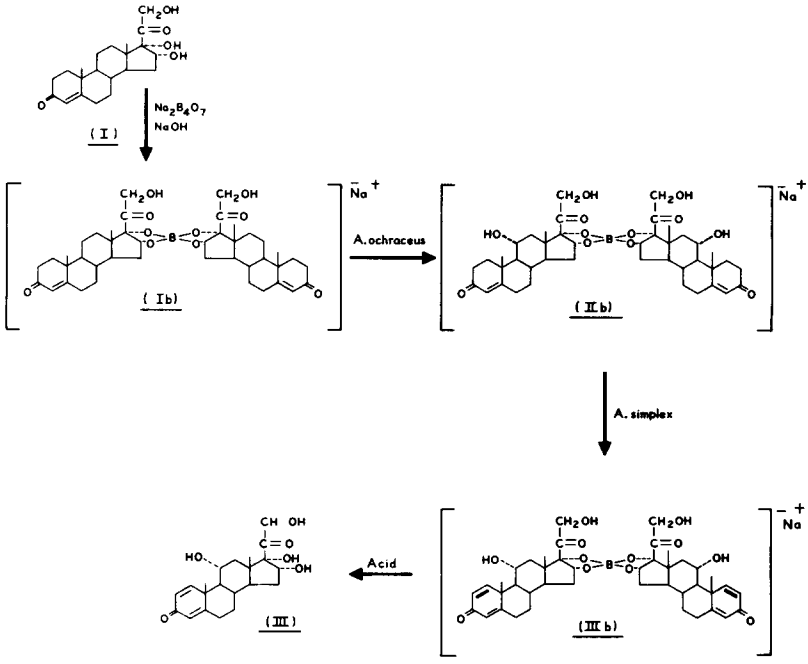

Fig. 1. Sequence of reactions converting I to III by formation of Ib, 11α-hydroxylation by *A. ochraceus* culture, 1-dehydrogenation by acetone-dried *A. simplex* cells, followed by acidification.

Preparation of Cycloborate Ester Salt

A solution of the sodium salt of the cycloborate ester (Ib) of I was made by combining 1123.6 mg of I (3.10 mmole) with 2.37 ml of 0.1635 molar aqueous sodium tetraborate solution (0.39 mmole), 1.55 ml of 0.5 molar aqueous sodium hydroxide solution (0.77 mmole), and 1.68 ml of ethanol to give a final concentration of I of 200 mg/ml. The mixture was heated gently until a clear solution was obtained, resulting from the formation of Ib by the reaction illustrated in Figure 2. The ester salt remained in solution when added to the fermentation broth.

Addition of Steroid Substrate

When a growing culture of *A. ochraceus* was used for 11α-hydroxylation, I was added as a sterile solution of Ib, at 3, 24, and 48 hr,

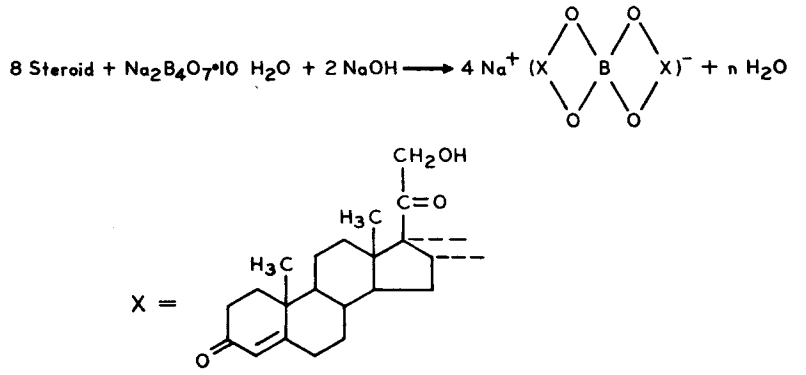

Fig. 2. Preparation of sodium salt of Ib by reacting 8 moles of the steroid, stoichiometrically, with 1 mole of sodium tetraborate and 2 moles of sodium hydroxide.

respectively, after inoculation of the organism. The concentration of I added to the broth each time was 1 mg/ml of broth. When resting cells of the organism were used, Ib was added at 0, 7, and 13 hr, each addition giving the same concentration of I as before.

11α-Hydroxylation

A. Preparation of Growing or Resting Cells. The spores of *A. ochraceus*, grown at 25°C on a glucose–yeast extract (Difco Laboratories, Detroit, Mich.) slant, were washed off the agar with 10 ml of sodium lauryl sulfate–saline solution (0.01 and 0.9% w/v, respectively, in distilled water). One-milliliter of the spore suspension was added to each inoculum flask. The inoculum of *A. ochraceus* was then developed at 25°C in one 24-hour stage, in a medium containing (% w/v) 0.6, cornsteep liquor; 1.0, dextrose; 0.3, $NH_4H_2PO_4$; 0.25, yeast extract, in distilled water. For use as growing cells, 5 ml of the developed inoculum was transferred into 100 ml of fresh medium of the same composition contained in a 500-ml Erlenmeyer flask. For use as resting cells, 45 ml of the inoculum was mixed with 45 ml of 0.05 molar phosphate buffer (K_2HPO_4—KH_2PO_4, pH 7.0) in a 500-ml Erlenmeyer flask.

B. Preparation of Spores. A 1-ml quantity of the spore suspension was added to each 500-ml Erlenmeyer flask that contained 15 g of

corn coated with 30 ml of 7% w/v cornsteep liquor. The flasks were shaken gently to place the cells uniformly in contact with the surface of the corn, and the preparation was incubated for 5 days at 25°C. The spores were washed off the corn particles with 25 ml of the sodium lauryl sulfate–saline solution, and collected by centrifugation. The spores were washed twice with an equal volume of the 0.05 molar phosphate buffer, then stored at −10°C.

C. Transformation and Isolation. To accomplish hydroxylation, 45 ml of the developed inoculum of *A. ochraceus* was mixed with an equal volume of the 0.05 molar phosphate buffer in each of two 500-ml flasks, and Ib was added, as indicated. After 27 hr of incubation at 30°C, with shaking in a 2-in. diameter circle at 280 cycles/min on a Gyorotory G-52 machine (New Brunswick Scientific Co.), the broth from the two flasks was harvested, combined and filtered. The empty flasks and mycelial cake were washed with 30 ml of 0.15% aqueous sodium tetraborate solution to minimize loss of steroid. The filtrate, combined with the borate washing, had a total volume of 180 ml. The filtrate was acidified to pH 3.9 with 10% H_2SO_4 and extracted five times with 110-ml portions of ethyl acetate. Acidification broke the steroid–borate complex and permitted extraction of the free steroid. The combined ethyl acetate extracts were concentrated to dryness on a flash evaporator, and the resultant crude II was recrystallized from methanol.

1-Dehydrogenation

A. Preparation of Growing Cells. An inoculum of *A. simplex* was developed in a manner similar to that employed with a culture of *A. ochraceus*, using the same medium.

B. Preparation of Acetone-Dried Cells. The methods described by Erickson *et al.*[5] were used.

C. Product Formation and Isolation. 11α-Hydroxylation was first effected as described previously; then 0.9 ml (40 mmole) of ethanolic menadione solution and 0.9 g of acetone-dried *A. simplex* cells were added to 90 ml of the 11α-hydroxylated broth in each of two 500-ml flasks. After 10 hr incubation at 30°C to allow dehydrogenation to take place, the resulting fermentation broth was worked up as it had been for the isolation of the 11α-hydroxylated intermediate.

Preparation of Samples for Steroid Measurement

Samples of 1–5 ml were drawn at appropriate intervals. Each sample was subsequently diluted with distilled water to a final concentration of steroid of about 300 μg/ml. The diluted sample was adjusted to pH 3 to 4 with 10% H_2SO_4, then extracted with 2 ml of methyl–isobutyl–ketone (MIBK).

Paper Chromatography

Steroid samples (0.1 ml of the MIBK extracts) were spotted and developed by descending chromatography on Whatman No. 1 paper in benzene–ethanol–water solvent (2:1:2, by vol.) at 25°C.

Steroid Identification and Measurement

After development of the chromatograms, steroid spots were detected by ultraviolet scanning. For identification, reliance was placed on comparative mobility (RF) values. These values, established with authentic materials, were 0.64, 0.03, and 0.015 for I, II, and III, respectively. For quantitative estimation, detected spots were cut out and eluted with ethanol (U.S.P. 190 Proof—95%); their absorption at 240 mμ was read on a Beckman DU spectrophotometer. The concentration of steroid in each sample was estimated by comparison with a standard solution equilibrated with MIBK, chromatographed, and further tested in an identical manner.

It should be noted that, for simplicity of presentation, all results are reported on the basis of the uncomplexed steroid, even though the cycloborate ester of I was the favored and most frequently used substrate.

RESULTS

Effect of Solubilization on Efficiency of Hydroxylation

The ability of resting cells of *A. ochraceus* to introduce an 11α-hydroxyl group into I was studied in two systems. I was added only once at 0-time, either as a sterile suspension* in Tween 80 or as a sterile solution of the cycloborate ester, Ib, to give a concentration of I of 1 mg/ml of broth. As shown in Figure 3, the rate of 11α-hydroxylation of Ib was three times greater than that obtained with

*Steroid was heated at 121°C for 10 min and then added to sterile aqueous Tween 80 (0.1% V/V).

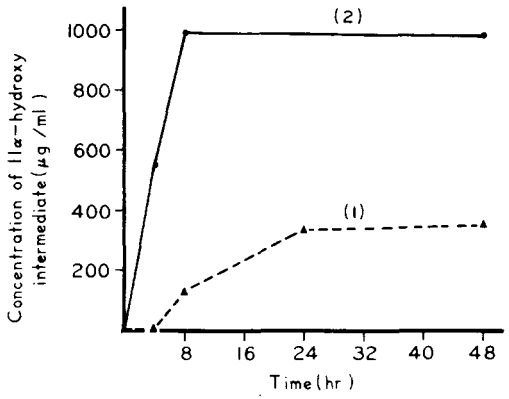

Fig. 3. Effect of solubilization of 16α-hydroxycortexolone (I) on 11α-hydroxylation by *Aspergillus ochraceus:* (1) Tween 80 suspension of I; (2) Solution of Ib.

the Tween 80 suspension of I. The maximal conversion of substrate achieved in the latter system was less than 40%, as compared with quantitative conversion in the former.

Comparison of Growing Cells and Resting Cells of A. ochraceus

Growing and resting cell systems of *A. ochraceus* were prepared and Ib was added once at 0-time to give a concentration of I of 1 mg/ml. As shown in Figure 4, the rate of conversion of steroid by resting cells

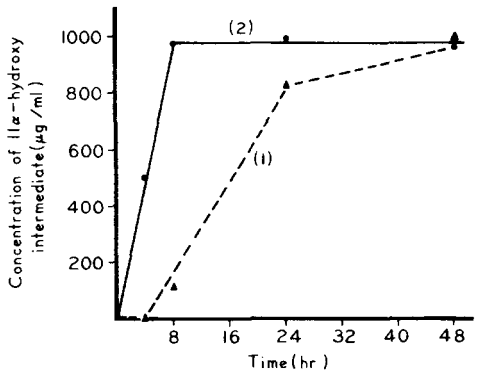

Fig. 4. Effect of type of cell on rate of 11α-hydroxylation of Ib by *Aspergillus ochraceus* (Substrate concentration = 1 mg/ml as I) (1) Growing cells, (2) Resting cells.

was considerably faster; almost quantitative conversion was accomplished in 8 hr using resting cells, as compared with the 48 hr required when growing cells were used.

When the culture broth was diluted either greater or lesser than 1:1 with phosphate buffer, steroid transformation was inferior.

Sequential 11α-Hydroxylation and 1-Dehydrogenation

A. Time-Course Study. Ib was added in three increments to a resting-cell preparation of *A. ochraceus.* After completion of hydroxylation, 1-dehydrogenation was initiated at 24 hr by adding to the reaction mixture 0.5 g of acetone-dried *A. simplex* cells and 0.5 ml (40 micromole) of ethanolic menadione solution. Dehydrogenation was completed in 5 hr. The changes with time in concentrations of substrate, intermediate, and product are shown in Figure 5. In the absence of menadione, the dehydrogenation proceeded more slowly, with only 60% conversion occurring.

B. Isolation of Products. II and III were isolated from representative broths before and after the dehydrogenation step. From a total substrate input of 540 mg in each case, 434 mg of II and 400 mg

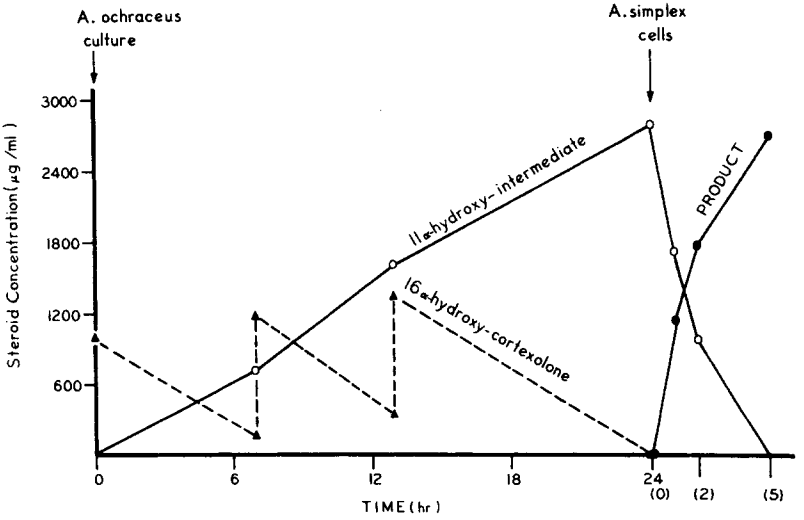

Fig. 5. 11α-Hydroxylation of Ib by resting cells of *Aspergillus ochraceus,* followed by 1-dehydrogenation of the resultant IIb by acetone-dried *Arthrobacter simplex* cells.

of III were obtained, respectively. Relative mobility values and melting points for II and III were comparable to those obtained with authentic samples.

11α-Hydroxylation and 1-Dehydrogenation in Mixed Systems

Three systems potentially capable of two-step conversion of Ib to IIIb were studied: a growing culture of *A. ochraceus* combined with acetone-dried cells of *A. simplex*; a mixed growing culture of *A. ochraceus* and *A. simplex*; and a nongrowing mixture of spores of *A. ochraceus* and acetone-dried cells of *A. simplex*. All gave less satisfactory results than did the sequential system already described.

A. Mixture of *A. ochraceus* Culture and Acetone-Dried *A. simplex* Cells. 5 ml of *A. ochraceus* culture broth and 0.5 g of acetone-dried *A. simplex* cells were added, at 0-time, to 100 ml of the cornsteep liquor medium. I was added, as a sterile solution of Ib, at 3, 24, and 48 hr, respectively, to produce a broth concentration of 1 mg/ml. As illustrated by comparing Figures 5 and 6, transformation of Ib to IIIb was substantially inferior to that obtained in the sequential procedure. From a total substrate input of 3 mg/ml, only 1.08 mg of III/ml was found in the broth after 120 hr of incubation. Substantial levels of unconverted Ib, IIb, and 1-dehydro Ib (IIdb) were detected in the harvest broth.

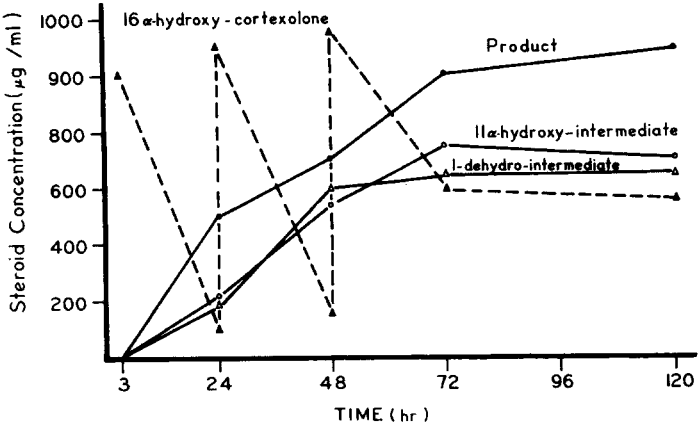

Fig. 6. Multiple transformation of Ib by a mixture of growing cells of *Aspergillus ochraceus* and acetone-dried *Arthrobacter simplex* cells.

B. Mixed Culture of *A. ochraceus* and *A. simplex*. When 5 ml each of *A. ochraceus* and *A. simplex* cultures from the appropriate inoculum stage were mixed at 0-time and incubated with 1 mg of I/ml, added as a sterile solution of Ib, Ib was converted to IIb, but no evidence of dehydrogenating activity was seen. The results suggested that the dehydrogenase was either repressed by substance(s) present in the fermentation medium or by metabolite(s) formed during the growth of the mixed culture.

C. Mixture of *A. ochraceus* Spores and Acetone-Dried *A. simplex* Cells. *A. ochraceus* spores (2% wet wt./vol.) were mixed with acetone-dried *A. simplex* cells in a phosphate buffer–glucose solution (Vézina *et al.*[21]). To this was added a sterile solution of Ib to give a concentration of I of 1 mg/ml, and the mixture incubated at 30°C. The results of this experiment are shown in Table II. Multiple transformation of the steroid substance to the desired product was not accomplished. Disappearance of all substrate in the reaction mixture resulted in the appearance of three compounds: IIb, IIIb, and an unidentified compound with an R_F of 0.96. The last compound was also observed in trace quantities when Ib was exposed to *A. ochraceus* spores alone. Interestingly, little transformation of

TABLE II

11α-Hydroxylation of Ib by *Aspergillus ochraceus* Spores, and Multiple Transformation of the Same Steroid Cycloborate Complex by a Mixture of the Spores and Acetone-Dried *Arthrobacter simplex* Cells

Sample[a]	Sampling Time (hr)	16α-Hydroxy-cortexolone (I) (0.64)[b]	11α-Hydroxy intermediate (II) (0.03)[b]	Δ¹-Intermediate (IId) (0.51)[b]	Product (III) (0.015)[b]	Unknown (0.96)
1	24	+++	+/−	−	−	−
	48	+++	+/−	−	−	+/−
	168	+++	+/−	−	−	+/−
2	24	−	−	++	+	+/−
	48	−	−	+	+	+
	168	−	−	+	+	+

[a] Sample 1: 11α-Hydroxylation by spores; Sample 2: Multiple transformation by the mixture.

[b] Relative mobility (R_F).

Ib to IIb was obtained, contrary to what one would expect from the results of Vézina *et al.*[21]

DISCUSSION

For transformation of 9α-fluorohydrocortisone to triamcinolone by a mixed culture of *A. simplex* and *S. roseochromogenes*, a medium with soybean meal as nitrogen source was developed to induce 1-dehydrogenase and 16α-hydroxylase activities and selectively to repress the appearance of undesirable 20-keto-reductase activity.[14] Selective induction of 1-dehydrogenase and 11β-hydroxylase and repression of 20-ketoreductase also were achieved by use of a medium containing cornsteep liquor, with resultant multiple conversion by a mixed culture of *A. simplex* and *Curvularia lunata* of 16α-hydroxycortexolone 16, 17-acetonide to its 1-dehydro-11β-hydroxy derivative.[17] In contrast, we have not been able to develop a medium suitable for converting Ib to IIIb in a mixed system. The best means for effecting the two-step transformation involves a sequential approach whereby Ib is first hydroxylated with resting cells of *A. ochraceus* and the resultant IIb is then dehydrogenated with acetone-dried *A. simplex* cells.

11α-Hydroxylation of I proceeds slowly because of its low solubility in water (<10 μg/ml); in contrast, the cycloborate complex of the steroid (Ib) remains soluble in the aqueous fermentation system up to concentrations greater than 200 mg/ml, a level of solubility more than 20,000 times as great. Thus the markedly enhanced hydroxylation rate is attributed directly to this increased solubility associated with the formation of the borate complex.

Menadione (2-methyl-1,4-naphthoquinone) participates in the electron-transport pathways of some strains of bacteria by substituting for the naturally occurring quinones.[1,7,13] When air-dried cells of *A. simplex* are used for dehydrogenation, the oxidation of steroid substrate proceeds rapidly.[20] With acetone-dried cells, however, the transformation ability is substantially lost. This property can be restored by the addition of menadione,[5] a hydrogen acceptor that has demonstrated superiority for this purpose.[19]

Vézina *et al.*[21] failed to demonstrate 11α-hydroxylase as an adaptive (inducible) enzyme from their work with *A. ochraceus* spores. We found no hydroxylating activity when a growing culture of *A. ochraceus* was exposed to cycloheximide prior to the addition of

Ib, which was to serve as inducer as well as substrate; however, when cycloheximide was added after exposure of the organism to Ib, the desired enzyme activity was obtained. These results suggest that 11α-hydroxylase is an inducible enzyme.

In contrast to the situation with the 1-dehydrogenase, a requirement for artificial cofactors by the 11α-hydroxylase has not been reported. We found, however, that hydroxylation of Ib with *A. ochraceus* can best be achieved by using a mixture of its developed culture broth and phosphate buffer. The broth to buffer ratio of 1:1 (vol./vol.) may provide a culture dilution that is low enough to ensure adequate levels of cofactor, yet high enough to limit the nutrients required for primary cellular metabolism of the organism.

When resting cells of *A. ochraceus* are used for transformation, fully grown culture broth is mixed with an equal volume of phosphate buffer. Cells are not expected to grow under such nutrient-limiting conditions, consequently the mycelial weight probably remains constant throughout the period of hydroxylation. On the other hand, when a growing culture is used, growth and hydroxylation occur simultaneously, with maximal growth occurring in the first 24 hr. In the growing culture the ultimate cell weight is about twofold that of the buffer system; thus, the reduced transformation experienced in the growing culture system could be attributed to suboptimal oxygenation. Whatever the explanation, the resting-cell system not only gives better transformation, but also favors ultimate recovery of steroid because of the lower concentration of medium ingredients.

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